

MUSCLE DEVELOPMENT OF LIVESTOCK ANIMALS

Physiology, Genetics and Meat Quality

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Preface

Meat is a major part of human nutrition containing essential protein components. Before muscle becomes meat (i.e. while the animal is alive), skeletal muscle tissue fulfils the important task of maintaining body stature and locomotion. Furthermore, as muscle tissue constitutes over 40% of lean body weight, growth and development of skeletal muscle tissue is a major component of body growth. During the lifetime this highly plastic tissue can adapt in many ways to its function and activity. Exercise in particular can change muscle appearance and physiology dramatically. Therefore knowledge about (livestock) muscle development is of great importance. This book describes the development, growth and adaptation of livestock muscle tissue in various ways, ending with the description of skeletal muscle-specific factors affecting post-mortem meat quality. Therefore the book is divided into three sections: Physiology, Genetics and Meat Quality.

The Physiology section first describes the mechanism of muscle fibre development in the mammalian fetus and the importance of high muscle fibre numbers for muscle mass and meat quality (Chapter 1), followed by the different morphological appearances of postnatal muscle fibres (Chapter 2) and possibilities for influencing the development of higher numbers of muscle fibres prenatally (Chapter 3). This is followed by a number of chapters covering factors affecting postnatal changes to muscle tissue: growth and exercise (Chapter 4), general nutrition level of the animal and specific essential amino acids (Chapter 5), and minerals and micronutrients (Chapter 6). Systemic regulation of skeletal muscle function by hormones affecting energy status (Chapter 7) and growth (Chapter 8) and mechanisms of remodelling the tissue (Chapter 9) conclude the Physiology section. The Genetics section starts again with prenatal skeletal muscle development, but this time at the genome level (Chapter 10), followed by a description of methods to describe the skeletal muscle transcriptome (Chapter 11) and the chromosomal localization of genes affecting skeletal muscle tissue development and meat quality (Chapter 12). The new field of proteomics for skeletal muscle is covered in Chapter 13 and the section ends with a description of two genes that have major effects on muscle tissue mass by affecting skeletal muscle fibre numbers (Chapter 14) and skeletal muscle fibre size (Chapter

15). Many of these chapters also cover specific effects on components of meat quality. The book ends with a short section describing specific aspects of general Meat Quality: intramuscular fat (Chapter 16), post-mortem skeletal muscle protein breakdown (Chapter 17) and water-holding capacity (Chapter 18).

We expect that this book will become a standard work for those interested in skeletal muscle biology and meat quality, and wish the readers many hours of pleasant reading.

Dr Marinus F.W. te Pas
Professor Maria E. Everts
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1

Number and Size of Muscle Fibres in Relation to Meat Production

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1.1 Introduction

The understanding of growth and development of skeletal muscle is one of the most important goals in animal and meat science and is also related to particular aspects of human medicine. The major component of a given muscle is the constituent muscle fibres. Muscle mass is therefore largely determined by the number of muscle fibres and the size of those fibres. Although being essential functional components within the muscle as a physiological unit, fat cells, connective tissue, capillary network and nerve fibres are of lesser importance in the determination of muscle size.

Current research suggests that animals with greater numbers of muscle fibres of moderate size produce a higher quantity and quality of meat. During myogenesis, the extent of muscle cell multiplication largely determines how many muscle fibres are formed. Therefore, the number of muscle fibres is mainly determined by genetic factors and those environmental factors that are capable of influencing prenatal myogenesis. Postnatal growth of skeletal muscle is realized by increases in length and girth of the muscle fibres, but not, with some exceptions, by increases in muscle fibre number. Muscle fibre hyperplasia in mammals is largely completed during gestation and fixed by about the time of birth, while many factors postnatally will affect the size of fibres. The purpose of this review is to describe the principles of muscle fibre growth and to demonstrate the influence of some genetic and environmental factors on muscle fibre number and size such as species, gender, selection, nutrition, physical exercise and selected growth promoters. We wish also to highlight the importance of muscle fibre number and size for lean growth and meat quality/stress susceptibility and how muscle fibre traits can be considered in selection to improve these special characteristics of animal performance.

1.2 Principles of Skeletal Muscle Growth

1.2.1 Prenatal myogenesis

During embryonic development, myoblasts develop from myogenic precursor cells, which are of mesodermal origin (Fig. 1.1). These cells are destined to enter the myogenic lineage and are able to proliferate and divide to establish a pool of myoblasts. Special signals cause the myoblasts to exit the cell cycle, to stop dividing and to differentiate. They begin to express muscle-cell specific proteins and finally fuse to form multinucleated myotubes.

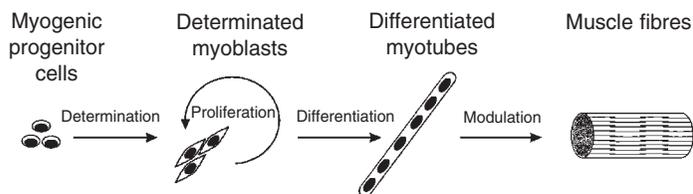


Fig. 1.1. Basic events of myogenesis (Rehfeldt *et al.*, 2000).

During myogenesis, muscle fibres develop from two distinct populations. Fibres, which form during the initial stages of myoblast fusion, are primary myofibres, which provide a framework for the larger population of smaller secondary fibres (Wigmore and Evans, 2002). These are formed from fetal myoblasts during a second wave of differentiation. The number of secondary fibres around each primary varies and can be over 20 in large mammals such as the pig (Fig. 1.2).

Another population of myoblasts does not form fibres but stays close to the myofibres; these are termed satellite cells and they are able to divide and serve as the source of new myonuclei during postnatal growth (Moss and Leblond, 1971; Schultz, 1974). They contribute to growth of the fibres and also participate in regeneration processes. Myonuclei themselves remain mitotically quiescent.

The importance and relations of the different myogenic lineages (primary and secondary) for the maturation into different muscle fibre types are not yet clarified. It seems, however, that the lineages are not very closely related to later fibre type composition. Postnatal fibre maturation seems to be more dependent on other factors such as innervation, contractile activity, growth factors, hormones and nutrition.

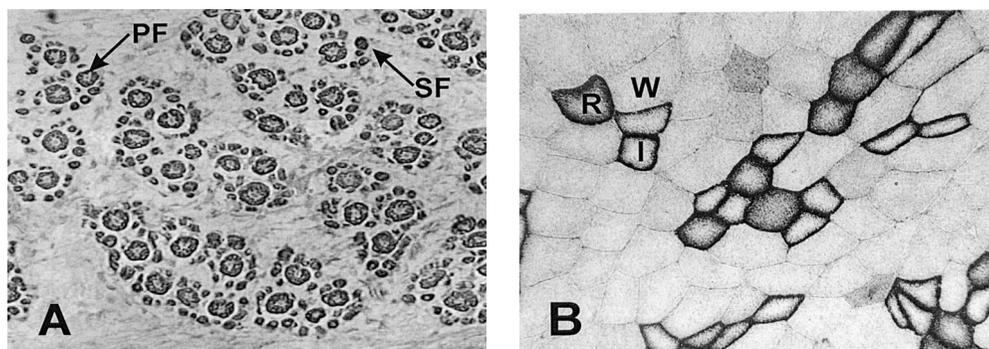


Fig. 1.2. Pig muscle cross sections at day 62 of gestation (A) and day 260 of postnatal age (B). (A) Semitendinosus muscle with primary (PF) and secondary (SF) fibres; aniline-blue/orange G staining; (B) longissimus muscle with red (R), intermediate (I) and white (W) fibres; NADH tetrazolium reductase staining.

1.2.2 Postnatal muscle growth

During postnatal growth, the increase in skeletal muscle mass is mainly due to an increase in muscle fibre size (hypertrophy). This process is accompanied by the proliferative activity of satellite cells, which are the source of new nuclei incorporated into the muscle fibres. After birth, total muscle fibre number has been reported to remain unchanged in mammals and birds by most authors. However, in fish the number of muscle fibres increases throughout life, although in later life, hypertrophy of fibres becomes increasingly important to growth (Stickland, 1983). Some reports have indicated increases in muscle fibre number after birth in rodents, chickens and pigs. In most studies, fibre counts were done on histological transverse sections. It is possible that the increase in fibre number during the first days of postnatal life is a result of maturation and elongation of the existing myotubes rather than production of new fibres (Ontell and Kozeka, 1984). This may also be true for pig muscle, since fibre formation is considered to be established between 70 and 95 days of gestation (Wigmore and Stickland, 1983a; Swatland, 1984), but increases in fibre number are apparent shortly after birth (Rehfeldt *et al.*, 2000). It is easy to imagine that myoblast alignment to form fibres does not lead to fibres of full length from the beginning. Intrafascicularly ending fibres, which have been reported to occur in growing chicken and pig muscle, provide further evidence that these may contribute to increases in the apparent total number of fibres determined per muscle cross section.

Figure 1.3 depicts the postnatal development of muscle fibre thickness and muscle fibre number in the semitendinosus muscle of pig and cattle. Muscle fibres grow in size towards a plateau, whereas fibre number remains almost constant. Initial increases shortly after birth are observed in pig muscle. Some decreases in fibre number with ageing have also been reported. These are possibly related to a reduction in physical exercise as discussed in Section 1.3.5.

1.2.3 Inverse relationship between muscle fibre number and size

The principles of muscle fibre growth and development reveal that the number of muscle fibres markedly determines the capacity of postnatal muscle growth. On the other hand, postnatal fibre hypertrophy mainly determines postnatal growth rate but it is limited by physiological and genetic reasons. Therefore, at this point, an interesting phenomenon of muscle growth should be emphasized. Postnatal muscle fibre hypertrophy is inversely correlated with the total number of muscle fibres within a muscle (Rehfeldt *et al.*, 2000). The postnatal growth rate of the individual muscle fibre is lower when there are high numbers of fibres and higher when there are low numbers of fibres. This can be concluded from the fact that muscle fibre number is inversely correlated with muscle fibre thickness as determined at the end of the intensive growth period (Table 1.1). On the other hand, both fibre number and fibre thickness are positively correlated with muscle cross-sectional area as summarized in Fig. 1.4. An explanation for this clear antagonism between fibre thickness and fibre number would be that nutritional energy is distributed evenly among all fibres. However, the correlation coefficient is not -1.0 , which means that some animals exhibit fast-growing fibres despite high fibre numbers.

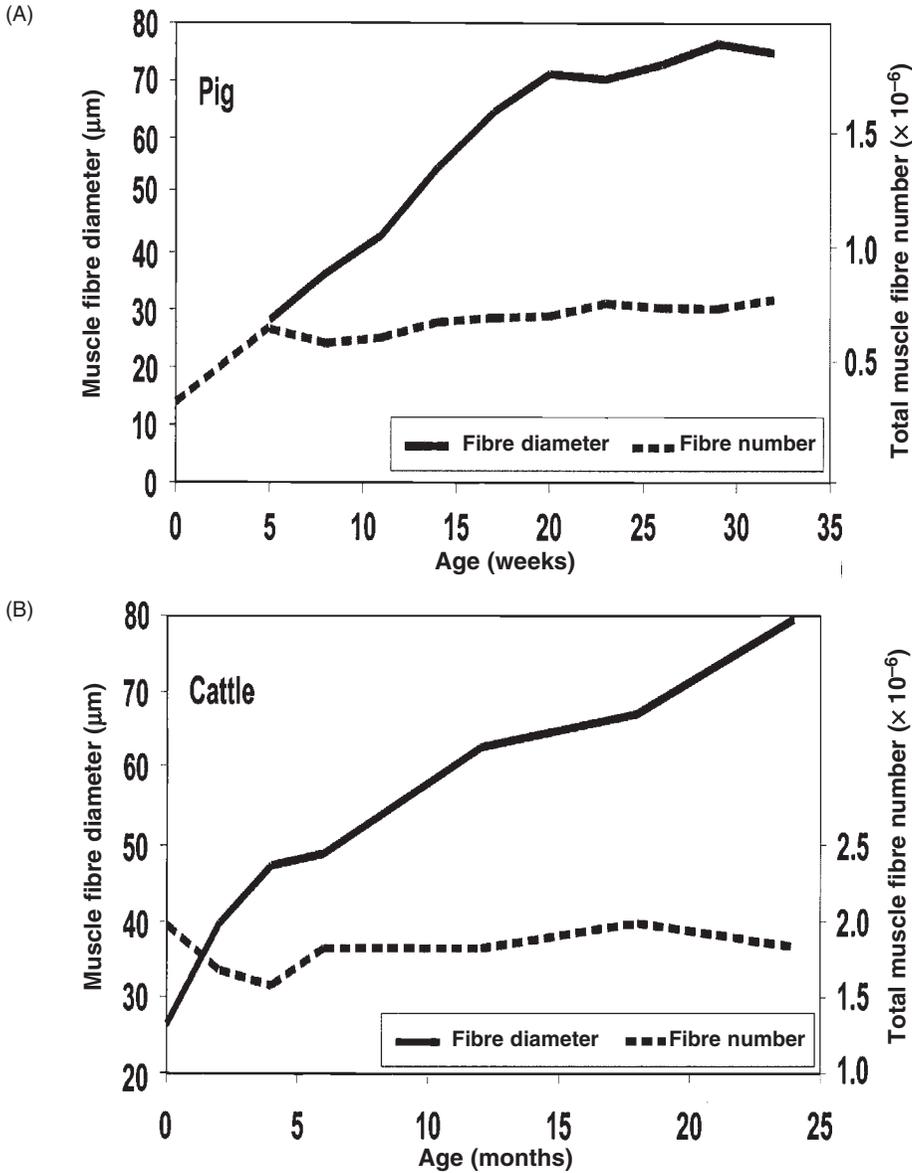


Fig. 1.3. Postnatal development of fibre diameter and total fibre number per muscle cross section in the semitendinosus muscle. (A) German Landrace pigs (Fiedler, 1983; Rehfeldt *et al.*, 1987); (B) Holstein Friesian cattle (Wegner *et al.*, 2000).

1.2.4 Measurement of muscle fibre number and size

Measurements of muscle fibre number and size can be carried out by various techniques. Fibre size is mostly determined by measurements of the diameter or cross-sectional fibre area from histological cross sections and not by measurements of fibre length. For that purpose modern image analysis systems are currently used.

Table 1.1. Phenotypic and genetic correlation coefficients (r_P , r_G) between fibre number, fibre size (area or diameter) and muscle cross-sectional area (MCA).

Trait pair	Species	r_P	r_G
Fibre number/MCA	Mouse	+0.4 / +0.5	+1.1
	Chicken	+0.3	+0.7
	Pig	+0.3 / +0.5	+0.4 / +0.7
Fibre size/MCA	Mouse	+0.6	+0.5
	Chicken	+0.5	+0.7
	Pig	0 / +0.3	0 / +0.5
Fibre number/ fibre size	Mouse	-0.3 / -0.6	-0.4
	Chicken	-0.4	-0.4
	Pig	-0.8	-0.7 / -0.8

Mouse: Rehfeldt *et al.* (1988); pig: Staun (1972), Fiedler *et al.* (1997); chicken: Locrniskar *et al.* (1980).

Incorrect results may arise by obliquely cut fibres, which can be overcome by the measurement of the smallest fibre diameter. In addition, the time of muscle sampling may also significantly influence the results of measurement by the state of fibre contraction (e.g. *rigor mortis* and subsequent relaxation). To obtain comparable results, it is therefore very important to standardize the conditions of muscle sampling.

Determination of muscle fibre number includes a series of technical problems, and differences in the results obtained may depend, at least in part, on the fibre counting technique used. Fibre counting from histological cross sections is still the technique most frequently used (Fiedler and Branscheid, 1998). Either all fibres per muscle cross section are counted (small muscles) or the fibre number per unit area is extrapolated to the whole muscle cross section to determine total fibre number. Inaccuracies arise when muscles are of the multipennate type or when fibres terminate intrafascicularly. The number of fibres has also been calculated by direct counts of individual fibres dissected from nitric acid-treated muscle (Gollnick *et al.*, 1981).

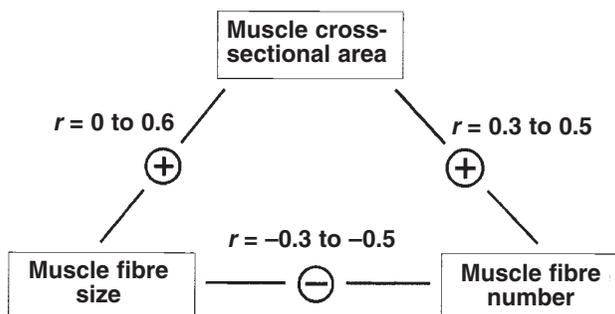


Fig. 1.4. Relationships by linear phenotypic correlation coefficients between muscle cross-sectional area, muscle fibre size (diameter or cross-sectional area) and muscle fibre number per cross section (Rehfeldt *et al.*, 2000).

The nitric acid digestion technique seems to be more accurate if breaks in fibres can be excluded and very thin fibres are not overlooked. Finally, fibre number can be determined by measuring fibre fragments derived from transverse sections by a cell particle analyser (Coulter counter; Thompson *et al.*, 1979), but this method has not been used frequently.

1.2.5 Related regulatory systems

Muscle cell growth and differentiation is under multifactorial control (Brameld *et al.*, 1998; Sabourin and Rudnicki, 2000; Maltin *et al.*, 2001) (see Houba and te Pas, Chapter 10, and Maltin and Plastow, Chapter 13, this volume). At least two families of transcription factors are involved. The muscle regulatory factors (MRF) consisting of a family of nuclear proteins termed MyoD family (MyoD, myf-5, myogenin, MRF-4) and the myocyte enhancer factor 2 (MEF2) have been shown to regulate muscle cell determination and differentiation. Other families of transcription factors, e.g. nuclear factors of activated T cell (NFATs) proteins whose activation is controlled by calcineurin, or cellular oncogenes have also been implicated in muscle development. Moreover, a number of growth factors, metabolic and steroid hormones, and nutrients are involved in controlling the processes of muscle cell proliferation and differentiation (see Dauncey *et al.*, Chapter 5, and Goldspink, Chapter 8, this volume). It has been suggested that nutrients may elicit their effects via changes in the local production of growth factors or changes in growth factor activity.

1.3 Factors Influencing Muscle Fibre Number and Size

Within an individual of the same species, the composition of a muscle is primarily influenced by its type, location and function. In addition, a number of genetic and environmental factors are important in influencing the major structural components of skeletal muscle.

1.3.1 Species

Body size and body weight of vertebrates are known to be remarkably different. Differences in muscle mass are related to muscle fibre number or size. A collection of data showing the longissimus muscle fibre diameters from small and large animals (poultry and mammals) indicates that the differences in body size are not sufficiently reflected by differences in muscle fibre size (Table 1.2). The mean diameters range among the species from about 20 to 80 μm , which is a factor of 4. However, when comparing the body weight between the shrew and the whale, there is a factor of 2.5 million. Interestingly, the largest fibres are not from the whale as the largest mammal, but from the pig. Further examples comparing identical muscles between rabbit, pig and cattle are given in Table 1.3. Pig longissimus muscle contains 6 to 7 times as many fibres as rabbit longissimus but fibre diameters only twice as large. Cattle exhibit 3 to 4 times as many fibre numbers as pigs, but same size or even

Table 1.2. Muscle fibre diameter of longissimus muscle in adults of different species.

Species	Fibre diameter (μm)	References
Chicken	20	Kumar <i>et al.</i> (1976)
Goat	22	Kumar <i>et al.</i> (1976)
Shrew	19	Joubert (1971)
Rabbit	35	Vigneron <i>et al.</i> (1976)
Sheep	25–50	Kumar <i>et al.</i> (1976); Hawkins <i>et al.</i> (1985); Koochmaraie <i>et al.</i> (1995); Carpenter <i>et al.</i> (1996)
Pig (wild)	58–85	Bader (1983); Rahelic and Puac (1981); Müller <i>et al.</i> (2002)
Pig (domestic)	40–80	Staun (1963); Kumar <i>et al.</i> (1976); Fiedler (1983); Rahelic and Puac (1981)
Fallow deer	19	Salomon <i>et al.</i> (1992)
Reindeer	45	Essén-Gustavsson and Rehbindler (1985)
Cattle	55–67	Joubert (1971); Vestergaard <i>et al.</i> (1991); Wegner <i>et al.</i> (2000)
Buffalo	26	Kumar <i>et al.</i> (1976)
Zebu	78	Wegner (personal communication)
Yak	70	Wegner (personal communication)
Elephant	51	Joubert (1971)
Whale	55	Joubert (1971)

smaller muscle fibres. In conclusion, species-specific differences in muscle mass are primarily due to differences in the total number of muscle fibres. Possibly the evolutionary increase in muscle fibre size is limited by physiological reasons in that normal cell function is maintained only as long as a certain limit in cell size is not exceeded.

1.3.2 Gender

Intact males mostly exhibit larger muscle fibres than females or male castrates (Seideman and Crouse, 1986), although for pigs partially larger fibres have been reported for females than entire males (Petersen *et al.*, 1998). Contradictory results have been reported concerning the determination of the number of muscle fibres by gender. A number of differences in the total number of muscle fibres seem to exist

Table 1.3. Muscle fibre number ($\times 10^{-6}$) in muscles of rabbit, cattle and pigs.

Species	Semitendinosus	Longissimus
Rabbit ^a	–	0.09 – 0.17
Pig ^b	0.55 – 0.83	0.66 – 1.09
Cattle ^c	1.70 – 3.36	2.51 – 3.77

^aVigneron *et al.* (1976).

^bStaun (1963); Fiedler (1983).

^cWegner *et al.* (2000).

between female and male muscles in that male muscles exhibit higher fibre numbers than female muscles. Sex-related differences in the number of muscle fibres have been found for mouse rectus femoris (Rehfeldt, unpublished) and rat levator ani (Joubert *et al.*, 1994), cattle longissimus (Papstein *et al.*, 1999) and chicken extensor hallucis longus (Rehfeldt *et al.*, 1997) muscles. Conversely, no sex-related differences were reported for various mouse and rat muscles (Rowe and Goldspink, 1969; Timson, 1982; Rehfeldt *et al.*, 1994), dog pectineus muscle (Ihemelandu, 1980) and pig longissimus muscle (Staun, 1963; Otto and Wegner, 1976; Fiedler *et al.*, 1989; Rehfeldt and Weikard, 1995). Influences of castration have not been considered.

Sex differences in muscle fibre number and size are primarily under the control of testosterone. Testosterone treatment in later postnatal periods is able to stimulate muscle fibre hypertrophy in a direct or indirect manner (Spencer, 1985; Florini, 1987) by stimulating satellite cell proliferation and muscle protein synthesis without increasing fibre number. Differences in muscle fibre number between males and females can arise by hormonal action if differences in androgen hormones are sufficiently high during the period of prenatal fibre formation. Testosterone may also stimulate the longitudinal growth of existing myofibres and in this way increase the fibre number per muscle cross section. Additionally, differences in fibre number and size have been related to differences in physical activity between the sexes.

1.3.3 Selection and breed

Within a species of farm animals, one of the most important factors that influence muscle fibre number and size is selection and breeding with the aim of improving animal performance.

Whether and to what extent a biological trait is inherited and can be changed by selection largely depends on its genetic variability, heritability and genetic correlation to the criteria used in selection.

1.3.3.1 Genetic variability/heritability

The cellular and molecular diversities of mammalian skeletal muscle fibres are the basis for the genetic variability of muscle fibre traits (Pette and Staron, 1990). As shown in Table 1.4, about half to two-thirds of the phenotypic standard deviations in muscle fibre number or muscle fibre size are of genetic origin. This proportion is relatively high as compared with performance traits commonly used in selection of

Table 1.4. Phenotypic and genetic variation coefficients (CV_P , CV_G ; %) of muscle fibre number and size (cross-sectional area or diameter).

Species/muscle	Fibre number		Fibre size	
	CV_P	CV_G	CV_P	CV_G
Mouse/extensor digitorum longus ^a	18.9	9.3	17.8	8.2
Pig/longissimus ^b	25.9	17.1	13.6	7.6

^aRehfeldt *et al.* (1988).

^bFiedler and Dietl (1992).

Table 1.5. Estimates of heritability (h^2) for muscle structure traits.

Species Muscle	Heritability (h^2)		Reference
	Fibre number	Fibre size (area or diameter)	
Mouse			
Extensor digitorum longus	0.23–0.24	0.16–0.21	Rehfeldt <i>et al.</i> (1988)
Soleus	0.44–0.68	0.07	Nimmo <i>et al.</i> (1985)
Chicken			
Pectoralis superficialis	0.12–0.49	0.00–0.26	Locniskar <i>et al.</i> (1980)
Pig			
Longissimus	0.66–0.88	0.17–0.31	Staub (1968)
	0.43–0.48	0.30–0.50	Staub (1972)
	0.28–0.41	0.22–0.34	Fiedler <i>et al.</i> (1991), Dietl <i>et al.</i> (1993)
	0.22	0.34	Larzul <i>et al.</i> (1997)
Cattle			
Longissimus	ND	0.29	Gravert (1963)
	0.35	0.74	Osterc (1974)
	ND	0.39	Andersen <i>et al.</i> (1977)

Ranges arise from the application of different methods of heritability estimation. ND, not determined.

farm animals. Several studies have been conducted to estimate the heritability of muscle fibre traits by use of different procedures. Heritability has been defined as the extent to which individual variation of a population is genetically determined or the extent to which the individual variation within a population is passed on to the next generation (Falconer, 1981). The coefficients of heritability estimated for muscle fibre number range from 0.12 to 0.88, most lying between 0.2 and 0.5 (Table 1.5). These results demonstrate that muscle fibre number is not exclusively determined genetically as has been previously presumed owing to its relative constancy during postnatal life (see Section 1.2.2). Probably, maternal factors (environmental and genetic) are significant determinants of muscle fibre number as the formation of

Table 1.6. Muscle fibre number and muscle fibre cross-sectional area (LSmeans \pm SE) in the semitendinosus muscle of wild type (WP) and domestic pigs (DP^a) at 7 and 20 weeks of age (Rehfeldt *et al.*, 2000).

		7 weeks	20 weeks
Fibre number ($\times 10^{-3}$)	WP	611 \pm 38	554 \pm 29
	DP	908 \pm 54*	860 \pm 54*
Fibre cross-sectional area (μm^2)	WP	407 \pm 36	1440 \pm 136
	DP	1082 \pm 51**	3855 \pm 255**

* $P < 0.01$, ** $P < 0.001$ for differences between DP and WP.

^aDP are German Landrace.

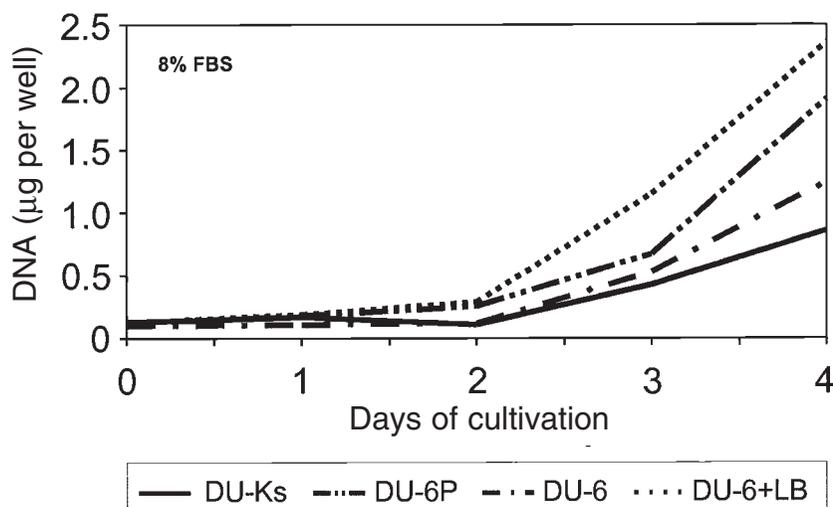


Fig. 1.5. DNA accumulation in cultures of satellite cells isolated from mouse lines selected for 6 week protein accretion (DU-6P), body weight (DU-6) or an index from body weight and treadmill performance (DU-6+LB) over 70 generations, and from a control (DU-Ks). FBS: fetal bovine serum (Rehfeldt *et al.*, 2000, 2002). Differences between the lines at day 4 of cultivation are (DU6+LB = DU-6P) > DU-6 > DU-Ks ($P < 0.05$).

fibres occurs prenatally. However, there is a lack of studies on maternal effects on fibre number and size in farm animals. Heritability estimates for muscle fibre size range from low to high, but most lie between 0.2 and 0.3.

1.3.3.2 Selection

Differences in muscle mass obtained by breeding and selection result from changes in both muscle fibre number and muscle fibre size. This can be concluded from a series of selection experiments for large body size or rapid growth rate with several species, including the mouse (Luff and Goldspink, 1967; Penney *et al.*, 1983; Rehfeldt and Büniger, 1990; Brown and Stickland, 1994), pig (Wicke, 1989; Brocks *et al.*, 1998), chicken (Aberle and Stewart, 1983; Remignon *et al.*, 1994), quail (Fowler *et al.*, 1980) and turkey (Cherel *et al.*, 1994). The results of long-term selection are most obvious when comparing wild and domestic types of the same species (Bader, 1983; Weiler *et al.*, 1995; Müller *et al.*, 2002). As an example, the European domestic pig, which was derived from the European wild pig, exhibits larger fibres but also higher numbers of fibres (e.g. semitendinosus muscle, Table 1.6).

Growth selection leads to increases in myoblast and/or satellite cell proliferation rates as indicated by higher myonuclear numbers, higher DNA synthesis rate and higher total muscle DNA content (Fowler *et al.*, 1980; Penney *et al.*, 1983; Jones *et al.*, 1986; Brown and Stickland, 1994; Mitchell and Burke, 1995). This has also been demonstrated by the *in vitro* growth of satellite cells derived from growth-selected lines of mice (Rehfeldt *et al.*, 2000; Fig. 1.5), chickens (Ridpath *et al.*, 1984; Duclos *et al.*, 1996) and turkeys (Merly *et al.*, 1998). According to the principles of skeletal muscle growth, higher proliferation rates contribute to the formation of higher muscle fibre numbers and, postnatally, to the accumulation of more myofibre nuclei. There is some evidence that selection for growth or body weight mainly stimulates

Table 1.7. Muscle fibre number and diameter (mean \pm sd) in the longissimus muscle of different pig breeds (Fiedler *et al.*, 1989; Kuhn *et al.*, 1998).

Pig breed (n)	Fibre number ($\times 10^{-6}$)	Fibre diameter (μm)
German Landrace (694)	1.041 \pm 0.280	68.9 \pm 9.5
German Large White (137)	1.016 \pm 0.251	70.0 \pm 8.4
Leicoma (1052)	1.061 \pm 0.275	68.6 \pm 9.4
Schwerfurter (77)	1.109 \pm 0.309	68.9 \pm 10.7
Pi�train (26)	1.107 \pm 0.178	71.3 \pm 8.8
Saddle Back (17)	0.909 \pm 0.178	67.1 \pm 7.8

myoblast proliferation and muscle fibre formation without a change in muscle DNA:protein ratio (Penney *et al.*, 1983). In contrast, for modern meat-type chickens (Jones *et al.*, 1986; Mitchell and Burke, 1995) and pigs selected for meat content (N stvold *et al.*, 1979) and mice selected long-term for protein content (Rehfeldt and B nger, 1990), decreased muscle DNA:protein or nuclear:cytoplasm ratios have been reported. Although there is a lack of sufficient information, these changes seem to result from modifications both in the hormonal system and in intrinsic properties of the muscle cells themselves.

1.3.3.3 Breed

The influence of growth selection on muscle fibre number or size is also apparent from differences between animals of different breeds. Clear differences in muscle fibre number, but not in muscle fibre thickness, were reported between Large White and Miniature pigs of the same age (Stickland and Handel, 1986). No marked differences in muscle fibre number and size of the longissimus muscle were apparent between different modern meat-type pig breeds and crosses in contrast to the ‘older’ fatty Saddle Back breed, which has a lower fibre number and size (Table 1.7). When comparing the same muscle of several European pig breeds 40 years ago, Staun (1963) found differences in muscle fibre size and number. Possibly, in some modern meat-type pig breeds such as Pi train, fibre number and size are at the limits of their correlated responses to selection for leanness, and new strategies must be applied to attain further changes.

Table 1.8. Muscle fibre number and size (LSmean \pm sd) in semitendinosus muscle of bulls of various cattle breeds kept under almost identical conditions (Wegner *et al.*, 2000).

Cattle breed	6 months		18 months	
	Fibre number ($\times 10^{-6}$)	Fibre size (μm^2)	Fibre number ($\times 10^{-6}$)	Fibre size (μm^2)
Galloway	1.89 \pm 0.24 ^a	2244 \pm 381	1.73 \pm 0.40 ^a	5005 \pm 756 ^a
German Angus	1.66 \pm 0.35 ^a	1682 \pm 259	1.84 \pm 0.29 ^a	4272 \pm 763 ^b
Holstein Friesian	1.82 \pm 0.52 ^a	1888 \pm 415	2.00 \pm 0.41 ^a	3562 \pm 611 ^c
Belgian Blue	3.06 \pm 0.83 ^b	1963 \pm 521	3.46 \pm 0.67 ^b	5179 \pm 1125 ^a

Significant differences between breeds are indicated by different letters ($P < 0.05$).

There are no obvious differences in muscle fibre number in most of the cattle breeds; meat-type cattle in part tend to have higher fibre numbers (Osterc and Zagozen, 1977; Wegner *et al.*, 2000; Table 1.8) Differences in fibre size have been found at 18 months of age; Holstein Friesian (dairy-type) bulls exhibit smaller fibres compared with the German Angus (beef-type) and Galloway (hardy type). An exception, however, are double-muscled cattle with about double the number of muscle fibres and somewhat larger fibres. The phenomenon ‘double-muscling’ is related to mutations in the myostatin gene, which is explained in more detail in Section 1.4.3 (see Kambadur *et al.*, Chapter 14, this volume).

In sheep, differences were found in the microstructure of the longissimus muscle in crossbreds between two wild-type crossbreds and crosses between wild-type (Pramenka and Sardinian) and domestic breeds such as Merino-Württemberg (W) and East Friesian (EF) (Gjurcevic-Kantura *et al.*, 2000). Fibres with small diameters were prevalent in muscles of the W and EF crossbreds, having higher body weight and meatiness than the wild-type crossbreds. However, no differences in the average fibre diameter were found. This is indicative of greater total fibre number in the W and EF crossbreds that was not analysed in this study. Vigneron *et al.* (1984) compared fibre size and total fibre number of two muscles of female pure Merino breed and Berrychon du Cher × Merino crossbreds. No differences were found in total fibre number between the genotypes, but the crossbreds had larger fibres in the sternohyoideus muscle, which was accompanied by higher live weight at slaughter and increased muscle weight compared with the pure Merino breed.

1.3.4 Nutrition

1.3.4.1 Prenatal nutrition

Adequate nutrition is essential for normal skeletal muscle development. Prenatal undernutrition in pigs has been shown to be related to lower muscle fibre numbers and myonuclear numbers or DNA content as evidenced by the comparison of small with large weight piglets (Wigmore and Stickland, 1983a,b; Handel and Stickland, 1987). Also, experimentally induced undernutrition during pregnancy has been reported to decrease muscle fibre number and/or myonuclear number in rats (Glore and Layman, 1983), in guinea pigs (Dwyer *et al.*, 1995) and in pigs (Buitrago *et al.*, 1974). The lasting negative effect of prenatal undernutrition on postnatal growth is commonly recognized (Pond *et al.*, 1985). In contrast, doubling of maternal feed intake of sows during gestation tended to increase semitendinosus muscle fibre number and lead to narrowing in the distribution of fibre number (Dwyer *et al.*, 1994). A dramatic increase in the nutrient availability to the embryos, which has been achieved by growth hormone treatment of sows during early pregnancy, was followed by significant increases in muscle fibre numbers in low and middle weight littermates (Rehfeldt *et al.*, 1993, 2001). Mechanisms responsible for the influence of maternal nutrition on fetal myogenesis may involve alteration in insulin-like growth factor levels or in placental morphology and efficiency. The influence of prenatal nutrition is discussed in more detail in Stickland *et al.* (Chapter 3, this volume).

1.3.4.2 Postnatal nutrition

It is well known that malnutrition during postnatal growth reduces body weight including skeletal muscle weight. Feed restriction both in quantity and in quality

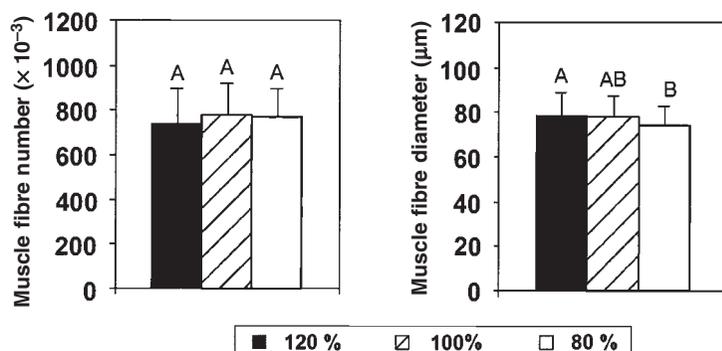


Fig. 1.6. Muscle fibre diameter and fibre number in pig longissimus muscle of a German Landrace crossbred ($n = 15$ per group) fed at different levels of energy at 32 weeks of age (Fiedler, 1985). Different letters indicate significant differences ($P < 0.05$).

(protein and energy deficient diets) has been reported to lead to decreases in muscle fibre diameters in various rodents (Hansen-Smith *et al.*, 1978; Goldspink and Ward, 1979; Hegarty and Kim, 1981; Rehfeldt *et al.*, 1991), pig (Staun, 1972; Stickland *et al.*, 1975), sheep (Joubert, 1956), cattle (Osterc and Zagozen, 1977; Beerwinkle *et al.*, 1979), rhesus monkey (Deo *et al.*, 1965) and humans (Cheek *et al.*, 1970) (see Dingboom and Weijjs, Chapter 4, this volume). The decreases in muscle fibre size are accompanied by a reduction in muscle nuclear number or DNA content but with increased DNA/protein or nuclear cytoplasm ratios (Stickland *et al.*, 1975; Tanaka *et al.*, 1992; Schadereit *et al.*, 1995).

In contrast to muscle fibre size, fibre numbers have been reported not to be influenced by postnatal malnutrition in post-weaning mice (Goldspink and Ward, 1979; Rehfeldt *et al.*, 1991) and rats (Bedi *et al.*, 1982; Tanaka *et al.*, 1992; Schadereit *et al.*, 1995), in pigs (Staun, 1972; Stickland *et al.*, 1975), cattle (Osterc and Zagozen, 1977) and humans (Cheek *et al.*, 1970). However, decreases in muscle fibre number have been reported in rodents in response to undernutrition during lactation or to temporary postnatal starvation (Layman *et al.*, 1981; Bedi *et al.*, 1982). Examples of nutritional influences on muscle fibre size and/or number for pigs and cattle are given in Figs 1.6 and 1.7.

In summary, whether postnatal malnutrition is able to induce muscle fibre loss seems to depend both on the intensity and on the time period (developmental stage and duration) of dietary restriction. Only severe restriction (starvation) seems to cause fibre loss, whereas moderate undernutrition exclusively affects fibre hypertrophy by means of reduced nuclear and protein accumulation. However, it seems to be important whether the lactational period is included into longer periods of undernutrition. Probably, the elongation of existing myotubes which leads to an increase of apparent fibre number during early postnatal growth cannot be completed when the animals are undernourished during early postnatal life. From studies with fetal sheep it has been concluded that lower fibre numbers will result when the longitudinal growth of intrafascicularly terminating fibres is inhibited by undernutrition during late gestation (Swatland and Cassens, 1973). The prenatal period of muscle development is much more sensitive to nutritional deficiencies, because this period includes muscle fibre formation. Maternal nutrition and/or nutrient availability are suggested to be key factors in the regulation of myogenesis.

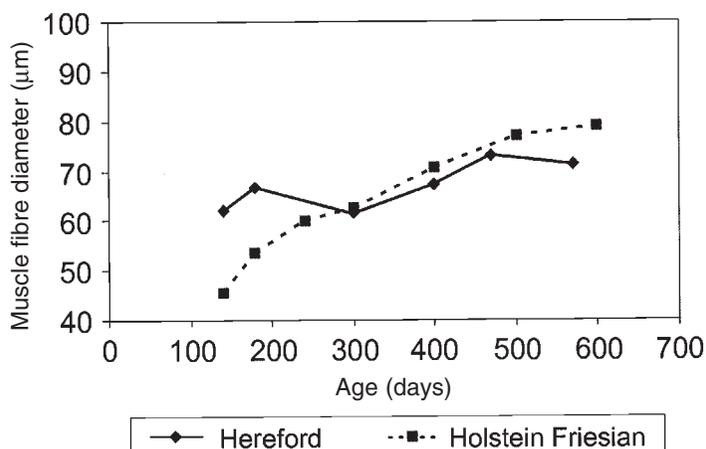


Fig. 1.7. Growth of muscle fibres as measured in biopsy samples from semitendinosus muscle of Hereford and Holstein Friesian (HF) bulls under extensive (low) and intensive (high) feeding conditions, respectively (Wegner and Matthes, 1994; Wegner, unpublished). In Hereford cattle, which were kept on pasture, fibre growth was almost stagnating after weaning (at 180 days of age) and during winter season (180 to 400 days of age; $P > 0.05$ for differences), whereas the muscle fibres of HF bulls were continuously growing in size ($P < 0.001$).

1.3.5 Physical activity

It seems to be important to what extent environmental conditions allow or force physical exercise, because the individual state of physical activity has to be considered as a relevant determinant of skeletal muscle structure. Physical activity is an important factor that influences adaptations in muscle. Different housing systems allow different degrees of physical activity for farm animals; however, targeted exercise programmes are not commonly applied in animal production.

Studies on the effects of exercise have been mostly carried out with laboratory animals such as rodents and quails. Activity-induced muscle growth has been reported to be accompanied by changes in muscle fibre size and number. Daily training for endurance running on a treadmill has been shown to increase fibre number in the cross section of plantaris muscle in guinea pigs (Faulkner *et al.*, 1972) or of rectus femoris muscle in mice (Rehfeldt and Bunger, 1983). On the other hand, it has long been recognized that adult skeletal muscle can achieve tremendous increases in size and strength in response to weight-lifting exercise. Several studies have suggested that compensatory hypertrophy (Rowe and Goldspink, 1968), tonic stretching (Sola *et al.*, 1973) and muscle growth induced by weight-lifting exercise (Gonyea *et al.*, 1986) may be the result of hypertrophy of existing fibres and/or an increase in fibre number. Other studies have demonstrated that increased muscle mass results primarily from an enlargement of muscle fibres without increases in their number (Gollnick *et al.*, 1981). Although for different models of activity-induced muscle growth there is evidence of muscle fibre hyperplasia and hypertrophy or of hypertrophy alone, clearly, skeletal muscle fibres appear to possess the appropriate mechanisms for both responses.

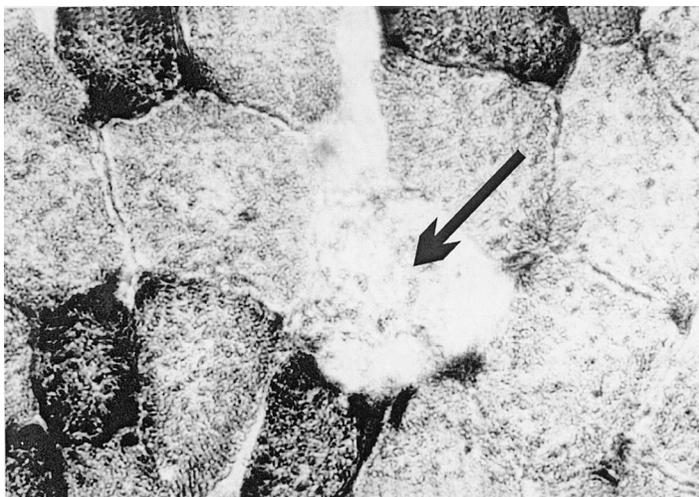


Fig. 1.8. Signs of muscle fibre degeneration in rectus femoris muscle obtained from untrained laboratory mice after running to exhaustion on a treadmill (NADH tetrazolium reductase staining) (Rehfeldt *et al.*, 1999).

Exercise-induced increases in fibre number are obviously different from the regular postnatal pattern of muscle growth under normal loading conditions in mammals and birds (see Section 1.2.2). What are the mechanisms that could result in exercise-induced increases in muscle fibre number? Severe overloading seems to induce fibre splitting (Hall-Craggs and Lawrence, 1970) but this seems to be of minor importance. On the other hand, muscle fibre degeneration, necrosis, apoptosis and loss of fibres (Schumann, 1972; Lexell *et al.*, 1992; Jacobs *et al.*, 1995) have been observed in response to overloading (Fig. 1.8). Eccentric running, flying or weight overloading produces focal injury of myofibres at multiple levels in the muscle. The damage is expressed by alterations of the morphological structure of the muscle and by an increase of muscle-specific proteins in the blood. Satellite cells are activated to proliferate and form new myotubes that regenerate the muscle. It is assumed that the leakage of mitogens contributes to satellite cell stimulation. By the immunohistochemical detection of nascent fibres in response to overload degeneration or experimentally induced myonecrosis (Lexell *et al.*, 1992) it has been shown that lost fibres can be effectively replaced. Figure 1.9 summarizes the events in activity-induced changes in fibre number and fibre size by a simplified hypothetical model. A more detailed model has been presented previously by Taylor and Wilkinson (1986).

Further evidence for the importance of physical activity on fibre hyperplasia and fibre hypertrophy is demonstrated by immobilization and denervation as experimental negative models in contrast to activity-induced growth. Immobilization results in a decrease in muscle mass, which is accompanied by decreases in muscle fibre cross-sectional area (Young *et al.*, 1982) and fibre number (Booth and Kelso, 1973); the latter is the result of focal degeneration of cell ultrastructure (Guba *et al.*, 1977).

There are only a few studies on pigs on the influence of physical activity on skeletal muscle by exercise or by comparisons between indoor and outdoor rearing systems. Outdoor rearing, which allows more physical activity, increases the aerobic

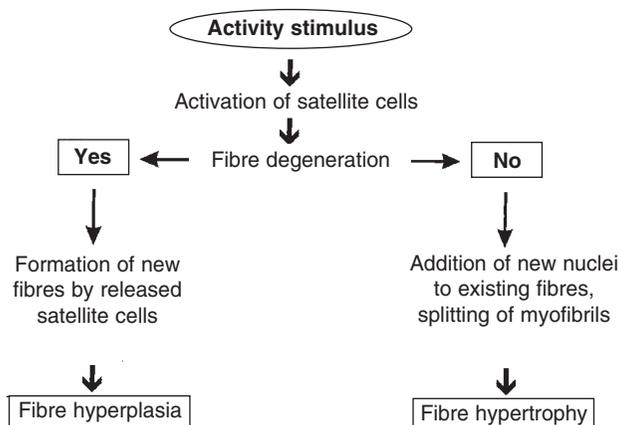


Fig. 1.9. Hypothetical model for activity-induced increases in muscle fibre number or muscle fibre size (Rehfeldt *et al.*, 1999).

capacity of the muscle and, in some muscles, leads to decreased muscle fibre diameters of type I and/or IIA (Bee, 2002; Gentry *et al.*, 2002) or increased type I diameters (Petersen *et al.*, 1998) (for details about muscle fibre types see Reggiani and Mascarello, Chapter 2, this volume). In other studies (Szentkuti and Schlegel, 1985) the fibre area ratio type II/I was reduced by exercise and increased by restricted movement in pigs. No differences in fibre diameter were found in semitendinosus muscle after 3 weeks of endurance exercise (Salomon *et al.*, 1984), whereas 10 weeks of exercise training could increase fibre size but did not affect the same muscles (Petersen *et al.*, 1998). Wiklund *et al.* (1998) have reported a trend towards increasing fibre area of type I and IIB fibres in selected muscles of cows grazing on pasture compared with cows kept tied in a stable. Essén-Gustavsson (1993) suggested that an adaptation as a result of physical activity is only seen in those muscles that are involved during work and in those fibres that are recruited. Nevertheless, exercise-training studies seem to be unsuitable for predicting effects of spontaneous physical activity occurring in outdoor rearing systems. No results have been reported on the influence of physical activity on the number of muscle fibres in pig or bovine muscle.

In summary, the occurrence of muscle fibre hyperplasia and hypertrophy differs amongst experimental models of activity-induced growth. Whether or not fibre hyperplasia occurs may result from differences in the magnitude and duration of the stimulus on the one hand and in the capacity of the fibres to adapt on the other hand. In contrast to activity-induced muscle growth, chronic disuse of muscle causes muscle atrophy associated with reductions in fibre size and number. In the pig, increased physical activity has been reported to be associated with both decreases and increases in fibre size; information on fibre number is not available.

1.3.6 Growth-promoting agents

Growth is known to be regulated substantially by the neuro-endocrine system as well as paracrine and autocrine actions of hormones and growth factors, and the influence of environmental stimuli on the individual is widely mediated by the induction

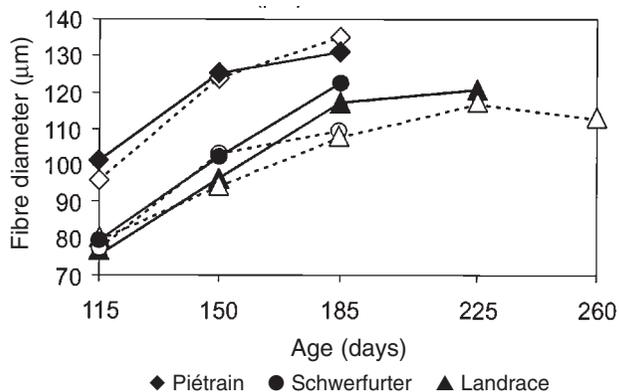


Fig. 1.10. Growth of longissimus muscle fibres and its response to long-term treatment with 4 mg/day porcine somatotrophin (pST) in various pig breeds (Rehfeldt and Ender, 1995; Rehfeldt *et al.*, 1996). Schwerfurter and Landrace pigs are castrates only, whereas Piétrain pigs include females and castrates with $P > 0.10$ for the influence of sex. Open symbols represent control pigs; filled symbols represent pST-treated pigs.

of hormonal actions. Based on this knowledge, growth-promoting agents that can influence muscle growth in farm animals have been derived. In particular, the effects of growth hormone (GH), β -agonists and steroids have been studied.

1.3.6.1 Growth hormone

Postnatal application of GH increases lean growth and decreases fat deposition (Beermann and DeVol, 1991; Etherton and Bauman, 1998). It stimulates muscle fibre hypertrophy as has been shown in pigs and rats (Ullman and Oldfors, 1989; Beermann *et al.*, 1990; Solomon *et al.*, 1990; Lefaucheur *et al.*, 1992; Rehfeldt and Ender, 1993). The response to growth hormone in pigs largely depends on the dosage, sex and breed. An example is given in Fig. 1.10. In cattle, the stimulating response in fibre size was largely dependent on muscle and fibre type (Maltin *et al.*, 1990; Vestergaard *et al.*, 1995; Ono *et al.*, 1996; Vann *et al.*, 2001). Mainly acting via IGF-I, GH is able to stimulate both satellite cell proliferation and protein synthesis in muscle. Little information is available about postnatal GH action on muscle fibre number. In two experiments with pigs it was found that porcine somatotrophin (pST) treatment did not significantly change the apparent muscle fibre number in longissimus muscle calculated from fibre number per unit area and muscle cross-sectional area (Rehfeldt and Ender, 1993). Nevertheless, there is some evidence that GH contributes to new fibre formation by stimulation of satellite cell proliferation in regenerating muscle (Ullman and Oldfors, 1989).

1.3.6.2 β -agonists

Growth-promoting effects on muscle by stimulating fibre growth are also known to be exerted by β -adrenergic agonists in various species (Maltin *et al.*, 1986, 1990; Beermann *et al.*, 1987; Sainz *et al.*, 1993; Rehfeldt *et al.*, 1997). As for GH, β -adrenergic agonists are able to increase lean growth and to decrease fat deposition in cattle, sheep, pig and poultry (Hanrahan, 1987; Moloney *et al.*, 1991). Muscle fibre hypertrophy seems to be achieved mainly by reduction of proteolytic activity after

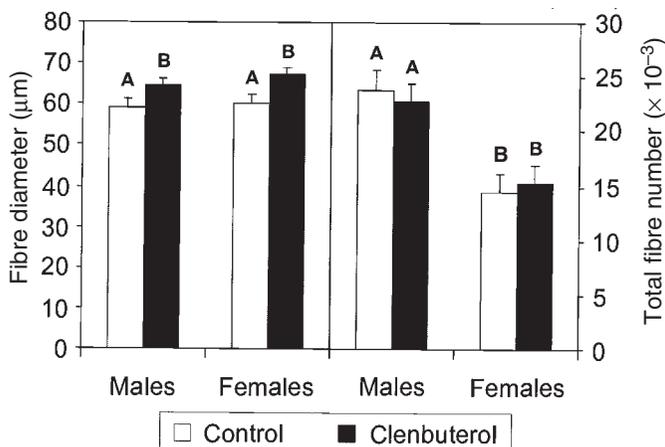


Fig. 1.11. Responses of fibre diameter and fibre number of extensor hallucis longus muscle to the β -adrenergic agonist clenbuterol (1 mg/kg feed from day 28 to 49) in broiler chickens (Rehfeldt *et al.*, 1997). Different letters indicate significant differences ($P < 0.05$).

long-term application. There is also some evidence for increases in protein synthesis as a short-term response to β -adrenergic agonists. In contrast to GH, most of the β -adrenergic agonists do not stimulate satellite cell proliferation. Consistent with GH action, the number of muscle fibres was not increased in response to clenbuterol in rats (Maltin *et al.*, 1986; Rehfeldt *et al.*, 1994) and in broiler chickens (Rehfeldt *et al.*, 1997). An example for clenbuterol action on fibre number and fibre size in broiler chickens is given in Fig. 1.11.

1.3.6.3 Oestrogens and androgens

These steroids are anabolic hormones whose growth-promoting effects are well known in cattle, whereas limited effects are known from swine (Hancock *et al.*, 1991). They have been widely used over years in the USA to improve the efficiency and product quality of meat animals. However, studies on the effects of steroids on the micro-morphology of muscle are rare. Despite increased lean accretion, increases in muscle fibre girth per each of three types have been found in only one of six muscles after combined administration of oestradiol and progesterone to beef steers (Ono *et al.*, 1996). Another study reported only increases in slow-twitch fibre area in muscle of steroid-implanted steers (Clancy *et al.*, 1986). Although average fibre diameters were not reported, the increase in muscle mass by steroids seemed to result mainly from transformation of small size fibre types to larger size fibre types and therefore increases in average fibre size. Changes in fibre number in response to steroid treatment have not been reported.

In conclusion, the hypertrophic response of postnatal muscle to growth hormone, β -agonists or steroids is based on muscle fibre hypertrophy, but not on muscle fibre multiplication. This mechanism represents the common growth pattern of healthy postnatal muscle under normal requirements. Recently, several attempts have been made to influence prenatal growth by different growth promoters. The available results are presented and discussed in Stickland *et al.* (Chapter 3, this volume).

1.4. Significance of Muscle Fibre Number and Size for Animal Performance

1.4.1 Lean growth

From the principles of skeletal muscle growth, it becomes clear that lean growth depends on the number of the prenatally formed fibres and on the degree of their postnatal hypertrophy. This has been confirmed by significant positive phenotypic and genetic correlation coefficients of muscle mass or lean meat percentage with both fibre number and size (Staun, 1972; Dietl *et al.*, 1993; Henckel *et al.*, 1997; Larzul *et al.*, 1997). An example is given in Table 1.9. Even in fish, it has been shown that fish with more muscle fibres at hatching grow better in the initial posthatch period than fish with fewer muscle fibres (Nathanailides *et al.*, 1995). On the other hand, it seems to be important to what extent each of the two fibre characteristics contribute to lean growth. The potential for lean growth of an animal largely depends on the number of the prenatally formed muscle fibres, because the postnatal increase in muscle fibre size is limited by genetic and physiological reasons. The latter is supported by the following results. A 15-week treatment with porcine somatotrophin, which repartitions nutrients to muscle, was able to accelerate fibre growth in Landrace pigs, but the ultimate fibre size did not exceed that of the control pigs attained 5 weeks later (Fig. 1.10). In Piétraains, representing the pig breed with the largest muscle fibres, exogenous pST was not capable of increasing fibre size at all. However, pigs are mostly slaughtered before their potential for lean growth is exhausted, so that in most cases the rate of lean growth seems to be of greater interest than the potential of lean growth. Lean growth rate in turn depends mainly on the rate of muscle fibre hypertrophy ($r_G = 0.47$) and not on fibre number ($r_G = 0.08$) as shown by Larzul *et al.* (1997).

Although within-breed correlation coefficients are mostly insignificant, there is some evidence that pigs with more muscle fibres exhibit less fat (Stickland and Goldspink, 1975; Kuhn *et al.*, 1998). This may be related to the fact that the plateau of muscle fibre growth is achieved earlier at lower fibre numbers, and that afterwards

Table 1.9. Genetic correlation coefficients ($r_G \pm SE$) of longissimus muscle fibre number and fibre diameter with traits of growth and pork quality estimated from data of half- and full-sib groups ($n = 1997$) from 514 sires and 1078 dams of four pig genotypes (Rehfeldt *et al.*, 2000; Fiedler *et al.*, unpublished).

Trait	Fibre number	Fibre diameter	Percentage of giant fibres
Average daily gain ^a (g/day)	0.46 ± 0.15	0.03 ± 0.19	0.47 ± 0.18
Backfat thickness (mm)	-0.05 ± 0.11	-0.12 ± 0.18	0.24 ± 0.23
Lean meat content (%)	0.38 ± 0.12	0.52 ± 0.10	0.06 ± 0.00
Drip loss (%)	-0.05 ± 0.19	0.64 ± 0.25	0.77 ± 0.17
Reflectance (%)	-0.05 ± 0.14	0.32 ± 0.14	0.79 ± 0.11
pH 45 min post mortem	0.13 ± 0.14	-0.37 ± 0.19	-0.78 ± 0.11

^aRelated to carcass weight.

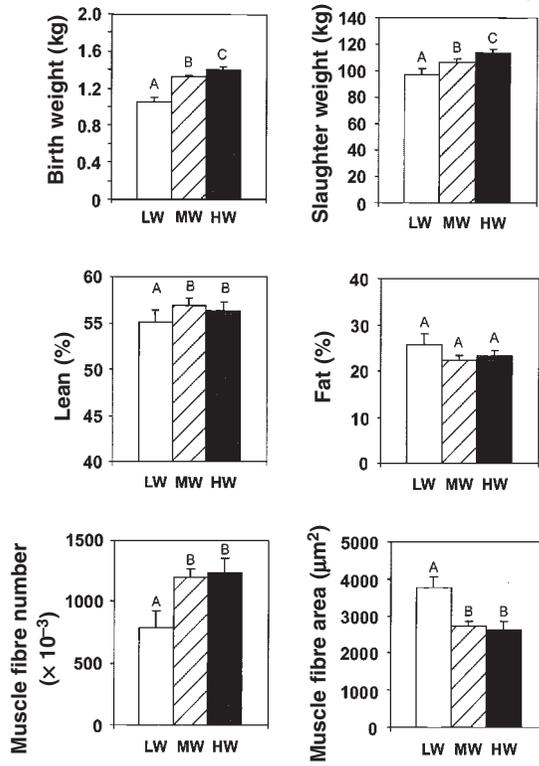


Fig. 1.12. Characteristics of growth performance and associated longissimus muscle fibre number and size as followed up to slaughter by different birth weight classes as low (LW), middle (MW) and heavy (HW) weight in German Landrace pigs (Kuhn *et al.*, 2002). Different letters indicate significant differences ($P < 0.05$).

available nutrients are preferentially used for fat deposition. In a recent study (Kuhn *et al.*, 2002) that followed piglets of three different birth weight classes until slaughter, these relationships become more obvious. Piglets of low birth weight, which exhibit a low number of muscle fibres (Handel and Stickland, 1987; Rehfeldt *et al.*, 2001), accrete relatively more fat and less meat during postnatal growth when compared with their littermates of middle and high birth weight despite *ad libitum* feeding (Fig. 1.12). Obviously, the pigs of low birth weight and low fibre number have attained their limit in individual fibre growth much earlier as seen by the extremely large fibre cross-sectional areas. This may also explain why the highest lean meat percentage cannot be expected at the maximum fibre size and why muscle fibre number or size alone are not more closely correlated with lean growth or fat deposition. As seen by the contour plots derived from 260 Landrace pigs at slaughter (Fig. 1.13), extreme meat percentages can be associated with very high fibre number at low fibre size and vice versa. However, there is a third group of pigs that realizes a very high meat percentage by moderate fibre number and moderate fibre size. The relationships discussed above may further explain why the correlations of fibre size and number with average daily gain, which includes gains in lean *and* fat mass, are in part contradictory shown by coefficients ranging from $r = -0.49$ to $+0.46$ for fibre

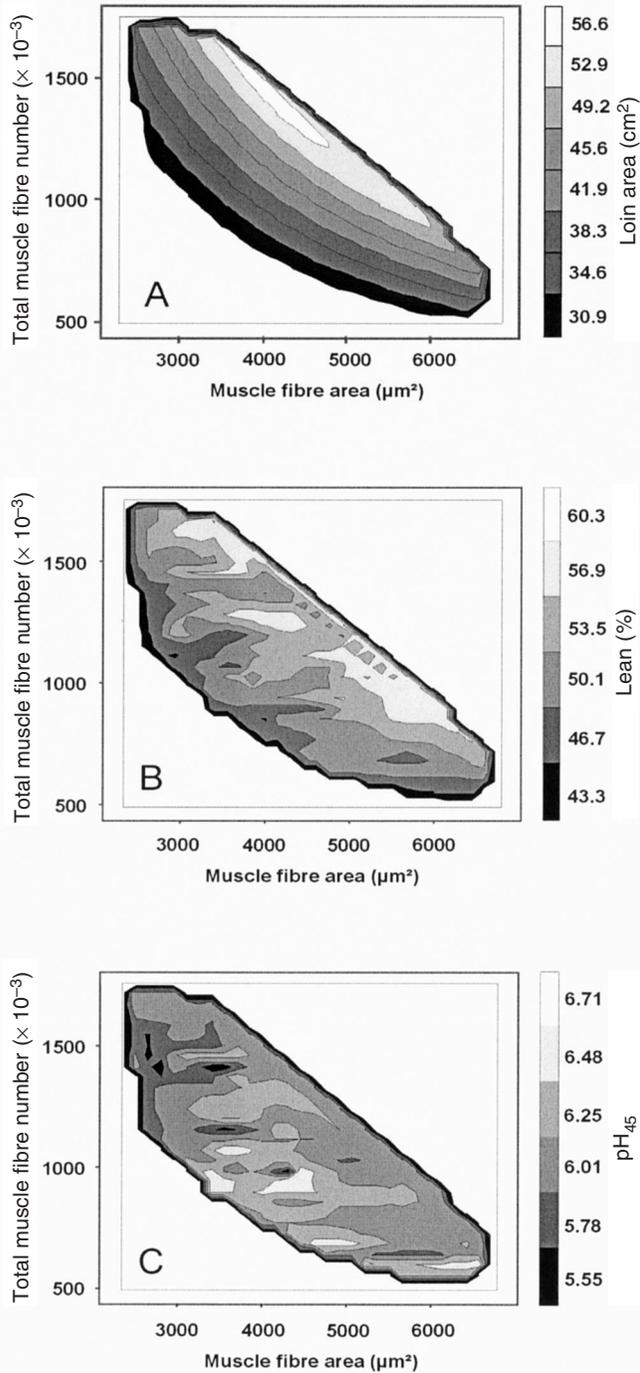


Fig. 1.13. Contour plots on the relationship between longissimus muscle fibre number and fibre area showing different levels of performance traits such as loin area (A), lean percentage (B) and pH_{45} value (C) in 260 Landrace pigs slaughtered at 105 kg.

number and from $r = 0.03$ to 0.74 for fibre size (Staun, 1972; Dietl *et al.*, 1993; Dwyer *et al.*, 1993; Larzul *et al.*, 1997).

1.4.2 Meat quality/stress susceptibility

It was discussed above that low muscle fibre number correlates with fibres that exhibit greater hypertrophy. However, strong fibre hypertrophy seems to reduce the capacity of the fibres to adapt to activity-induced demands, which in turn may be associated with stress susceptibility and poor meat quality in modern meat-type pig and poultry breeds (Klosowska *et al.*, 1979; Sosnicki *et al.*, 1989; Wegner and Ender, 1990; Henckel *et al.*, 1997; Fiedler *et al.*, 1999). This has been shown by low to moderate phenotypic and genetic correlation coefficients between fibre size and various meat quality characteristics, such as drip loss, lightness and pH value (Table 1.9). In most cases, the genetic correlations are numerically higher than the phenotypic ones. The genetic correlation between fibre number and meat quality is less clear, but the individual coefficients bear the opposite sign, which appears logically consistent because of the antagonistic relationship of fibre number and size.

Different reasons for the detrimental influence of extreme fibre hypertrophy are under discussion. Energy and oxygen supply are reduced with increasing fibre size due to lowered capillary density (Cassens and Cooper, 1971; Fiedler *et al.*, 1993; Henckel *et al.*, 1997), and nuclear control of cellular processes may be impaired in large fibres, which often exhibit enlarged nuclear domains as indicated by low nuclear:cytoplasm ratios (Cheek *et al.*, 1970; Rehfeldt and Ender, 1995). Particularly in modern meat-type pigs and poultry, larger fibres tend to have lower numbers of mitochondria, and they belong to the white, fast-contracting type. In pigs, higher white fibre percentages have been shown to correlate with the PSE (pale, soft, exudative) meat condition (Linke, 1972; Larzul *et al.*, 1997; Fiedler *et al.*, 1999) and with stress susceptibility (Nelson and Schochet, 1982; Fiedler *et al.*, 1993, 1999). These fibres produce energy for contraction mainly by the glycolytic pathway and, under energy-demanding conditions (before slaughter), their metabolism contributes to a very fast pH decline by over-production of lactate, which cannot be removed, and subsequent denaturation of proteins. This in turn is related to the PSE condition after slaughter. From other reports (Maltin *et al.*, 1997) it may further be suggested that strong muscle fibre hypertrophy also contributes to poor tenderness of pig muscle.

When the relationship between muscle fibre number and pH value of pig longissimus muscle and meat percentage was investigated, it was found that there is a range of optimum muscle fibre number which guarantees both high meat percentage and good meat quality at a moderate fibre size (Lengerken *et al.*, 1997). This 'optimum hypothesis' is also supported by the contour plots of Fig. 1.13. First, from all graphs the inverse relationship between fibre number and size (see also Section 1.2.3) becomes clearly apparent. Secondly, the largest loin area is seen at an optimum balance of fibre size and fibre number. Thirdly, high drip losses or low pH values are apparent at both extreme fibre size *and* extreme fibre number, whereas lower drip losses and higher pH values are found at moderate fibre number and moderate fibre size. Pigs that realize their high meat percentage by this balanced fibre composition exhibit the better meat quality. These relationships may be the

Table 1.10. Meat characteristics (mean \pm SD) dependent on the total muscle fibre number of longissimus muscle in Piétrain pigs (Rehfeldt *et al.*, 2000).

	Class of muscle fibre number ($\times 10^{-3}$)		
	Low 800 to 1000	Middle >1000 to 1200	High >1200 to 1600
Number of animals	9	9	8
Total fibre number ($\times 10^{-3}$)	908 \pm 56	1112 \pm 57	1325 \pm 110
Fibre diameter (μm)	86.0 \pm 8.2	77.5 \pm 8.6	67.1 \pm 5.4
Lean meat (%)	60.0 \pm 2.3	59.8 \pm 2.4	59.1 \pm 3.4
Loin muscle area (cm^2)	54.9 \pm 2.0	57.2 \pm 6.0	58.2 \pm 7.5
pH 45 min post mortem	5.95 \pm 0.36	6.01 \pm 0.44	6.20 \pm 0.39
Reflectance (%)	48 \pm 3	49 \pm 4	46 \pm 3
Drip loss (%)	4.01 \pm 2.21	4.39 \pm 2.85	2.91 \pm 1.45

Halothane status as the number of homozygous negative/homozygous positive pigs: low, 3/6; middle, 4/5; high, 2/6.

reason why significant within-breed correlations between fibre number, fibre size and meat quality were scarcely presented. Also, within various cattle breeds, correlations between these fibre characteristics and meat quality traits are mostly insignificant (Ozawa *et al.*, 2000; Vestergaard *et al.*, 2000; Wegner *et al.*, 2000). The importance of muscle fibre size and number for meat quality becomes more obvious when comparing genotypes or groups of animals that differ extremely in lean percentage or meat quality. An example of extreme pigs is shown in Table 1.10. Piétrain pigs with the highest number of low-size fibres in the longissimus muscle tended to exhibit the best meat quality without significant differences in lean meat percentage and loin muscle area. Examples of extreme genotypes are presented in the following section (1.4.3).

A special case of extreme fibre size that is closely related to meat quality is the phenomenon of the so-called giant fibre. Giant fibres have been observed in cross sections of post-mortem muscle samples, but not in biopsy samples, from chickens, turkeys, pigs and cattle (Klosowska *et al.*, 1979; Handel and Stickland, 1986; Solomon and Eastridge, 1987). They are usually oval or round in shape, often exhibit extremely large (giant) cross-sectional areas, and are mostly located at the periphery of the fascicle (Fig. 1.14). These structurally abnormal fibres are considered to arise from hypercontraction of the muscle fibre or parts of it that are not able to undergo normal relaxation after initial *rigor mortis*. The molecular mechanisms that underlie the ethology of the giant fibres are not yet clarified.

In muscle without problems in meat quality, giant fibres occur at a low frequency of less than 0.5%. Higher frequencies of giant fibres have been shown to be associated with impairment in meat quality in the longissimus muscle of Piétrain pigs (Fiedler *et al.*, 1999) and in the pectoralis muscle of chickens (Klosowska *et al.*, 1979). Close genetic relationships between the frequency of hypercontracted giant fibres and various meat quality characteristics have been found in pigs (Table 1.9). High percentages of giant fibres correlate with high drip loss, light colour and low pH value in meat as indicators of poor meat quality. The results suggest that giant fibres represent a morphological indicator of muscle fibre dysfunction and their increased

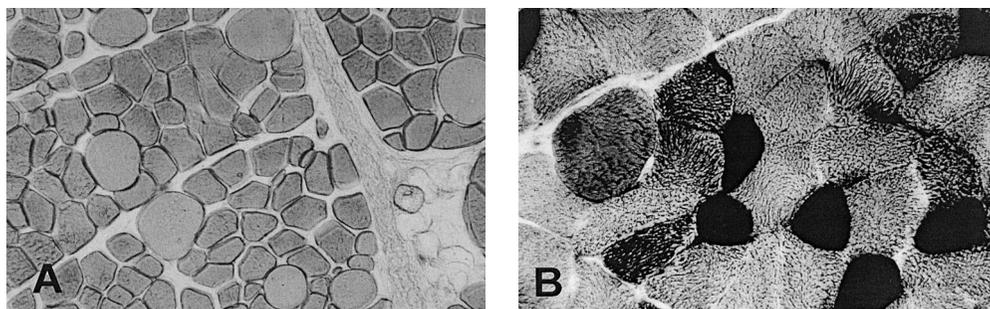


Fig. 1.14. Giant fibres in muscle cross section of pig longissimus (A) and broiler chicken gastrocnemius (B) muscles. (A) Eosin staining; (B) combined acid stable ATPase and NADH tetrazolium reductase.

occurrence is related to accelerated glycolysis and deficiencies in meat quality. Giant fibres can originate from muscle fibres of both the slow oxidative and the fast glycolytic types (Handel and Stickland, 1986; Wegner and Ender, 1990; Wiklund *et al.*, 1998; Fiedler *et al.*, 1999, 2001). Besides hypercontracted fibres with intracellular disruptions, additional fibre abnormalities characterized by fundamental lesions and interstitial necrosis have been described for the turkey (Sosnicki *et al.*, 1989). The occurrence of those abnormal changes was associated with deficiencies in meat quality as dry stringy texture and a grossly oedematous appearance.

Overall, extreme fibre hypertrophy and increased occurrence of giant fibres are strong indicators for the development of insufficient meat quality. An optimum balance between the number of fibres and their postnatal hypertrophy may be the key to meet the demands for both lean growth and meat quality.

1.4.3 Influence of major genes

In various species, mutations of several major genes influence muscle fibre number and (or) muscle fibre size and fibre type composition of skeletal muscle. These mutations, which are associated with extreme muscular hypertrophy and changes in meat quality, are the *ryanodine receptor (RyR)* gene in the pig, the *myostatin* gene in cattle and the *callipyge* gene in sheep. Another major gene termed *RN* (Rendement Napole) occurring in pure Hampshire pig breed and crosses has shown to be associated with higher lean meat content and a larger proportion of ham. The importance of these genes will also be the subject of later chapters (see Kambadur *et al.*, Chapter 14, and Freking *et al.*, Chapter 15, this volume).

1.4.3.1 Myostatin gene

The relationship between muscle fibre number and lean growth becomes very obvious by the example of double-musled cattle. The condition of double-muscling (DM) seen in several cattle breeds such as Belgian Blue and Piedmontese is a particular case of excessive muscle fibre formation (hyperplasia). Skeletal muscles of DM cattle contain almost double the number of fibres compared with other cattle breeds, whereas the fibres are of the same size or slightly larger at greater age (Ouhayoun

and Beaumont, 1968; Holmes and Ashmore, 1972; Wegner *et al.*, 2000; Table 1.8). This tremendous increase in muscle fibre number is associated with increases in muscle mass of $\geq 20\%$ (Shahin and Berg, 1985; Wegner *et al.*, 2000). However, double-muscled cattle exhibit paler meat and higher proportions of glycolytic fibres compared with other cattle breeds (Holmes and Ashmore, 1972). This suggests that fibre size and fibre metabolism may also be independently related to ultimate meat quality.

Prenatal studies with DM cattle suggest that the higher number of muscle fibres is a consequence of delayed differentiation and extended myoblast proliferation (Picard *et al.*, 1995). The double-muscled phenotype arises from mutations in the myostatin gene. Myostatin is a growth and differentiating factor (GDF-8) that belongs to the transforming growth factor-beta (TGF- β) superfamily and has been identified as an important negative regulator of muscle development in a mouse model of gene deletion (McPherron *et al.*, 1997). In Belgian Blue cattle an 11 base pair deletion in the coding region of the myostatin gene prevents the expression of the myostatin protein, whereas Piedmontese cattle have a G to A transition in the same region. In a study investigating the myostatin gene in samples from ten European cattle breeds, seven DNA sequence polymorphisms were identified in the coding region, of which five would be predicted to disrupt the function of the protein (Grobet *et al.*, 1998). An autosomal recessive inheritance has been suggested for the DM condition. It has been reported that proliferation of myoblasts decreased with increasing levels of myostatin, when C₂C₁₂ myoblasts were incubated with myostatin. However, the detailed physiological mechanisms by which myostatin affects muscle development remain to be investigated.

1.4.3.2 Callipyge gene

The callipyge condition in sheep as a further example of extreme muscular hypertrophy is associated with extensive muscling in the loin and hindquarters. It is caused by a mutation of the callipyge gene located on ovine chromosome 18 (Cockett *et al.*, 1994). Expression of this phenotype is the only known case in mammals of paternal polar overdominance gene action. A single base-change mutation causing the callipyge muscle hypertrophy phenotype has been identified recently (Freking *et al.*, 2002). In contrast to the double-muscled condition in cattle, muscular enlargement in callipyge lambs seems mainly to result from muscle fibre hypertrophy, particularly of the fast-twitch oxidative and fast-twitch glycolytic types (Carpenter *et al.*, 1996). Extreme muscle fibre hypertrophy in callipyge lambs has been reported to be associated with poor meat quality such as reduced tenderness and juiciness (Shackelford *et al.*, 1997). The influence of the callipyge genotype on muscle fibre number has not been investigated so far.

1.4.3.3 Ryanodine receptor (*halothane*) gene

In 1991, Fujii and co-workers identified a mutation in the porcine *ryanodine receptor* (*RyR*) gene that was associated with the malignant hyperthermia syndrome (MHS) in response to halothane anaesthesia. In skeletal muscle, this mutation leads to higher calcium release rates that accelerate glycolysis, resulting in rapid, early post-mortem pH decline, protein denaturation and loss of protein functionality. Consequently, carriers of the halothane sensitivity allele (nn) produce pale, soft and exudative meat. On the other hand, homozygous nn pigs exhibit higher carcass yield and lean per-

centage. At the microscopic level, these changes are associated with increased muscle fibre cross sections, decreased densities of capillaries and higher frequencies of fibre abnormalities such as giant fibres (Essén-Gustavsson *et al.*, 1992; Rehfeldt and Ender, 1995; Aalhus *et al.*, 1997; Fiedler *et al.*, 1999, 2001). Only few results are available on differences in muscle fibre number in dependency on the halothane genotype. As the increases in fibre girth were larger than the increase in meat percentage (Essén-Gustavsson *et al.*, 1992) or loin area (Rehfeldt and Ender, 1995), it has been assumed that the total muscle fibre number is lower in halothane sensitive pigs. This was confirmed by studies with Piétrain pigs (Giesel, 1997; Wicke *et al.*, 1998) or Piétrain crossbreds (Pedersen *et al.*, 2001), which showed that pigs from both the heterozygous nN and homozygous nn exhibited a lower number of fibres in longissimus or semitendinosus muscles (down to 80%) than non-carriers (Table 1.11). In conclusion, prenatal muscle fibre formation is negatively influenced by the disturbed RyR function and may contribute to extraordinary fibre hypertrophy in halothane sensitive pigs.

Table 1.11. Lean growth and meat quality characteristics (mean \pm sd) in Piétrain crossbreds of different halothane genotypes (Giesel, 1997).

Trait	Genotype		
	NN	Nn	nn
Lean meat (%)	49.9 ^a \pm 3.3	52.8 ^b \pm 3.3	57.0 ^c \pm 4.5
Loin area (cm ²)	32.2 ^a \pm 2.9	34.4 ^b \pm 2.8	36.5 ^c \pm 2.6
Intramuscular fat (%)	2.1 ^a \pm 0.8	2.0 ^a \pm 0.9	1.6 ^b \pm 0.5
Reflectance (%)*	28.4 ^a \pm 5.3	29.1 ^{ab} \pm 10.6	35.0 ^b \pm 10.2
pH 45 min post mortem*	6.43 ^a \pm 0.43	5.91 ^b \pm 0.39	5.58 ^c \pm 0.32
Conductivity (mS/cm)*	3.37 ^a \pm 0.62	4.29 ^{ab} \pm 2.29	15.49 ^b \pm 9.61
Fibre diameter (μ m)*	74.2 ^a \pm 8.7	85.8 ^b \pm 10.3	86.3 ^b \pm 10.2
Fibre number index ($\times 10^{-3}$)*	699.9 ^a \pm 172.1	563.0 ^b \pm 130.7	591.0 ^b \pm 131.1

NN, halothane-free ($n = 22$); Nn, halothane heterozygous ($n = 64$); nn, halothane homozygous ($n = 49$). *Longissimus muscle.

1.4.3.4 RN gene

The *RN* gene affecting a measure of cured-cooked ham processing yield (Rendement Napole) and first suggested by Naveau (1986) exists as two alleles; one is recessive (rn^+) and one is dominant (RN^-). Hampshire pigs with the RN^- allele realize higher daily gain and leaner carcasses (Enfält *et al.*, 1997). However, the RN^- allele is associated with a dramatic increase in the *in vivo* muscle glycogen content. The higher glycolytic potential, reflectance and water content, but lower ultimate pH and water-holding capacity of fresh meat are responsible for the negative technological properties such as lower Napole yield (Lundström *et al.*, 1996). The *RN* gene has been mapped on chromosome 15 and identified as a mutation in the *PRKAG3* gene (Milan *et al.*, 2000). Average muscle fibre size is not influenced by the *RN* genotype (Feddern *et al.*, 1995; Lebret *et al.*, 1999). However, homozygous RN^- carriers have been shown to exhibit larger fast-twitch oxidative fibres in longissimus muscle, but no differences between heterozygous carriers and non-carriers were found (Feddern *et al.*, 1995;

Lebret *et al.*, 1999). The influence of the RN genotype on muscle fibre number has not been investigated so far.

1.4.4 Genetic improvement of animal performance by muscle fibre traits

Considerable progress has been made in improving pig growth through the application of biological technologies. However, improvements are still needed in the areas of meat quality, production efficiency and disease resistance. The genetic improvement of farm animals for meat production by selection strategies aims at increased muscle growth potential, adequate leanness and good meat quality at slaughter. Selection could be more efficient if later carcass and meat characteristics could be predicted in the live animal, allowing an earlier selection of breeding stock than is currently possible after the progeny test.

First suggestions to use number and size of fibres of a single muscle as indicators for muscle mass date back to the 1970s (Staun, 1972; Stickland and Goldspink, 1975; Stuhlec *et al.*, 1976). Later, from genetic correlation coefficients and results of selection experiments, it has been derived that increases in muscle mass solely through muscle fibre hypertrophy are, at least in pigs and poultry, associated with problems in stress adaptability and meat quality. In contrast, selection for high fibre numbers at a moderate fibre size are presumed to be advantageous in achieving both high meat content and good meat quality. That it is possible to produce more meat and better meat quality by high muscle fibre numbers has been shown by a selection experiment with pigs (Table 1.12). Divergent selection on high or low muscle fibre diameter in the longissimus muscle, with each of them associated with a low backfat:muscle ratio, increased muscle thickness in equal terms but produced meat of extremely different structure and quality. The quality was poor in the high line and good in the low line and the proportion of stress-susceptible (halothane-positive) pigs declined from about 50% to zero in the low line and increased to 70% in the high line.

Moreover, it has been demonstrated by simulated selection with mouse and pig data that, if muscle structure traits were included in selection indices, selection responses in commonly used performance traits could be markedly improved. Recent studies on simulated selection with pig data using total fibre number and frequencies of glycolytic and giant fibres combined with traditionally used criteria such

Table 1.12. Results of a four-generation divergent selection on high and low longissimus muscle fibre diameter together with a low backfat:muscle ratio in German Landrace pigs (Wicke, 1989).

Trait	High (<i>n</i> = 31)	Low (<i>n</i> = 68)	Difference low/high (%)
Muscle thickness (mm)	49.3	49.4	0
Fibre diameter (μm)	90.0	81.6 ^a	91
Fibre number index ($\times 10^{-3}$) ^b	310	379 ^a	122

^aSignificant differences at $P < 0.05$.

^bCalculated from ultrasound-measured muscle thickness and fibre number per unit area in muscle biopsy sections.

Table 1.13. Direct and correlated responses to simulated selection for loin area without (A) and with (B, C) longissimus muscle fibre traits (Fiedler *et al.*, unpublished).

Selection criteria ^a	Direct	Correlated	
	Loin area (cm ²)	pH value	Drip loss (%)
A			
(+) Loin area	+2.04	-0.02	+0.41
B			
(+) Loin area			
(+) Total fibre number	+1.14	+0.01	+0.23
(-) Frequency of white ^b fibres			
C			
(+) Loin area			
(+) Total fibre number	+0.87	+0.03	-0.08
(-) Frequency of white fibres			
(-) Frequency of giant fibres			

^aIntensity of selection = 10%.

^bGlycolytic fast-twitch fibres.

(+) Positive selection to select animals with the highest values.

(-) Negative selection to select animals with the lowest values.

as loin area revealed a simultaneous improvement of loin area as well as pH value and drip loss (Table 1.13). These results are of importance with regard to a possible use of muscle structure characteristics in farm animal selection. However, before fibre traits can be practically applied in breeding schemes to improve animal performance, the most suitable selection criteria should be determined, and selection indices are to be constructed, optimized and tested within selection experiments. It should also be proven whether the incorporation of marker-assisted selection by the use of quantitative trait loci or candidate gene approaches into new selection strategy can be combined with muscle fibre trait analysis to predict animal performance, because the same muscle sample derived by biopsy can be used to analyse both kinds of traits in the live animal.

1.5 Conclusions

The number of muscle fibres formed during prenatal myogenesis and the degree of postnatal fibre hypertrophy are significant in the determination of lean growth and ultimate meat quality after slaughter. Achieving an optimum balance of sufficiently large numbers and sizes of muscle fibres and eliminating fibres of abnormal structure may be important steps in producing both high quantity and quality of meat in farm animals. From the discussed environmental and genetic influences on pre- and postnatal skeletal muscle growth, strategies can be derived to develop practical approaches to altering muscle structure. In farm animal production these will be based mainly on genetic selection or environmental modulation of prenatal myogenesis and postnatal muscle cell growth.

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2

Fibre Type Identification and Functional Characterization in Adult Livestock Animals

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2.1 Introduction: Definition of 'Fibre Type'

The contractile performance of vertebrate skeletal muscles is greatly variable: a comparison of muscles that accomplish different functions or a comparison between

corresponding muscles in different species reveals a large diversity in parameters such as tension development, rate of tension development, power output, shortening velocity and fatigue resistance. Functional diversity also appears when single fibres inside a muscle are individually studied. The structural basis of the functional heterogeneity is represented by a distinct molecular architecture: the molecules that are used to build muscle fibres might be deeply diverse.

Molecular diversity is the result of two mechanisms of gene expression regulation combined.

1. Quantitative regulation of gene expression: many genes are up- or downregulated in different fibres.
2. Qualitative regulation: expression of different isoforms of the same protein in different muscle fibres.

The term 'isoforms' indicates proteins that are similar, but not identical, in their structure and exhibit distinct functional properties. Isoforms may derive from the same genes through alternative processes of RNA maturation: for example, different myosin light chain (MLC) isoforms can be obtained by alternative splicing, or may derive from isogenes, closely related genes belonging to the same multigene family (e.g. eight different genes code for various myosin heavy chain (MHC) isoforms). Isoforms are similar enough to replace each other, but also diverse enough to give the fibre distinct functional properties. A recent comparison based on microarray hybridization techniques on a set of 6519 genes has shown that 177 genes, i.e. about 3%, are differentially expressed in slow and fast muscles of the mouse (Campbell *et al.*, 2001).

The two mechanisms of gene expression regulation identified above might in principle produce an infinite number of combinations. This number is, however, limited by the constraints set by functional or structural requirements and by the fact that many muscle-specific genes share similar regulatory mechanisms and their expression is therefore regulated in a coordinated way. These limitations strongly reduce the number of the actually occurring combinations that might arise from isoform selective expression and quantitative regulation of gene expression. These 'actually occurring' combinations identify specific phenotypes of skeletal muscle fibres or, more simply, fibre types. In conclusion, a fibre type is the result of a specific profile of gene expression, which means for some genes either up- or down-regulation and for other genes selective isoform expression.

The phenotype of a given muscle fibre is specified during muscle development, but is generally open to changes during the life of the fibre: adult skeletal muscle fibres display plasticity, i.e. they can change their type in response to changes of functional demands. Fibre phenotype can be altered by variations in neural discharge patterns, mechanical load or hormonal stimulation. Fibre type transformations represent one of the best occasions to identify the features of fibre types. In spite of the asynchrony produced by the different thresholds and turnover rates of the various muscle proteins, it is possible to observe that when muscle fibres undergo transformations, over the long run they move from one phenotype to the other (type transition). This is the result of the rules which coordinate the expression of various genes and modulate various functions (energy production, energy consumption, calcium metabolism, etc.) in muscle fibres. Fibre type transformations have been studied par-

ticularly in small mammals such as rat or rabbit (see Pette and Staron (1997) for a review). Data on muscle fibre plasticity in large animals (for example induced by training or by disuse) are also available and substantially confirm what is known from studies on small animals. Among the determinants of fibre type the neural discharge pattern has a central role (Windisch *et al.*, 1998). As a consequence all fibres of a motor unit, i.e. all fibres innervated by the same motor neurone and receiving the same pattern of stimulation exhibit the same phenotype or, in other words, are of the same type (Kugelberg and Edstrom, 1968; Burke *et al.*, 1971, 1973; Kugelberg, 1973).

The simplest functional classification of fibre types is based on the following:

- The parameters of the contractile response, which are determined by protein composition of the myofibrils and sarcoplasmic reticulum. Fibres can be slow or fast depending on their speed of contraction, i.e. on the rate at which tension develops during contraction and disappears during relaxation.
- The ability to cope with energy consumption due to contractile activity with adequate ATP production. Fibres can be resistant to fatigue or fatiguable depending on their ability to maintain the contractile performance during repetitive stimulation.

With these two criteria three main types of fibres can be identified (see Table 2.1):

1. S: slow and fatigue resistant.
2. FR: fast and fatigue resistant.
3. FF: fast and fast fatiguable.

Recent work has aimed to identify the mechanisms of gene regulation that determine fibre type.

Table 2.1. Fibre types as identified from motor unit studies.

Motor unit	Time to peak tension	Twitch/tetanus	Tetanus tension	Fatigue resistance	ATPase activity
Slow and fatigue resistant	Long	Low	Low	High	Low
Fast and fatigue resistant	Short	High	High	Intermediate	High
Fast and fast fatiguable	Short	High	High	Low	Very high

Several signalling pathways seem to be involved in mediating the action of neural discharge patterns, mechanical load during contraction and hormonal stimulation. Even a short bout of muscle contractile activity can strongly activate three of the mitogen-activated kinase (MAP) signalling pathways (ERK1/2, JUNK and P38). These pathways lead to the activation of transcription factors such as CREB, Elk, c-Jun, c-Myc and Mef2 (for a recent review see Sakamoto and Goodyear, 2002).

Calcineurin, a calcium-calmodulin activated phosphatase, is the most likely can-

didate to mediate the action of the neural pattern of activity in regulating the slow type gene expression (Chin *et al.*, 1998). Two of the known effectors of the calcineurin pathway are Mef2 and Nfat (see Houba and te Pas, Chapter 10, this volume): the transcription factors belonging to these two families are de-phosphorylated by calcineurin and in de-phosphorylated form enter the nucleus and activate transcription of genes specific to the slow phenotype (Delling *et al.*, 2000). Upstream of calcineurin, calcium conveys the information related to neural discharge pattern: the specific patterns of the motor neurones characterized by repetitive low frequency activity in slow motor units and by high frequency bursts in fast motor units dictate the levels of intracellular calcium not only during contractions but also during the resting state between contractions. The high level of calcium of slow fibres is likely to be sufficient to activate calcineurin via calmodulin (Chin *et al.*, 1998).

Distinct signalling pathways regulate the genes involved in mitochondrial biogenesis and control the expression of metabolic enzymes (Sakamoto and Goodyear, 2002; Hoppeler and Fluck, 2003). The increased energy consumption during contraction implies an increase in the cytosolic concentration of AMP, which can activate AMP kinase. Several other kinases such as GSK3, Akt (also called PKB, protein kinase B) and p70-S6 kinase are also activated during contractile activity. AMP kinase has been shown to control transcription of NRF-1 (nuclear respiration factor 1), which in turn controls TFAM, a transcription factor acting on mitochondrial DNA. PGC-1 α (peroxisome proliferator activated receptor gamma coactivator 1) is a transcription factor which activates the programme of mitochondrial biogenesis and increases expression of slow type myofibrillar proteins in cooperation with Mef2 transcription factors (Jin *et al.*, 2002). Which factors act upstream of PGC-1 α is still unknown.

2.2 Fibre Types and Myosin Isoforms

Among all proteins whose expression varies from one fibre type to another, myosin isoforms are generally considered as the molecular marker of the fibre type: fibre types are often indicated using the name of the myosin isoform that is expressed. There are several reasons that support this choice. In particular myosin is: the most abundant protein; the motor protein and thus the determinant of both mechanical performance and energy consumption; and the determinant of the histochemical ATPase reaction after alkali and acid pre-incubation, historically the first fibre type classification.

Each myosin is associated with specific kinetics of acto-myosin interaction and ATP hydrolysis: these properties determine the mechanical properties of the muscle fibres such as speed of actin filament sliding, mechanical power output and ATP consumption rate (see Schiaffino and Reggiani, 1996).

Myosin is a hexamer, composed of two heavy chains (MHC), two alkali light chains and two regulatory light chains (MLC). MHC are large molecules (MW *c.* 220 kDa) composed of a globular part close to the N terminus (the head) and a filamentous part (the tail) towards the C terminus. In each myosin molecule the filamentous parts of two MHCs form a coiled helix, which contributes to the backbone of the thick filament. The myosin head contains the site of actin interaction, the catalytic site for ATP hydrolysis and the converter region, which transforms the small con-

formational changes related to the ATP catalytic cycle in larger movements of the filamentous part. Two MLCs, one alkali and one regulatory, are wrapped around the filamentous part of MHC in the proximity of the head. Myosin structure in relation to function has recently been reviewed by Geeves and Holmes (1999).

There are many isoforms of MHC present in the genome of each organism. The MHC gene superfamily includes at least 19 classes and each class comprises several isogenes (Sellers *et al.*, 1997). The myosin isoforms expressed in striated muscles and contributing to the sarcomeric architecture, i.e. the sarcomeric myosins, all belong to class II and, from the information presently available on human and murine genome, are coded for by eight genes. Two genes are located in tandem on chromosome 14 (human) or 11 (murine), whereas the other six are clustered on chromosome 17 (human) or 14 (murine) (Weiss *et al.*, 1999). Two genes code for the isoforms MHC-emb and MHC-neo, which are expressed during development and, in adult life, only during regeneration, in intrafusal muscles and, in some species, in masticatory muscles. Four genes, i.e. *MHC-1* or β /*slow*, *MHC-2A*, *MHC-2X*, *MHC-2B*, are expressed in extrafusal fibres of limb and trunk muscles. Among them *MHC-1* or β /*slow* is also expressed in ventricular myocardium. *MHC- α* is expressed in cardiac muscle and, in some species, in masticatory muscles and in a few slow fibres. A specific isoform, *MHC-exoc*, is expressed in extraocular muscles. Which MHC isoforms are expressed in intrafusal fibres is not yet completely defined. Studies on MHC isoforms have been extensively reviewed by Pette and Staron (1990), Schiaffino and Reggiani (1994, 1996) and Bottinelli and Reggiani (2000).

Light chains also exist in several isoforms: at least four genes coding for regulatory MLC (*MLC2 fast*, *MLC2 slow/cardiac*, *MLC2 atrial*, *MLC2 masticatory*) and four genes coding for alkali MLC (*MLC1-fast*, *MLC1-slow-a*, *MLC1-slow-b* or *MLC1-slow-ventricular*, *MLC1-embryonic/atrial*) are expressed in sarcomeric muscles, i.e. muscles where contractile proteins are organized in sarcomeres (skeletal and cardiac muscles; see for a review Schiaffino and Reggiani, 1996). From the genes coding for alkali MLC1 fast two isoforms (*MLC1fast* and *MLC3*) are obtained by alternative splicing (Peryasamy *et al.*, 1984). In many species strict association or coordination rules control expression of MLC and MHC isoforms, so that slow MHC combines with slow isoforms of alkali and regulatory MLC, whereas fast MLC isoforms combine with various fast MHC isoforms (Salviati *et al.*, 1982a; Wada and Pette, 1993).

The rules of coordinated expression which determine the association between MLC and MHC hold also for most of the myofibrillar proteins (see below): thus, each MHC isoform is accompanied by a specific set of myofibrillar proteins and this will determine the functional properties, for example specific values of maximum shortening velocity or specific parameters of the force–pCa curve. The rules of coordinated expression do not seem to apply so strictly to metabolic enzymes. Although a general association may also exist between MHC isoform expression and metabolic enzyme expression (for example slow myosin is generally associated with high aerobic oxidative activity), the connection is weak as suggested also by the fact that distinct signalling pathways are involved in control of myofibrillar and metabolic genes (see above).

2.3 Methods for Fibre Type Identification in Various Mammalian Species

The interest in reliable methods of fibre type identification extends from small laboratory animals where it is essential for all studies on muscle plasticity and on the correlation with contractile performance, to human muscles where identification of muscle fibre types is important for diagnosis of muscle diseases as well as for performance evaluation in athletes, and to livestock animals where several studies have tried to correlate fibre type composition with meat quality, growth index and the effect of breed crossing on muscle growth and quality. Differences in fibre type composition among breeds and between males and females have been reported and modifications of the fibre type composition following hormonal or alimentary treatments have been shown.

In the pig variations of fibre type composition have been reported comparing different breeds (da Costa *et al.*, 2002): a specific association between abundance of fast glycolytic fibres and expression of the halothane gene, i.e. a mutated RYR calcium channel, has been found (Depreux *et al.*, 2002). Indications of relations between parameters of meat quality such as tenderness or post-mortem muscle pH and proportion of fibre types have been published (reviewed by Depreux *et al.*, 2002). In bovine muscles the analysis of fibre type composition has contributed to identifying the mechanisms related to the development of the 'double-muscle' phenotype, which is caused by a mutation of the myostatin, a factor of the TGF beta family (see Kambadur *et al.*, Chapter 14, this volume). Equine muscles have been studied mainly in relation to race performances either comparing different breeds or evaluating the effects of training protocols. Muscle analysis is generally carried out on biopsy samples and gluteus is the most studied muscle.

2.3.1 Histochemistry

In accordance with early studies on small laboratory animals, fibre types of livestock animals have been classified on the basis of myosin ATPase (m-ATPase) activity and metabolic glycolytic and oxidative enzymes (Fig. 2.1). The extension of the protocols defined in rat and rabbit muscles for determination of m-ATPase after alkali or acid pre-incubation to other species has required adjustments of pH and molarity values and of incubation times.

In canine muscles (Latorre *et al.*, 1993) acid and alkali resistance seem to depend on the method used and may give opposite results for the same type of fast fibres. In bovine and equine muscles the ranges of pH that can discriminate between fibre types are more narrow than in laboratory species.

These difficulties make the use of m-ATPase staining restricted to separation between type 1 (slow) and type 2 (fast) fibres, whereas determination of oxidative enzyme activity has been used to distinguish between two subgroups (2A and 2B) of fast fibres. Classifications based on combined use of metabolic enzyme determination and m-ATPase reaction lead to the identification of three types, beta-R, alpha-R and alpha-W (Ashmore and Doerr, 1971), or SO, FOG and FG (Peter *et al.*, 1972) or four types, 1, 2A, 2B oxidative, 2B non-oxidative (Lopez *et al.*, 1992). The combined use of these classifications is, however, limited by the large variability in metabolic activities inside each fibre type and has become less frequent.

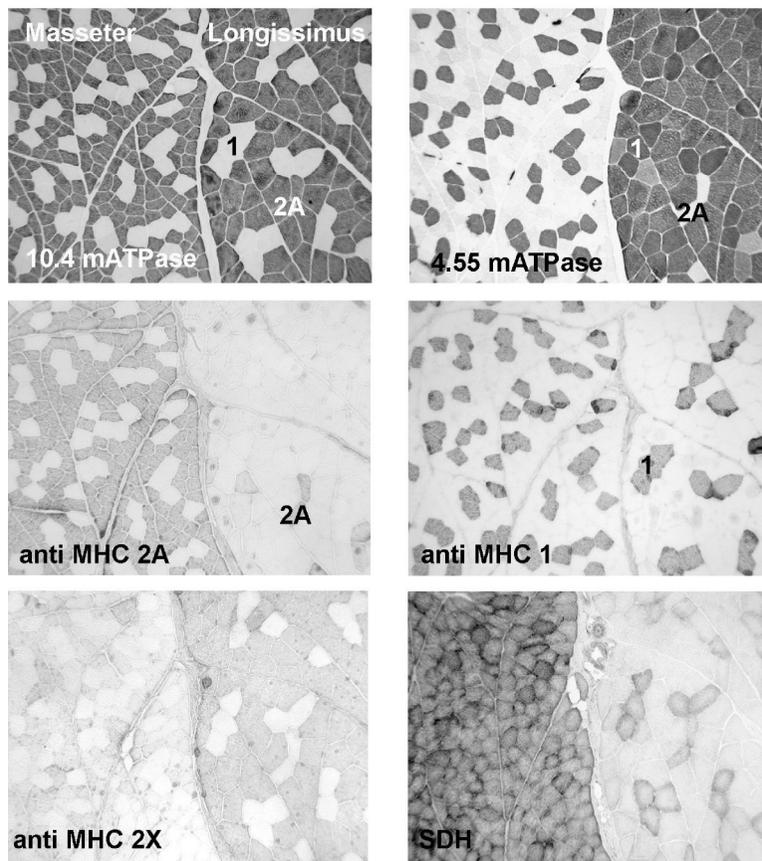


Fig. 2.1. Histochemistry and immunohistochemistry of pig masseter and longissimus muscles. Note: (i) the different fibre type composition between the two muscles: masseter is composed of type 1 and type 2A; longissimus is composed mainly of pure 2X fibres and hybrid 2X/B fibres, which cannot be taken apart by means of histochemistry and immunohistochemistry; and (ii) the highly oxidative character of masseter (succinate dehydrogenase staining).

2.3.2 Immunohistochemistry

Some of the above difficulties have been overcome by the use of antibodies directed against MHC isoforms, which are generally considered as markers of fibre type. A list of often used and commercially available antibodies is reported in Table 2.2. Some antibodies are very effective in identifying corresponding MHC isoforms in different species (Table 2.2 and Fig. 2.2). This can be explained by the fact that MHC primary structure is more similar between corresponding isoforms in different species (orthologue isoforms) than between isoforms in the same species (paralogue isoforms, see below).

Reactivity with specific antibodies shows, for example, that fibres histochemically identified as type 1 or 2A in livestock animals express a slow MHC or, respectively, a 2A MHC similar to those expressed in the slow or 2A fibres of rat or rabbit muscles.

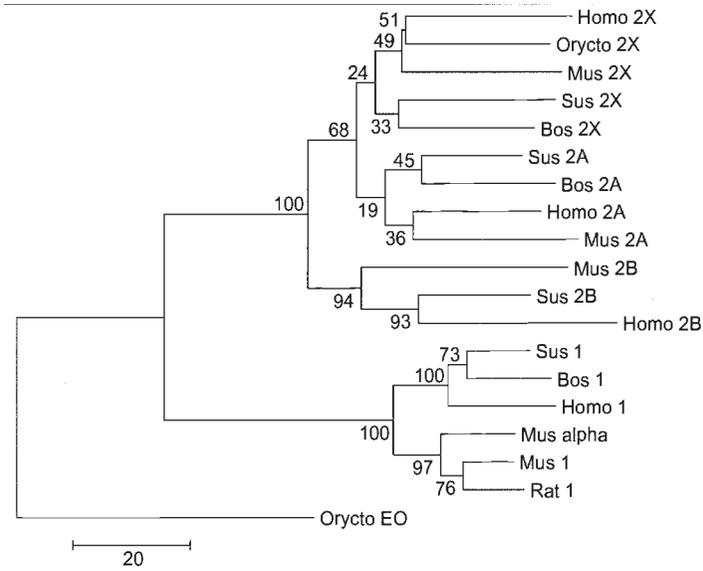


Fig. 2.2. Homology tree of MHC isoforms in mouse, rat, rabbit, human, pig and cow, based on alignment of nucleotide sequence (cDNA) of MHC isoforms. Note that the distance between orthologue isoforms is shorter than the distance between paralogue isoforms.

Table 2.2. Reactivity of monoclonal antibodies specific to MHC isoforms in various mammalian species and often used for fibre type identification.

	Rat (1)				Pig (2)				Llama (3)				Horse (4)			
	1	2A	2X	2B	1	2A	2X	2B	1	2A	2X	2B	1	2A	2X	2B
BA-F8	+				+				+				+			
NLC-MHC					+											
SC-75		+	+	+						+	+	+		+	+	
SC-71		+				+	+/-			+	+			+		
S5-8H2	+		+	+	+		+	+	+	+	+	+	+			+
BF-35	+	+		+					+	+			+	+		
BF-G6				+								+	+	+		
BF-F3								+/-								

	Bovine (5)				Goat (6)			
	1	2A	2X	2B	1	2A	2X	2B
BA-D5	+							
BA-F8					+			
SC-75		+		+	+	+		
SC-71		+		+	+	+		
S5-8H2	+			+	+	+		
BF-35	+	+		+	+	+		
BF-G6							+	
BF-F3							+/-	

Data source: (1) rat: Schiaffino *et al.*, 1989; (2) pig: Lefaucheur *et al.*, 2002; (3) llama: Graziotti *et al.*, 2001; (4) horse: Rivero *et al.*, 1996b, 1999; Graziotti *et al.*, 2001; (5) cow: Duris *et al.*, 2000; (6) goat: Arguello *et al.*, 2001.

Antibody reactivity, however, does not clarify completely the identification of the other two types of fast fibres, 2X and 2B, which are also characterized by glycolytic metabolism. This limitation was first found (Snow *et al.*, 1982) studying dog muscle: canine 2B fibres do not correspond to classical 2B fibres of rat and rabbit. Later, the discovery of 2X fibres in rabbit and rat muscles has contributed to clarifying that two distinct MHC isoforms can be expressed in the glycolytic fast fibres (Bar and Pette, 1988; Schiaffino *et al.*, 1989). Without any specific antibody for MHC-2X the precise identification of which fibres express 2X and which express 2B MHC remained uncertain, and the demonstration that the so-called 2B fibres in human muscles contain an MHC that is more similar to rat 2X than to rat 2B was made possible only by *in situ* hybridization with specific cDNA probes (Smerdu *et al.*, 1994).

The most recent attempts to classify fast fibres using antibodies in livestock animal muscles have met increasing difficulties because of the variable reactivity of antibodies directed against fast 2X and 2B MHC (see Table 2.2). Recently, a new battery of monoclonal antibodies able to identify in a highly specific way all three fast MHC variants has been published (Lucas *et al.*, 2000). Using these antibodies in mouse, rabbit and guinea pig muscles, all three fast isoforms have been identified, whereas only two isoforms (2A and 2X) have been found in cat and baboon muscles (Lucas *et al.*, 2000). Also in seven species of marsupials three fast MHC variants, which should correspond to MHC 2A, 2X and 2B in eutherian mammals, have been found (Zhong *et al.*, 2001). Unfortunately these antibodies are not yet commercially available.

The interpretation of the patterns obtained by histochemical and immunohistochemical methods is often made difficult by the presence of hybrid fibres containing more than one MHC isoform. Hybrid fibres show intermediate staining with m-ATPase and double staining with monoclonal anti-MHC antibodies. The problem is less relevant for small mammals where hybrid fibres in hindlimb muscles represent a minor population, for example 5–6% in tibialis anterior (Lucas *et al.*, 2000). In livestock animals the proportion of hybrid fibres is much larger, for example 21% in goat muscles (Arguello *et al.*, 2001) and more than 40% in llama muscles (Graziotti *et al.*, 2001). In order to completely identify all fibre types in each animal species it must be taken into account that each muscle contains only some fibre types: for example the masseter is composed only of slow fibres in ruminants, slow and 2A fibres in the pig, fast 2A fibres in rodents, and slow and type 2M in carnivores. Thus, more than one muscle must be examined in each species before arriving at the conclusion that all fibre types have been identified.

2.3.3 Gel electrophoresis and immunoblotting

If MHC isoforms are assumed to be the molecular markers of fibre types, the electrophoretic separation of MHC isoforms represents the most direct approach to attribute a single muscle fibre to a given type or to determine the composition of a given muscle. Immunoblotting, or Western blotting, provides a way to connect the electrophoretic separation with the reactivity with a specific anti-MHC antibody.

Separation of MHC isoforms was first achieved in small laboratory rodents (Danieli-Betto *et al.*, 1986) where the MHC isoforms can be separated in order of

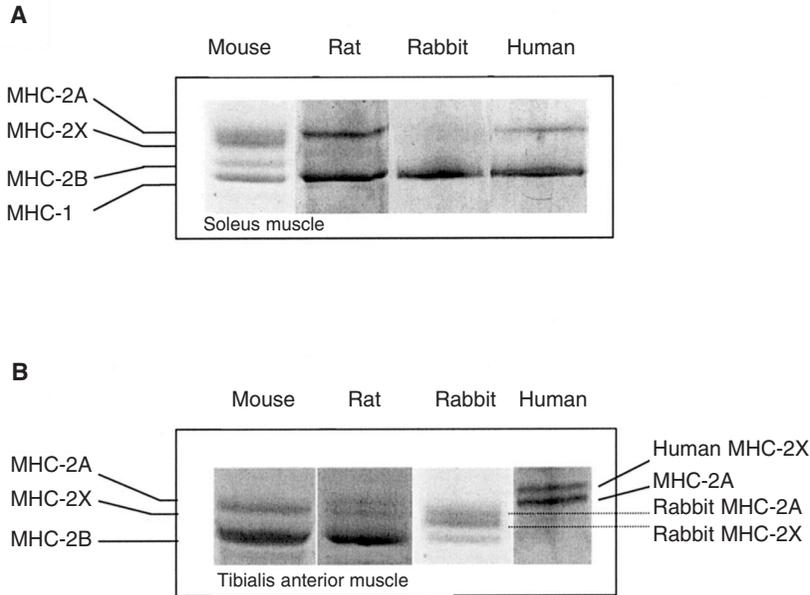


Fig. 2.3. Examples of electrophoretical separation of myosin heavy chain (MHC) isoforms in two functionally different muscles (A: soleus; and B: tibialis anterior muscle) of four different mammalian species. Soleus is mainly a postural muscle and tibialis is a phasic muscle. Note: (i) the lack of type 2 MHC in rabbit soleus and of type 1 (slow) MHC in the samples of tibialis anterior; (ii) the different migration of rabbit type 2 MHC.

decreasing migration rate from slow or MHC-1 (the fastest) to MHC-2B, MHC-2X and MHC-2A (the slowest) (Fig. 2.3). The separation of MHC-2A and MHC-2X is rather difficult and can be obtained only with specially designed electrophoretic protocols (Bar and Pette, 1988; Talmadge and Roy, 1993). The separation of MHC in human muscles reveals a different order with slow MHC as the fastest, followed by MHC-2A and MHC-2X as the slowest (Biral *et al.*, 1988) (Fig. 2.3).

In livestock animals electrophoretic separation has led to results that are still uncertain: in the horse, slow and 2A MHC co-migrate with the corresponding MHC isoforms of rat muscles, whereas a third MHC isoform identified as MHC-2X shows an intermediate rate of migration (Rivero *et al.*, 1996b, 1999). Also in the pig, three bands have been identified (Bee *et al.*, 1999): two of them co-migrate with slow and fast 2A MHC of rat muscles, while the third has an intermediate migration rate. In the cow, several protocols to find the optimal conditions for electrophoretic separation have been tested and under optimal conditions four MHC isoforms have been separated (Picard *et al.*, 1999). The fastest migrating band corresponds to slow MHC, whereas the three slowest correspond to fast isoforms; the identification of the latter is, however, still uncertain. In the sheep, three adult MHC isoforms have been separated (Maier *et al.*, 1992), whereas four bands have been found by Sayd *et al.* (1998): three fast MHC isoforms should correspond to the three slower migrating bands, but the authors are very cautious in defining which band corresponds to which isoform. Three bands corresponding to MHC isoforms have been detected in goat muscles (Arguello *et al.*, 2001). These bands have been attributed on the basis of

immunoblotting to MHC-2A, MHC-2X and slow MHC in order of increasing migration rate.

The electrophoretic approach seems to be powerful and useful only when adequately supported by immunoblotting and accompanied by single fibre studies. Only the slow MHC isoform seems until now to migrate consistently in all species, whereas fast MHC isoforms change their migration rate from species to species. Without immunoblotting, assuming that specific antibodies are available and without electrophoresis of single fibres, the doubts about which band corresponds to which isoform and the possible overlap of different MHC isoforms can never be completely removed.

2.3.4 Molecular biology

The precise and reliable identification of an MHC isoform can only be achieved by determining its nucleotide or amino acid sequence. As discussed above MHC isoforms form a multigene family with high similarity in the exon–intron structure and high conservation of the sequence. The identity of the sarcomeric MHC isoforms ranges between 78 and 98% and is higher when MHC orthologues (corresponding isoforms in different species) than when MHC paralogues (different MHC isoforms in a given species) are compared (Fig. 2.2). Some regions of the MHC molecule are completely identical, for example, parts of the catalytic site, the actin binding surface and the converter domain, whereas other regions are much more variable and for this reason can be better used to identify MHC isoforms: the surface loops on the myosin head in the translated part of the gene and two untranslated extremes (called 3' and 5' UTR) are among the variable parts. Using these variable parts it is possible to design specific primers for RT-PCR (Fig. 2.4) or probes for *in situ* hybridization.

Complete sequences of all sarcomeric MHC genes (*beta/slow*, *alpha*, *2A*, *2X*, *2B*, *exoc*, *emb*, *neo*) are available in humans (Weiss *et al.*, 1999) and all four adult skeletal muscle MHC (*slow*, *2A*, *2X*, *2B*) are available in the pig (Chikuni *et al.*, 2001). Fewer data are available for laboratory rodents: in the rat only the complete sequence of the *beta/slow* MHC and the sequences of the 5' and 3' UTR of other MHC isoforms, and in the mouse some complete sequences have been recently published. In the

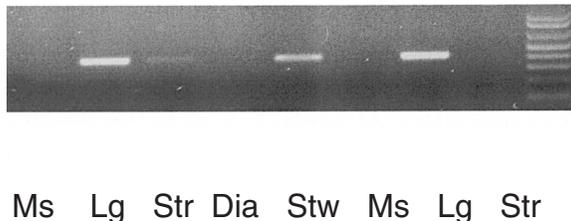


Fig. 2.4. RT-PCR expression analysis of pig muscles based on cDNA amplification in different pig muscles (Ms, masseter; Lg, longissimus; Str, semitendinosus red portion; Stw, semitendinosus white portion; Dia, diaphragm) using specific primers for MHC-2X. Note that MHC-2X is not expressed in masseter and diaphragm and expressed very little in semitendinosus red portion.

cow, cDNA of type 1, 2A and 2X MHC has been sequenced (Tanabe *et al.*, 1998) and fragments of the same three MHC isoforms have been sequenced in the sheep (Yamaguchi, unpublished, accession numbers AB 058898, AB 058896, AB 058897).

2.4 Coordinated Expression of the Myofibrillar Protein Isoforms Determines the Contractile Performance

Due to the constraints set by the precise molecular architecture of the sarcomere, the stoichiometric ratios between myofibrillar proteins cannot be changed. Diversity at myofibrillar level can only be based on isoform replacement and most, if not all, myofibrillar proteins exist in two or more isoforms, whose expression is coordinated with the expression of MHC isoforms. A list of the major myofibrillar proteins and their respective isoforms is reported in Table 2.3. The analysis of the contractile properties of single fibres, followed at the end of the functional experiments by gel electrophoresis and Western blotting, has provided reliable information on the impact of many myofibrillar protein isoforms on the contractile performance.

2.4.1 Calcium sensitivity

The sensitivity of the myofibrillar apparatus to Ca^{2+} is generally studied in permeabilized fibres where the steady-state isometric force is measured in activating solutions with different free calcium concentrations: in this way force–pCa relationships are obtained and fitted by sigmoidal Hill's equation. Two parameters that quantify calcium sensitivity are calculated from Hill's equation: pCa50%, which is an index of myofibrillar affinity for Ca^{2+} , and n , the Hill coefficient, which is an index of the steepness of the relationship and therefore of the cooperativity of myofibril activation. In small mammals, at room temperature, slow fibres have higher calcium sensitivity than 2B fibres, and 2B fibres higher calcium sensitivity than 2A fibres, whereas the Hill coefficient is lower for slow than for fast fibres (Stephenson and Williams, 1982; Danieli-Betto *et al.*, 1990). Measurements in human fibres are generally consistent with animal data (Ruff and Whittlesey, 1991; Lynch *et al.*, 1994; Bottinelli *et al.*, 1998), whereas no data are available on large livestock mammals.

Although Ca^{2+} sensitivity is mainly determined by the isoforms of the regulatory proteins tropomyosin and troponin (Danieli-Betto *et al.*, 1990), other proteins are surely involved as well. It is well known that myosin phosphorylation (Sweeney *et al.*, 1993) regulates Ca^{2+} sensitivity; MHC isoforms and C-protein isoforms might also play a part.

2.4.2 Maximum shortening velocity and power output

Many studies have investigated the rate of chemo-mechanical energy transduction in various muscle fibre types. Maximum shortening velocity and peak power output are the two mechanical parameters that, under conditions of maximal calcium activation, best define the rate of mechanical energy generation. Maximum shortening velocity (generally indicated as V_{max} or V_0) can be determined by extrapolating to

Table 2.3. Myofibrillar proteins and their isoforms.

Thick filament proteins	
Myosin heavy chain	Nine sarcomeric isoforms (from eight different genes): MHC-1 (or beta/slow), MHC-alpha, MHC-2A, MHC-2X, MHC-2B, MHC-extraocular, MHC-m (masticatory), MHC-embryonic, MHC neonatal (or fetal)
Alkali myosin light chain	MLC1-fast, MLC1-slow-a, MLC1-slow-b or MLC1-slow-ventricular, MLC1-embryonic/atrial
Regulatory myosin light chain	MLC2 fast, MLC2 slow/cardiac, MLC2 atrial, MLC2 masticatory
Titin	One gene which gives origin by alternative splicing to several isoforms with fibre type specific distribution
C-protein or MyBP-C	Three genes coding for three isoforms: MyBP-C-cardiac (MyBP-C-3) MyBP-C-slow (MyBP-C-1) MyBP-C-fast (MyBP-C-2)
Myomesin	Two genes coding for two isoforms: Myomesin-1 Myomesin-2
M protein	
H protein	
Thin filament proteins	
Actin	
Nebulin	
Troponin C	Two genes coding for two isoforms: Tn-C cardiac/slow: myocardium and slow fibres Tn-C fast: fast fibres
Troponin I	Three genes coding for three isoforms: Tn-I cardiac Tn-I slow Tn-I fast
Troponin T	Three genes which generate many isoforms by alternative splicing: Tn-T cardiac Tn-T slow Tn-T fast
Tropomyosin	Three genes coding for: TM-alpha-fast (TPM1) fast fibres TM-alpha-slow (TPM3) slow fibres TM-beta (TPM2) all muscle fibres
Z line proteins	
Alpha-actinin	Alpha-actinin-2 : all muscle fibres Alpha-actinin-3: fast fibres

zero load the force–velocity relationship, or with the simpler protocol referred to as the ‘slack test’ (Edman, 1979). Peak power output (W_{max}) is the highest value reached by the product force \times shortening velocity: it is therefore calculated from the force–velocity relationship. These two parameters are directly related to the myosin

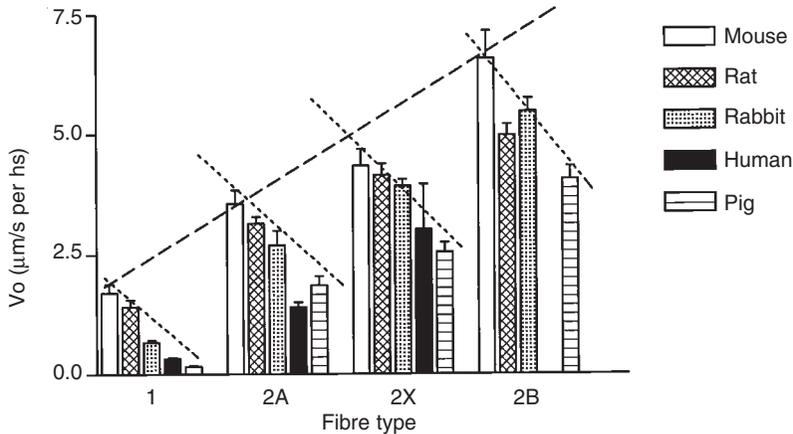


Fig. 2.5. Maximum shortening velocity (V_o) of single skeletal muscle fibres. V_o has been measured in four fibre types in five mammalian species and is expressed in $\mu\text{m/s}$ per half sarcomere (hs). Straight lines are superimposed to show two trends: dashed line indicates the increase of V_o comparing slow with fast fibres; dotted lines indicate that for each fibre type V_o decreases from mouse (the smallest animal studied) to rat, rabbit, humans and pig (the largest animal studied). Data for mouse, rat, rabbit and humans are from Pellegrino *et al.* (2003), data for pig are unpublished observations (Reggiani and Mascarello).

isoform expressed in muscle fibres: they depend in the first place on MHC isoforms (Reiser *et al.*, 1985; Sweeney *et al.*, 1988; Rome *et al.*, 1990; Bottinelli *et al.*, 1991). In all animal species examined to date, maximum shortening velocity and peak power show increasing values from fibres expressing MHC-slow to fibres expressing MHC-2A, MHC-2X and MHC 2B (Fig. 2.5). In fast fibres the ratio between the two isoforms of alkali MLC is also relevant (Sweeney *et al.*, 1988; Bottinelli *et al.*, 1994a). The difference in V_o between the fastest and slowest fibres ranges from four to five times in mouse and rat to eight times in man and more than ten times in pig (Pellegrino *et al.*, 2003). The difference in W_{max} among fibre types can be even greater than the difference in V_o . It is also important to observe that, when corresponding fibre types are compared in different animal species, both W_{max} (expressed relative to unitary muscle volume) and V_o (expressed in relative units, muscle length/s or $\mu\text{m/s}$ per half sarcomere) show a trend to decrease with increasing body size (Rome *et al.*, 1990; Seow and Ford, 1991; Pellegrino *et al.*, 2003) (Fig. 2.5).

In living animals skeletal muscles never shorten at the maximum shortening velocity, i.e. at zero load. There is evidence that they generally shorten at V_{opt} , or optimal velocity, which is the velocity where power output reaches its highest value (W_{max}). V_{opt} is determined by V_o and by the curvature of the force–velocity curve: thus V_o can be considered a determinant of V_{opt} and W_{max} , which are both physiologically relevant mechanical parameters.

2.4.3 Energy consumption

Differences between ATP consumption of slow and fast muscles have long been known (Barany, 1967). Later studies have shown that the ATPase activity of myosin

subfragment 1 is mainly modulated by the MHC component where the catalytic site is located (Wagner, 1981; Lowey *et al.*, 1993). More recently, refined techniques have allowed the detection of the tiny amount of ATP consumed by a single fibre (Potma *et al.*, 1995; He *et al.*, 1997), and ATPase activities of different fibre types from rat, rabbit and human muscles have been studied (Bottinelli *et al.*, 1994b; Sieck *et al.*, 1995; Stienen *et al.*, 1996).

In isometric conditions, the ATP splitting rate is three to four times slower in type 1 fibres than in type 2B fibres, type 2A and 2X fibres being intermediate. Tension cost, the ratio between ATPase and tension, indicates the amount of ATP used to develop and maintain a given amount of force. As specific tension of type 1 is only slightly lower than that of fast fibres, whereas ATPase is much lower in slow than in fast fibres, tension cost is several times lower in type 1 than in type 2A, 2X and 2B fibres (Bottinelli *et al.*, 1994b; Stienen *et al.*, 1996). All this implies that in isometric contractions type 1 fibres are energetically more economic than type 2A, 2X and 2B fibres.

During shortening the rate of energy liberation increases above isometric values, i.e. the 'Fenn' effect occurs. The Fenn effect has been generally studied in mouse intact muscles (Heglund and Cavagna, 1987; Barclay *et al.*, 1993) using calorimetric or oxymetric methods. Recent determinations in single fibres of rabbit and rat using a spectrophotometric method (Potma and Stienen, 1996; Reggiani *et al.*, 1997) and of human and rabbit using a fluorescent probe for detection of inorganic phosphate (He *et al.*, 1999, 2000) confirm that ATP consumption increases several times during shortening both in slow and in fast fibres.

From the amount (or rate) of extra energy liberated, and from the amount of work (or power) produced, efficiency can be determined. Contrasting results have been obtained comparing efficiency of slow and fast muscles of small mammals (Heglund and Cavagna, 1987; Barclay *et al.*, 1993). Single fibre experiments, however, show that efficiency varies little between slow and fast fibres, definitely much less than power output or energy consumption (Reggiani *et al.*, 1997; He *et al.*, 2000). Thus, when movement requires generation of high mechanical power, fast fibres are no less economic than slow fibres.

2.5 Contraction Speed is Related to the Excitation–Contraction Coupling

Distinct fibre types exhibit different contraction and relaxation speeds, i.e. develop force and relax at different rates, and respond differently to increases in the rate of stimulation, i.e. show different twitch/tetanus ratios and force–frequency relationships. At cellular level the main determinants of such fibre heterogeneity are: the sensitivity of myofibrillar apparatus to Ca^{2+} (see above), the rate of actin–myosin interaction and tension development, and the rate of Ca^{2+} uptake and release by the sarcoplasmic reticulum (SR). A difference between slow and fast fibres in the calcium transient following a single stimulation has been demonstrated in rat skeletal muscles (Eusebi *et al.*, 1980; Fryer and Neering, 1989). Calcium transient in fast fibres reaches a higher peak and declines faster than in slow fibres. The difference between slow and fast fibres in excitation–contraction coupling and calcium transients is based on both the mechanism

of isoform replacement and the mechanism of up- and down-regulation of gene expression.

Calcium release in skeletal muscle fibres follows the opening of sarcoplasmic reticulum calcium channels (generally indicated as ryanodine receptors or RyR), which are activated during action potential by sensors of sarcolemma potential represented by calcium channels located in the T tubules (di-hydropyridine receptors or DHPR). The density of the voltage sensors, DHPR, is three- to fivefold greater in fast twitch than in slow twitch fibres (rat: Delbono and Meissner, 1996; mouse: Renganathan *et al.*, 1998) and, in accordance with this, charge movement is much greater in fast than in slow fibres (Dulhunty and Gage, 1983). Two isoforms of the sarcoplasmic calcium release channel/ryanodine receptors are expressed in mammalian skeletal muscle fibres: RyR1 is the dominant one, whereas expression of RyR3, after a wide distribution during the postnatal period, is restricted to a few muscles in adult animals (Sorrentino and Reggiani, 1999). There is also evidence of a differential distribution of RyR3 in individual muscle fibres (Flucher and Sorrentino, 1999), but it is not yet clear whether expression of RyR3 is related to a specific fibre type. Ca release from SR in slow fibres is less inhibited by cytosolic Mg (Stephenson *et al.*, 1998): this contributes a lower sensitivity to fatigue as intracellular Mg concentration increases during advanced stages of fatigue. The explanation might be found in different RyR isoforms, in different RyR density or in greater concentrations of calcium inside the SR of slow fibres. This would also explain the greater effect of caffeine in slow fibres (Stephenson *et al.*, 1998). Actually, a greater sensitivity to caffeine of slow compared with fast fibres has been observed in rat (Fryer and Neering, 1989).

The end of the contractile response is determined by active calcium transport from cytosol back to the sarcoplasmic mechanism. The sarcoplasmic reticulum calcium pump is present in two isoforms, one of them expressed in fast fibres (SERCA1b) and the other expressed in slow fibres (SERCA2a) (Lytton *et al.*, 1992). The latter is regulated by the phosphorylation state of the regulatory subunit phospho-lamban (Hawkins *et al.*, 1994). The density of the pump is much greater (five- to sevenfold) in fast than in slow fibres (rabbit: Leberer and Pette, 1986; human: Everts *et al.*, 1989; rat: Wu and Lytton, 1993). This ensures a faster and more efficient removal of calcium in fast than in slow fibres. The rate of calcium uptake by SR is about twofold faster in fast than in slow human muscle fibres (Salviati *et al.*, 1982b). No significant difference has been reported between 2A and 2B fast fibre types.

There is evidence that the electrical membrane properties also differ between slow and fast fibres. The functional demands placed by the motor neurone discharge rates on membrane properties of muscle fibres are very different. Slow fibres must be able to generate action potentials during prolonged firing at low rate, without losing excitability despite the accumulation of potassium in extracellular space and particularly in T tubules. Fast fibres, and among them 2B fibres more than 2A fibres, need to recover excitability quickly after each action potential, but do not need to maintain excitability for long periods as fast motor unit discharge occurs in short bursts of high frequency (Henneman *et al.*, 1974; Monster *et al.*, 1978). Distribution and functional properties of ionic channels and ionic pumps on the sarcolemma probably reflect the need to comply with these requirements (Duval and Leoty, 1980; Ruff and Whittlesey, 1992; Everts and Clausen, 1992).

2.6 Fatigue Resistance is Related to the Balance Between Energy Production and Energy Consumption

The energy required to support the contractile activity is provided by ATP hydrolysis to ADP and inorganic phosphate (Pi). ADP is then re-synthesized to ATP from PCr through the creatin-kinase reaction and PCr is in turn regenerated from ATP produced by glycolytic processes in the sarcoplasm and by oxidative phosphorylation in the mitochondria. Glycolytic processes represent the initial stages of glycogen and glucose metabolism and lead to pyruvate or lactate production. Pyruvate, fatty acids and ketone bodies provide a supply of acetyl-CoA, which is the substrate for the mitochondrial oxidative processes.

The correlation between motor unit studies and histochemical determination of enzymatic activity showed that: (i) metabolic properties of the fibres belonging to the same motor unit are substantially homogeneous; and (ii) fatigue resistance is related to high oxidative activity and fatiguability to low oxidative activity (Kugelberg and Edstrom, 1968; Burke *et al.*, 1971, 1973; Kugelberg, 1973).

The micro-determinations of enzymatic activities on single fibres were first developed by Lowry and by Essen and provided a tool to quantitatively assay glycolytic and oxidative enzymatic activities in single muscle fibres (Essen *et al.*, 1975; Lowry *et al.*, 1978; Pette and Spamer, 1986) dissected from human muscle biopsies and from animal muscles.

The metabolic diversity among muscle fibre types has a structural basis: oxidative aerobic metabolism needs an oxygen supply from the capillary vessels, oxygen storage in myoglobin and oxygen utilization by the respiratory coenzyme chain. Capillary density is higher in muscles rich in oxidative fibres than in those rich in glycolytic fibres and the same is true for myoglobin concentration (Kayar *et al.*, 1988; Krenacs *et al.*, 1989). Mitochondrial content varies between fibre types and correlates with oxygen uptake and oxidative enzyme activities (Krenacs *et al.*, 1989; Taylor and Bachman, 1999). For example, in human muscles the fibre volume occupied by mitochondria ranges from 6% in type 1 fibres to 4.5% in type 2A fibres and 2.3% in type 2X fibres (Howald *et al.*, 1985).

A second source of diversity is the presence of specific isoforms of metabolic enzymes with different activities. For example, five isoforms of lactate dehydrogenase (LDH) are expressed in rabbit muscles: slow fibres express all isoforms with a marked predominance of LDH-1, LDH-2 and LDH-3, whereas in fast fibres LDH-5 is the predominant or, in some cases, the only isoform expressed (Leberer and Pette, 1984). In guinea pig muscles five isoforms have also been separated: H4, H3-M, H2-M2, HM3 and M4 (Peter *et al.*, 1971). The isoform M4 is predominant in glycolytic fibres, whereas in oxidative fibres H4 is predominant. A correlation between the abundance of M4 and the proportion of 2B fibres has also been found in lamb muscles (Sayd *et al.*, 1998).

Muscle fibres differ in the availability of energetic substrates and high-energy phosphates. All fibres contain stores of glycogen and lipids (see Gerbens, Chapter 16, this volume) to be used as substrates for energy production, in addition to glucose, fatty acids and amino acids that can be taken up from the blood supply. Glycogen content at rest is higher in fast than in slow fibres (Vollenstad *et al.*, 1984); on the other hand lipid content is greater in slow than in fast fibres (Howald *et al.*, 1985). In resting muscles PCr content is higher in fast than in slow fibres, whereas ATP

content is rather similar in slow and fast fibres. Pi content is higher in slow than in fast fibres: in small mammals the difference can reach 0–2 mM in fast muscles versus 3.6–6.7 in slow muscles (Kushmerick *et al.*, 1992).

The rate of energy consumption in muscle fibres is very low at rest and increases many fold during contractile activity; the total rate of energy consumption can be estimated from the sum of the ATP consumed by myofibrillar ATPase and ATP consumed by ionic transport (Stienen *et al.*, 1995) and, as discussed above, both these values are different in slow and in fast fibres.

The energy balance of the muscle fibres requires that ATP consumption rate is completely compensated by ATP resynthesis rate. The first and faster reaction is catalysed by creatin kinase, which allows conversion of ADP to ATP spending energy stored in PCr. Glycolytic and oxidative ATP synthesis allow PCr recovery: both processes show a clear difference between fast and slow fibres (Fig. 2.6). Probably activated by the increase of AMP and IMP concentrations, glycogenolysis promotes the PCr resynthesis based on the glycolytic pathway. Single fibre determinations during maximal contractions elicited with electrical stimulation show a clear-cut difference of metabolic power between slow and fast fibres: 0.18 mmol/kg dw/s in slow fibres vs. 3.54 mmol/kg dw/s in fast fibres (Greenhaff *et al.*, 1993). Aerobic oxidative ATP generation also reveals significant differences between skeletal muscle fibre types (Reichmann and Pette, 1982; Essen-Gustavsson and Henriksson, 1984). The relative contribution of anaerobic and aerobic processes to the maximal rate of ATP generation in human muscles is shown in Fig. 2.6.

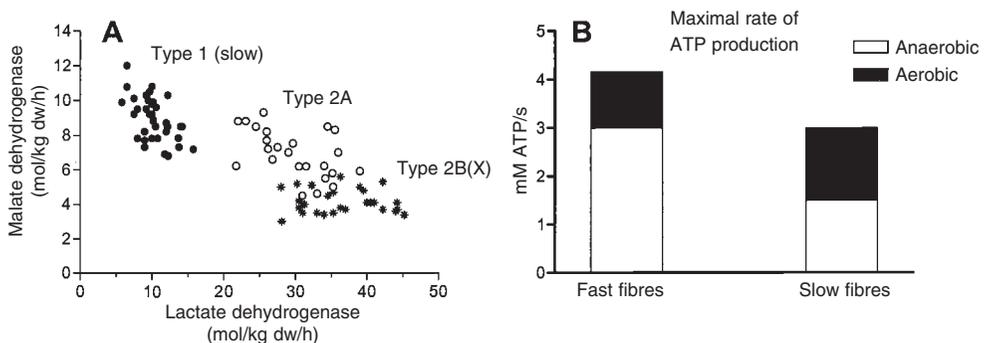


Fig. 2.6. Diversity of metabolic properties among fibre types in human muscles. (A) Inverse correlation between the activity of malate dehydrogenase (aerobic oxidative metabolism) and lactate dehydrogenase (anaerobic metabolism) in three fibre types (1, 2A and 2X): note the variability inside each fibre type (data from Rosser and Hochamba, 1993). (B) Metabolic power of human slow and fast fibres. The relative contributions of anaerobic and aerobic metabolism are indicated: note that the total power is higher in fast than in slow fibres, but in the latter the fraction of aerobic energy production is higher (data from Greenhaff *et al.*, 1993).

2.7 Muscle Fibre Type Composition in Adult Livestock Animals

The aim of this section is to review the information presently available on fibre type composition of some mammalian species of agricultural and veterinarian interest. The analysis will be restricted to adult muscles and nothing will be said about the

developmental phase, which may also be important for alimentary purposes, and about the progressive disappearance of immature muscle fibres, expressing embryonic and neonatal myosins.

2.7.1 Horse

In the horse, fibre type composition of muscles has received considerable interest in view of a possible relationship between sport performance and training. Several studies have shown that muscle fibre compositions of different breeds (for example quarter horse, Thoroughbred, Arab, heavy hunter, Shetland pony) were different and correlated with their sport performances (Snow and Guy, 1980; Roneus *et al.*, 1991, 1992; Snow and Valberg, 1994; Rivero and Henckel, 1996). Other studies have shown that training reduces the proportion of 2B (or 2X) fibres in favour of an increase of type I and 2A fibres (Roneus *et al.*, 1992; Rivero *et al.*, 1996a; Serrano and Rivero, 2000; Dingboom, 2002). An increased proportion of fast 2A fibres in gluteus medius has been reported by Dingboom *et al.* (1999) in a comparison between Dutch warmblood foals reared in box or trained with daily gallop sprints. These studies have been generally carried out on biopsy samples and gluteus medius is the most frequently studied muscle because of its implication in locomotion.

Early studies (Gunn, 1978; Snow and Guy, 1980) identified only two fibre types (type 1 or slow and type 2 or fast) using the m-ATPase reaction. Later studies (Essen *et al.*, 1980; Snow *et al.*, 1981) using improved m-ATPase reaction distinguished two subgroups of fast fibres, called 2A and 2B; however, only two fibre types were identified using antibody staining and only two bands were detected on electrophoretic gels (Sosnicki *et al.*, 1989). The combined use of m-ATPase and metabolic enzyme staining confirmed the presence of two subpopulations of fast fibres, one more glycolytic and one more oxidative (Lopez *et al.*, 1992). Sinha *et al.* (1992), on the basis of the lack of reactivity of specific monoclonal antibodies, questioned whether the most glycolytic fibres actually expressed 2B myosin. The results were re-interpreted assuming that the second fast isoform, expressed in the less oxidative fibres, was myosin 2X and not myosin 2B (Serrano *et al.*, 1996). The identification of the third myosin as 2X was also supported by the position of the electrophoretic band close to the 2A band and the lack of reactivity to the antibodies BF-G6 (selective for 2B myosin in small rodents) and BF-35 (reactive with all myosin except 2X in the rat).

This approach to fibre type identification is based on the correlation between histochemistry and immunohistochemistry comparing the results obtained in laboratory animal muscles with results obtained in equine muscles. The two possible limitations are that: (i) only one muscle (gluteus medius) has been analysed; and (ii) many hybrid fibres (1-2A and 2A-2X) are present and might become even more frequent with training and de-training. A significant presence of hybrid 1-2A and 2A-2X fibres has been reported (Rivero *et al.*, 1999; Dingboom *et al.*, 1999); this implies an underestimation of the frequency of type 2A and overestimation of type 2X when histochemistry is compared with immunohistochemistry. It is not clear whether these hybrid fibres represent transitional fibres or are stable fibres with mixed MHC isoform composition; in both cases, these observations underline the need to carefully reconsider all results obtained on training and de-training in equine muscles.

The determination of the sequence of the expressed myosin isoforms is the only

approach for an unambiguous interpretation, but this type of information is not presently available for equine muscles. An important contribution can come from the functional characterization of fibre types in equine muscles: in all mammalian species studied up to now, precise relationships exist among the values of maximum shortening velocity of various fibre types. In equine muscles maximum shortening velocity and isometric tension in three groups of single fibres dissected from soleus have been measured by Rome *et al.* (1990) and compared with values obtained in corresponding fibre types in rat and rabbit.

2.7.2 Ruminants

The classification of fibre types in ruminants on the basis of the m-ATPase reaction is rather difficult due to the narrow ranges of acid and alkali resistance of various fibre types. Fibres identified with m-ATPase staining can be then further classified as glycolytic or oxidative using metabolic enzyme histochemistry, but the results are uncertain.

2.7.2.1 Cattle

The interest in fibre type composition of bovine muscles is related to the assumption that it may play a part in determining meat quality and tenderness. For example, post-mortem meat tenderization might be influenced by the fibre type composition. Muscles with a large proportion of fast glycolytic fibres have a higher speed of ageing: fibre type composition varies with rearing conditions and genotype (Jurie *et al.*, 1995a). Physiological status and feeding might affect fibre type composition (Brandstetter *et al.*, 1998). Furthermore, the analysis of fibre type composition has contributed to the understanding of the mechanism of development of greater muscle mass in carriers of the phenotype 'double muscle' (Holmes and Ashmore, 1972; Hendricks *et al.*, 1973; Swatland and Kieffer, 1974).

m-ATPase staining has allowed the separation of slow and fast fibres and later on also the separation of subpopulations (called 2A and 2B) of fast fibres (Jurie *et al.*, 1995a). All more recently published studies agree on the identification of slow and type 2A fibres, but there are contrasting views on the identification of the second fast type. The difficulties arise from the fact that histochemical methods are not sufficient to discriminate and that no specific antibody is available to distinguish between MHC-2X or MHC-2B. Also electrophoresis does not provide a clear separation of fast MHC isoforms.

Picard and co-workers (Picard *et al.*, 1998) have compared several classification systems for bovine muscle fibres: (i) m-ATPase with modifications introduced by the authors, which led to the identification of 1, 2A and 2B; (ii) m-ATPase combined with enzyme histochemistry already used by the authors in a previous study (Jurie *et al.*, 1995b); and (iii) antibody staining. The different methods show discrepancies in classifying the hybrid fibres that are rather frequent (8% in semitendinosus, 11% in longissimus): hybrid fibres are differently classified depending on the method used.

Using gel electrophoresis only two bands (slow and fast) were identified in the early studies, but later studies have identified two fast MHC isoforms (Totland and Kryvi, 1991; Jurie *et al.*, 1995b). More recently, a modified protocol of SDS-PAGE has shown the presence of three fast MHC isoforms, characterized by a migration

rate slower than slow MHC (Picard *et al.*, 1999). The three fast isoforms, which may be identified as 2B, 2X and 2A, are present in all muscle examined (semitendinosus, triceps brachii, longissimus thoracis and biceps femoris) except the masseter, where only slow MHC is present. This seems to be clear evidence in favour of the expression of three fast MHC in bovine muscles.

The use of antibodies and/or gel electrophoresis to classify fibre types on the basis of the MHC isoforms has given contrasting results. Evidence supporting the view that the fibres originally called 2B express either 2X and/or 2B MHC has been given (Picard *et al.*, 1999; Duris *et al.*, 2000), but the antibodies used are not very effective to distinguish MHC-2X from MHC-2B. The antibody BF-35 generally reacts with all MHC except 2X MHC. In bovine muscles BF-35 seems to leave unstained fibres identified as 2B (Duris *et al.*, 2000): fibres reacting with BF-35 should therefore be identified as 2X (see Table 2.2). The antibody S5-8H2 reacts with all MHC, including 2X, but leaves 2A unstained, as shown in pig, horse and llama (see Table 2.2). In bovine muscles S5 8H2 stains slow and 2B fibres, therefore leaving 2A and 2X unstained. Such reasoning may lead to the conclusion that either the fibres generally called 2B form a double population containing 2X and 2B fibres or they all express only MHC-2X.

The expression of only two fast MHC isoforms (2A and 2X) in addition to the slow MHC isoform has been demonstrated by Tanabe *et al.* (1998) in bovine muscles with RT-PCR of the 5' non-coding regions. The three isoforms are expressed in semitendinosus and longissimus, whereas only slow and fast 2A are expressed in diaphragm and tongue, in agreement with Duris *et al.* (2000). However, in contrast with Picard's observation, no indication of expression of a 2B MHC isoform has been found (Tanabe *et al.*, 1998). In our view, a possible explanation is that the fibres identified as 2B by Picard and co-workers (Picard *et al.*, 1999) are 2X fibres and the third fast MHC found on gel electrophoresis is artefactual.

2.7.2.2 Goat and sheep

As for bovine muscles, using m-ATPase with a modified molarity of the buffer for acid pre-incubation, three fibre groups can be identified in ovine muscles. Two fast types are detectable: 2A fibres closely resemble those of other mammalian species as confirmed also by the use of specific antibodies, whereas 2B fibres exhibit features that might lead to their identification as 2X fibres (Mascarello and Rowleson, 1995). Gel electrophoresis also shows two closely adjacent bands, one of which should correspond to MHC-2A and the other might correspond to MHC-2X better than to MHC-2B (Maier *et al.*, 1992).

In a recent comparative study on different lamb breeds three methods have been combined: (i) m-ATPase after acid pre-incubation, which led to separate slow (strongly reactive), 2A (completely unreactive) and 2B (moderately reactive) fibres; (ii) SDH staining, which allowed a further distinction of 2B fibres in oxidative (2Bo) and non-oxidative (2Bno); and (iii) SDS-PAGE, which showed three slow migrating MHC bands in addition to slow MHC (Sayd *et al.*, 1998). According to the authors further studies are required to finally define how many MHC isoforms are expressed in sheep muscles.

In the semitendinosus muscle of the goat using SDS-PAGE, m-ATPase and monoclonal antibody staining the expression of three MHC isoforms has been recently demonstrated: one slow and two fast (Arguello *et al.*, 2001). Of the two fast MHCs one clearly appears to be a MHC-2A, whereas the second has been tenta-

tively identified as MHC-2X, on the basis of the electrophoretic migration rate and lack of reactivity with BF-35. The correlation between the indication of m-ATPase staining and antibody staining is high in pure fibres and low in hybrid fibres, which form a substantial population in goat semitendinosus: for example, 2A-2X represent 21% of the total.

2.7.3 Llama

In a recent study (Graziotti *et al.*, 2001) muscle fibres of the llama have been classified for the first time on the basis of their MHC isoform composition combining m-ATPase staining and monoclonal antibody staining. The reactivity for monoclonal antibodies in the llama muscles is peculiar: S5-8H2 reacts with all MHC except MHC-2A; SC-71, which is highly specific for MHC-2A in other species, reacts with both MHC-2A and MHC-2B; BF-35, which identifies MHC-2X for negative staining in many species, leaves both MHC-2X and MHC-2B unstained in the llama. The results show that four MHC isoforms (slow, 2A, 2X and 2B) are expressed in the semitendinosus, biceps femoris and vastus lateralis of the llama giving origin to four pure fibres and four hybrid fibres (1-2A, 2A-2X, 2X-2B, 2A-2X-2B), the latter representing about 40% of the total population. An organized pattern with islets of slow fibres surrounded by fast fibres has been reported. An electrophoretic analysis of MHC isoforms in llama muscles, which might confirm the presence of a third fast isoform, is still lacking.

2.7.4 Pig

The fibre type composition of porcine muscle has been the object of many studies aiming to describe the effects of the selective pressure and the differences among pig breeds. Four MHC isoforms are expressed in pig muscles as shown on the basis of RT-PCR amplification and sequencing of all four transcripts (Chang and Fernandes, 1997) and subsequently confirmed by histochemistry (combined use of m-ATPase and NADH staining; Gil *et al.*, 2001), immunohistochemistry using monoclonal anti-myosin antibodies (Lefaucheur *et al.*, 1998) and *in situ* hybridization (Lefaucheur *et al.*, 2002). The four genes coding for adult sarcomeric MHC isoforms have been completely sequenced (Chikuni *et al.*, 2001). However, up to now, only three bands have been separated using gel electrophoresis (Bee *et al.*, 1999).

Fibres of different types are organized in pig muscles in a typical pattern with islets of slow fibres surrounded by fast oxidative fibres (2A and 2X) and at the periphery by fast non-oxidative fibres (2B). The islet pattern is the result of subsequent waves of myogenesis (Lefaucheur *et al.*, 1995), but might have functional implications in view of the gradual recruitment of fibres in relation to the intensity of exercise (Lefaucheur *et al.*, 2002). The presence of hybrid fibres seems to be common in porcine muscles, particularly regarding 2X and 2B MHC (Lefaucheur *et al.*, 2002). Differences in fibre type composition among pig breeds have been reported (da Costa *et al.*, 2002) and a significant relationship between a high proportion of 2B fibres and the presence of the halothane gene (*n*, corresponding to mutated *RyR1* gene) has been described (Depreux *et al.*, 2002). The abundance of 2B fibres has also been correlated with lower post-mortem pH values, a factor which might reduce meat quality (Depreux *et al.*, 2002).

2.8 Conclusions

The concept of fibre type is evolving, as are the requirements for fibre type identification. Fibre types arise from a complex pattern of coordinated gene expression, but the main criterion for identification is still represented by the identification of the MHC isoforms expressed. Any method based on electrophoretic separation, histochemistry and immunohistochemistry may show its limitation when applied from one species to the other.

Histochemical methods, if well applied, can distinguish three fibre groups: slow, fast 2A and a third group, which contains 2X and/or 2B fibres; 2X and 2B fibres are virtually undistinguishable. Moreover the identification is made difficult by the presence of hybrid fibres. As shown by the direct comparison of antibody reactivity in different species (Table 2.2), immunostaining may also give contrasting results: for example, BF-35, generally used to identify 2X fibres by negative staining, seems to leave 2B fibres unstained in cow and llama muscles. On the other hand, SC-71, which is generally considered very specific for 2A fibres, seems also to react with 2X fibres in llama, cow, pig and goat. BF-F3, which is often used for 2B fibres, gives uncertain results even in species where the presence of 2B fibres is clearly demonstrated. The possible explanation of this variability in antibody reactivity is that minor variations among orthologue MHC isoforms are sufficient to cancel or alter the epitopes recognized by monoclonal antibodies. Another possible explanation is given by the presence of a very high proportion of hybrid fibres in muscles of large mammals: the presence of even small amounts of a second MHC can completely modify the reaction with antibodies.

Electrophoretic separation requires continuous adaptations to obtain reliable results in different species. Electrophoretic bands are often close to each other and can easily mask each other: this can, for example, explain why in pig muscles only three bands are detectable although four MHC isoforms are clearly documented. Moreover the sensitivity is low: an isoform present in a low proportion (say less than 3%) can easily remain undetected. This creates problems opposite to those determined by the high sensitivity of the antibody staining.

The only safe method is represented by identification and sequencing of the genes coding for MHC isoforms and analysis of their expression in muscle fibres. The identification of the genes allows the precise interspecies comparison; the homology between paralogue MHCs is much less than that between orthologue MHCs. The identification of the expressed mRNA either by *in situ* hybridization or by RT-PCR gives precise information on which isoform is expressed in a given fibre.

Single fibre studies, i.e. biochemical or functional analysis of individual fibres dissected from muscles, also give useful information. Many fibres express only, or predominantly, one MHC isoform: they are often indicated as 'pure fibres'. The electrophoresis and the immunoblotting on single pure fibres can be of great help for identification. Functional experiments on single fibres provide values of parameters, as V_0 , which are directly determined by MHC isoforms: in all species examined to date V_0 shows values increasing in the order $1 < 2A < 2X < 2B$.

Functional diversity among fibre types gives muscles the possibility of optimizing their responses: resistance to fatigue, power output and speed of contraction are the most relevant parameters to characterize fibre types. The fibre types most suited for each motor task are selectively recruited by nervous control mechanisms, and this

allows the optimization of the muscle performance for locomotion or for vegetative (eating, breathing) needs. The variations of the properties of each fibre type from species to species allow a further adaptation to the specific motor requirements of each animal. Finally, the stable presence of a high proportion of hybrid fibres probably gives a further opportunity for fine tuning of the contractile properties of many muscles.

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3

Manipulation of Muscle Fibre Number During Prenatal Development

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3.1 Introduction

Muscle mass is predominantly a function of muscle fibre size and number. Other constituents of muscle, such as connective tissue, are of lesser importance in determining muscle mass. Chapter 1 highlighted the positive relationship between total muscle fibre number within muscles and muscle mass, lean meat percentage and growth efficiency. There is often an inverse relationship between muscle fibre number and size. Although muscle fibre hypertrophy will contribute to muscle mass, it appears that enhanced fibre hypertrophy often has detrimental influences on meat quality (see also Kambadur *et al.*, Chapter 14, this volume). Taken overall, therefore, it would appear that, in terms of meat production, high muscle fibre number will be advantageous for optimizing lean meat percentage, growth efficiency and meat quality. However, for most livestock species, muscle fibre number is largely fixed by

about the time of birth. Postnatal muscle growth continues by muscle fibre hypertrophy. This means that any manipulation of muscle fibre number must be undertaken prenatally. It could be argued, in fact, that the meat potential of an animal is indeed determined before birth. The purpose of this chapter is to explain how prenatal muscle fibre hyperplasia is achieved and to explore possible methods for manipulation of muscle fibre number during prenatal development. Naturally occurring variations in hyperplasia will be discussed as well as various experimental methods for altering fibre number, such as prenatal nutrition and ‘growth promoters’. For fish species, the role of temperature on fibre hyperplasia will also be briefly explored.

3.2 Prenatal Muscle Development

Mammalian muscle tissue exhibits a biphasic pattern of development (Kelly and Zacks, 1969). Myoblasts (derived from myogenic precursor cells in the mesoderm) proliferate within the presumptive muscles. Some of the myoblasts then line up and fuse to form a population of large primary myofibres. These large primaries usually exhibit slow myosin ATPase activity (Fig. 3.1A). Other myoblasts then line up on the surface of the primaries and fuse to form a larger population of smaller secondary myofibres (see Rehfeldt *et al.*, Chapter 1, this volume). Some myoblasts will remain under the basal lamina of all muscle fibres as satellite cells, which enable fibres to hypertrophy. The secondaries do not normally exhibit slow myosin ATPase activity although some will become slow during later prenatal development and more will differentiate into slow as body weight increases postnatally (Handel and Stickland, 1987a). In the pig, the myofibres once formed remain in their original relative positions. This means that postnatal pig muscle is characterized by clusters of slow fibres surrounded by fast fibres (Fig. 3.1B). One of the fibres in each slow cluster would have been the primary myofibre during development. In most other species the myofibres become mixed so that this pattern is not seen. The number of secondary myofibres which form around each primary varies between species (and muscle) from one or two in the mouse to 20 or so in the pig.

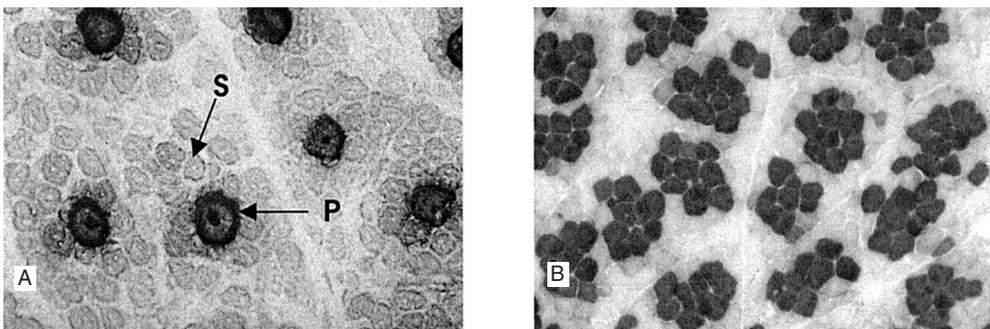


Fig. 3.1. (A) Section of porcine semitendinosus muscle stained for slow myosin ATPase at about 75 days gestation; P, primary myofibre; S, secondary myofibre. Slow fibres are black. (B) Section of adult porcine semitendinosus muscle (about 6 months) stained for slow myosin ATPase.

In the pig, the primary myofibres form up to about 60 days gestation and secondary myofibres from about 54 to 90 days' gestation (Wigmore and Stickland, 1983a) (full term is about 113 days). In the cow, primaries form up to about 125 days and secondaries from 100 days to near term (9 months) (inferred from Stickland, 1978). In the sheep the timing is 32 to 38 days for primary and 38 to 62 days for secondaries (term is 5 months) (Wilson *et al.*, 1992). In the sheep there also appears to be a third generation of developing muscle fibres. A small population of secondary fibres move away from the primaries and then support the formation of a third generation of myofibres (from 62 days' gestation), the tertiary myofibres (Maier *et al.*, 1992; Wilson *et al.*, 1992). In the sheep peroneus longus muscle myogenesis is completed by about 115 days' gestation (Greenwood *et al.*, 1999). Some authors also claim that the pig may show some tertiary myofibre formation (Mascarello *et al.*, 1992; Lefaucher *et al.*, 1995). In the chicken, primary myofibres form from about 6 days' incubation (Takeura *et al.*, 1994) and secondaries from 12 to 16 days (hatching is 21 days) (McLennan, 1983).

In fish such as rainbow trout, muscle fibres do not appear to form in the same biphasic manner, i.e. there are no identifiable primary and secondary myofibres. Most fish also exhibit indeterminate growth (i.e. continual growth throughout life) and this is reflected in the ability to produce new muscle fibres throughout life (Stickland, 1983). Three phases of muscle development and growth can be identified in the rainbow trout and other species. Initially muscle fibres form throughout the developing myotome and, in late larval stages, this is followed in many fish by a hypertrophic phase with negligible fibre hyperplasia (Koumans *et al.*, 1991). Fibre hyperplasia then commences again, particularly in dorsal and ventral caps of the somites (Lopez-Albors *et al.*, 1998) but new small fibres can also be identified throughout the myotome. The source of new muscle fibres is still not clear in fish but there is some evidence that a significant source of precursor cells exists within the myosepta (Stoiber and Sanger, 1996).

3.3 Natural Variations in Development

There are a number of naturally occurring factors that will influence the number of muscle fibres that form during prenatal development. These factors include species and breed. Larger species tend to produce more muscle fibres in their muscles than smaller species. Mature muscle fibre size, on the other hand, is not generally related to species size. There is also evidence that, within a species, there are breed differences in muscle fibre number parameters. For some muscles and in some species there would also appear to be an influence of gender on muscle fibre number. These factors are discussed in more detail by Rehfeldt *et al.* (Chapter 1, this volume).

Some situations of extreme muscle hypertrophy have been shown to be consequences of gene mutations. These mutations include the *myostatin* gene in cattle (with double-muscling), the ryanodine receptor gene (*RyR*) in the pig and the *callipyge* gene in sheep. These genes are discussed in detail in Chapter 1 (Rehfeldt *et al.*), Chapter 14 (Kambadur *et al.*) and Chapter 15 (Freking *et al.*) of this volume. Only in the case of the *myostatin* gene is there clear evidence for an increase in muscle fibre number.

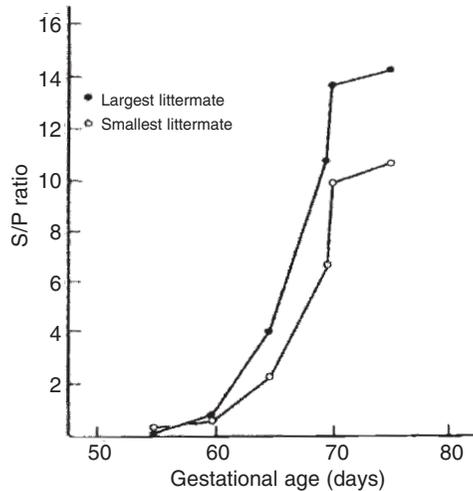


Fig. 3.2. Secondary:primary myofibre ratio (S/P) during gestation in large and small littermate pairs. Taken from Wigmore and Stickland (1983a) with permission from Blackwell Publishing Ltd.

In multiparous species such as the pig, intralitter variation in muscle fibre number has been demonstrated. Wigmore and Stickland (1983a) showed that large fetuses within porcine litters generally exhibited higher muscle fibre numbers in complete sections of the semitendinosus muscle than small fetuses. They concluded that most of the variation was due to a difference in the number of secondary myofibres which formed around each primary (Fig. 3.2). The diameter of the primary myofibres was greater in the larger fetuses and the larger primaries appeared to support more developing secondaries. The larger fetuses also contained more DNA in their muscles than the small fetuses (Wigmore and Stickland, 1983b). The importance of these variations in muscle fibre number between littermates is highlighted by the work of Handel and Stickland (1988), which showed that only piglets with high fibre number were able to exhibit postnatal catch-up growth. On average, postnatal growth and feed conversion efficiency, from 25 kg weight to slaughter, is positively correlated with muscle fibre number and not, for example, with birth weight (Dwyer *et al.*, 1993).

It is likely that much of the intralitter variation in both body size and fibre number is due to differences in nutritional supply. Indeed the U-shaped (McLaren *et al.*, 1960; Perry and Rowell, 1969) distribution of body size usually seen along the uterine horn, with the largest at each end, would tend to support this claim. Furthermore, the experimental work on maternal nutrition during pregnancy also supports this hypothesis, and is explained in the next section (3.4).

Studies on sheep have shown that fetal size (Greenwood *et al.*, 1999) or litter number (singletons versus twins) (McCoard *et al.*, 2000a,b) has no significant influence on muscle fibre number. However, when an added constraint, season, is considered, McCoard *et al.* (1997) found that fibre number was differentially affected by season and fetal number. In pigs, extremes in birth weights have been shown to relate to a difference in muscle fibre number (Handel and Stickland, 1987b).

3.4 Prenatal Nutrition

Several studies have shown that the number of muscle fibres in a given animal can be significantly altered by manipulating the maternal food intake during gestation (Bedi *et al.*, 1982; Wilson *et al.*, 1988; Ward and Stickland, 1991; Dwyer *et al.*, 1994, 1995) whereas postnatally, starvation can no longer affect this number. This is illustrated by studies carried out in various species including rat, rabbit, guinea pigs and hamster (Hegarty and Kim, 1981; Timson and Dudenhofer, 1990) that showed starvation in young animals induces a decrease in muscle fibre diameter but has no effect on the number of muscle fibres. The exception is the study by Layman *et al.* (1981), which showed that severe dietary restriction to 25% of *ad libitum* over a period of 9 days in weanling rats of 100 g caused a decrease in both number and diameter of muscle fibres in various muscles. However, the rat is very immature at birth and muscle fibre hyperplasia continues for a while postnatally (Condon *et al.*, 1990).

Inadequate maternal nutrition appears to lead to a reduction in the number of secondary myofibres that form, although primary fibres are not affected (Bedi *et al.*, 1982; Ward and Stickland, 1991). More recent work has been aimed at identifying the most critical period during gestation when fetal muscle development is most susceptible to maternal undernutrition. Experiments with guinea pigs have shown that feed restriction within the first half of gestation produced the same 25% reduction in secondary myofibre number in the offspring as restricted feeding throughout gestation (Dwyer *et al.*, 1995). If undernutrition occurs late in gestation, it has no effect on the muscle in the offspring. A study by Ezekwe and Opoku (1988) showed that pregnant gilts undernourished for either 7 or 14 days from the 100th or 107th day of gestation gave birth to fetuses with reduced DNA content in the gastrocnemius muscle but the reduction was not maintained in the muscle 49 days postnatally. Other parameters such as muscle mass, protein, RNA and protein/DNA and RNA/DNA ratios were unchanged in the gastrocnemius muscle of offspring from these undernourished mothers at birth or 49 days postnatally. The authors concluded that complete feed deprivation from 7 to 14 days before parturition in gilts did not cause permanent growth retardation.

Therefore there are crucial periods in gestation where maternal undernutrition alters the number of muscle fibres in the offspring. In the rat maternal feed restriction up to birth produced the same deficit in fibre numbers as restriction up to weaning (Bedi *et al.*, 1982; Wilson *et al.*, 1988). Therefore, in most mammals, quantitative nutritional restriction prior to the formation of the majority of secondary myofibres significantly affects the number of muscle fibres that form in the offspring.

Dwyer and Stickland (1994) showed that qualitative changes in a restricted diet could prevent the decrease in the muscle fibres that formed in the offspring. Their study carried out in the guinea pig showed that the number of muscle fibres that formed in the bicep brachii of the offspring from pregnant females fed a 60% restricted-fat supplemented diet was reduced by about 15%. In contrast, when this diet was supplemented with carbohydrates or proteins, the number of muscle fibres that formed in the offspring was restored to levels similar to control. Slow and fast muscles are differentially affected by maternal undernutrition. A study carried out in the guinea pig by Dwyer and Stickland (1992a) showed that skeletal muscles with a high proportion of slow type fibres such as the soleus muscle were less affected by maternal undernutrition than other muscles with higher proportions of fast fibres. A

study carried out in sheep (Palmer *et al.*, 1998) showed that an increased food intake well above maternal maintenance (15 kg per week instead of 5 kg) between days 4 and 104 of gestation reduced the mass, DNA, RNA and protein content in the plantaris muscle of the fetuses whereas these parameters were increased in the plantaris muscle of the mother. The detrimental effect of maternal overfeeding on muscle growth in the lamb can be explained by alterations in the placenta growth. Wallace *et al.* (2000) showed that over-nourishing pregnant sheep largely reduced the placental mass as well as the placental RNA, DNA and protein contents leading to gradual slowing of fetal organ growth including muscle tissue.

3.4.1 Insulin-like growth factors and nutrition

Many growth factors influence proliferation and differentiation during myogenesis (Florini *et al.*, 1991; Chambers and McDermott, 1996; Perry and Rudnicki, 2000). Of central significance, in terms of nutritional regulation of myogenesis, is the insulin-like growth factor (IGF) system. IGF-I and IGF-II actions are mediated by IGF binding proteins (IGFBPs 1–6) and the receptors IGFIR and IGFIIR. Growth hormone (GH) initiates the release of the major source of circulating IGF-I from the liver, to act on muscle and other tissues. IGF-I is an endocrine and autocrine/paracrine growth factor that has a wide range of effects, including stimulation of erythropoiesis, anabolism, cell growth, cell differentiation and satellite cell proliferation (Cohick and Clemmons, 1993; Frey *et al.*, 1995; Florini *et al.*, 1996; Johnson *et al.*, 1998; Brink *et al.*, 2001). IGF-I, as a regulator of myogenesis (Daughaday and Rotwein, 1989; Magri *et al.*, 1991; Jones and Clemmons, 1995), stimulates differentiation by inducing expression of the myogenin gene, thereby up-regulating myogenin necessary for terminal differentiation in myogenesis (Florini *et al.*, 1991). Although necessary for differentiation, it is also apparent that high levels of IGF-I can inhibit differentiation probably through inhibition of myogenin expression by cyclin D1, expressed by proliferating cells (Rao *et al.*, 1994).

Nutritional status is an important systemic modulator of IGF-I pre- and postnatally (Jones *et al.*, 1990; Rotwein, 1991; Cohick and Clemmons, 1993; Osgerby *et al.*, 2002). In adult starvation or maternal undernutrition, IGF-I levels quickly fall (Gluckman *et al.*, 1991 (in sheep and rats); Dwyer and Stickland, 1992b (in guinea pigs); Thissen *et al.*, 1999 (in adult humans)), as does hepatic IGF-I production, due to a reduction in GH receptors (Gluckman *et al.*, 1991). This decreases the amount of IGF-I accessible to bind to IGF-I receptors. This problem is counteracted by an upregulation in the number of IGF-I receptors in starvation (Thissen *et al.*, 1999; Brink *et al.*, 2001) and this may be one reason why satellite cells from small neonatal littermates proliferate more than cells from large littermates when grown *in vitro* in the same medium (Clelland and Stickland, 2001). IGF-II is also depressed by fetal malnutrition (Jones *et al.*, 1990 (in guinea pig)).

In the sheep, maternal overfeeding, which alters placental and fetal growth and reduces muscle mass in the offspring, is accompanied by a reduction in fetal plasma IGF-I concentration whereas these levels are higher in the ewe (Wallace *et al.*, 2000).

It is evident from the foregoing that maternal nutrition can significantly influence fibre number determination in the offspring. In commercial pig production maternal feed intake is often reduced to perhaps 50% of *ad libitum*, particularly in

early critical stages of gestation. Dwyer *et al.* (1994) showed that doubling feed intake during certain gestational periods could enhance muscle fibre number in the offspring as well as narrowing the intralitter variation in fibre number. The offspring also exhibited faster and more efficient postnatal growth.

3.5 Growth promoters

3.5.1 Growth hormone

Sows given porcine somatotrophin (pST) during early gestation (10–27 days) increased semitendinosus muscle fibre number in their offspring (Rehfeldt *et al.*, 1993, 2001b, 2002). Increases were especially observed in middle- and light-weight piglets within litters and were associated with higher birth weights (Fig. 3.3). The numbers of both primary and secondary fibres were higher; the secondary:primary ratio remained unchanged. This is different from the nutritional situation (see above). Associated increases in the expression of *myoD* and *myf-5* gestation and muscular DNA concentrations at birth suggest that elevated maternal GH concentrations induced increased myoblast proliferation and/or commitment. Under treatment higher circulating concentrations of maternal IGF-I as well as glucose and

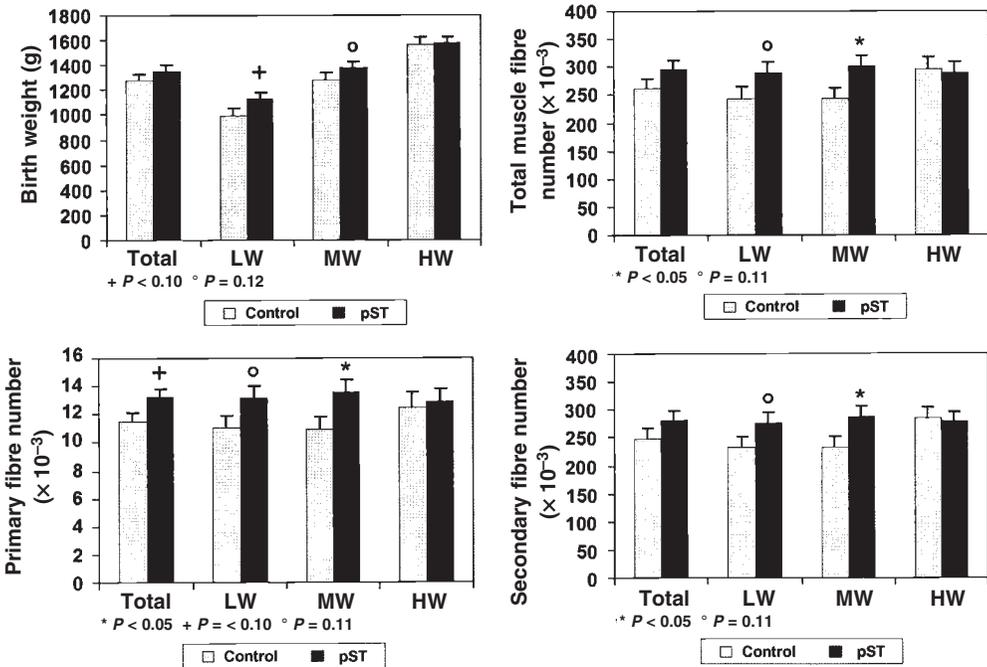


Fig. 3.3. Birth weight and semitendinosus muscle fibre number of newborn piglets born to gilts treated with porcine somatotrophin (pST) during early gestation (days 10 to 27; summary of two experiments, $n = 79$). Data are separately shown for the piglets of the lightest (LW, but > 800 g), middle (MW) and heaviest (HW) birth weight within the litter (Rehfeldt *et al.*, 2001b, 2002).

free fatty acids were observed (Schneider *et al.*, 2002). Also nutrient availability to the embryo was enhanced and placental growth was intensified (Rehfeldt *et al.*, 2001a). Possibly, the growth conditions for the smaller littermates are less than optimal and, consequently, only these may profit by additional maternal nutrients and growth factors. As a result of elevated circulating pST the litters are more balanced in terms of birth weight and muscle structure with higher potency for lean growth in those littermates exhibiting increased fibre numbers.

Similarly, Kelley *et al.* (1995) administered pST to gilts during days 28–40 of gestation. At day 41, at birth and 21 days postnatal, pST treatment increased embryonic crown–rump lengths ($P < 0.01$) but not body weight ($P > 0.1$). At 20 kg body weight, the progeny of pST-treated mothers had heavier ST muscles ($P < 0.1$) and larger longissimus muscle cross-sectional areas ($P < 0.05$). Sterle *et al.* (1995, 1998) recorded greater fetal weights, placental weights and maternal IGF-I concentrations when pST was administered. This may imply that pST enhances nutrient transfer by the placenta and/or uptake of nutrients by the fetus and this may compensate for intrauterine growth retardation.

Mitchell *et al.* (2002) used a retroviral delivery system (RCAS) to over-express IGF-I throughout the developing hind limb of stage 24 chicken embryos. There were significantly more muscle fibres in the IGF-I-injected muscles ($P < 0.05$). Four days postinjection, in the muscle of injected limbs, there was a 32% increase in myoblast to myofibre ratio, compared with the muscle in the contralateral non-injected control limbs ($P < 0.05$). Although maternal IGF-I cannot cross placental membranes, increased levels of this maternal growth factor may improve nutrient transfer across the placenta and thereby augment fetal IGF-I production, which promotes fetal growth (Hall *et al.*, 1986; Lassarre *et al.*, 1991).

3.5.2 β -agonists

Opposite effects to GH action were obtained in an experiment with rats, which were fed the β -adrenergic agonist clenbuterol during gestation and lactation (Maltin *et al.*, 1990). Muscle weights, protein, RNA and DNA content and total muscle fibre number were clearly reduced in the offspring. However, no effects on muscle growth, fibre size and fibre number were found after *in utero* exposure of lambs to a β -adrenergic agonist (Shackelford *et al.*, 1995).

3.6 Temperature

Temperature can have a marked effect on the rate of embryonic development in many cold-blooded animal species (Krogh, 1914) including the Atlantic salmon (*Salmo salar* L.) (Hayes *et al.*, 1953). More recent studies have investigated the cellularity of muscle tissue from fish embryos and larvae reared at different temperatures. For the Atlantic salmon it has been shown that higher temperatures produce, by the time of hatching, larger but fewer muscle fibres and fewer muscle nuclei than lower temperatures (Fig. 3.4) (Stickland *et al.*, 1988; Usher *et al.*, 1994). This situation has been found in other species. However, Vieira and Johnston (1992) showed that the opposite was true for herring embryos (*Clupea harengus* L.) in that a higher rearing

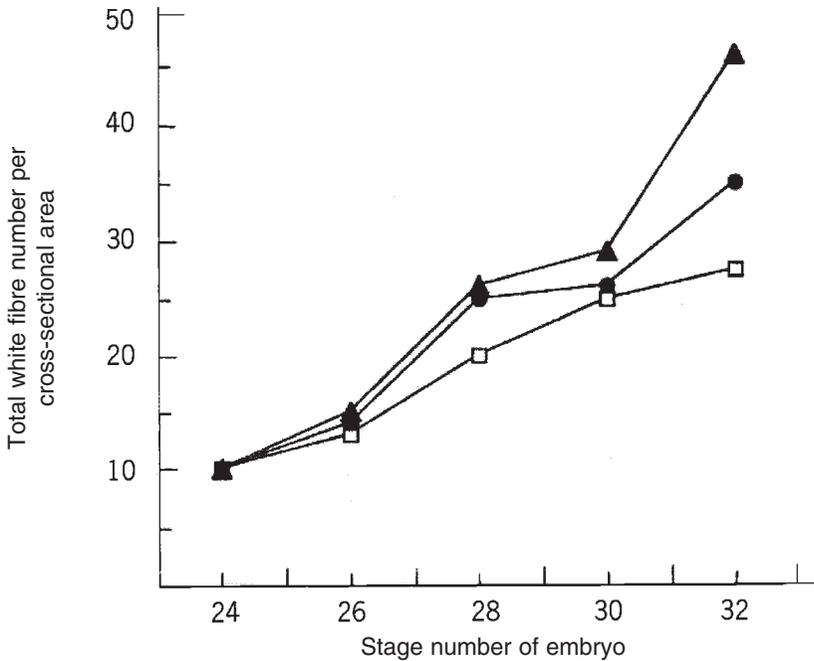


Fig. 3.4. White muscle fibre number (per cross-section) in Atlantic salmon embryos reared at 5°C (▲), 8°C (●) and 11°C (□) and at different stages (stage 33 = hatching). Taken from Usher *et al.* (1994) with permission from Blackwell Publishing Ltd.

temperature produced more but smaller muscle fibres. It has been suggested that the balance between hyperplasia and hypertrophy will depend on available energy supply. Cheek and Hill (1970) concluded that reduced energy intake compromised nuclear division but not necessarily cell hypertrophy. Fibre hypertrophy may be a more energy efficient method of increasing body size to a viable survival size at hatching. In this context it has been shown that physiological hypoxia may be the driving force behind the temperature effects on muscle development (Matschak and Stickland, 1995). This will be more of an issue with large egg species, such as Atlantic salmon, with a relatively low surface to volume ratio. When rainbow trout eggs were incubated at different temperatures, Xie *et al.* (2001) found that muscle differentiation (as detected by fast myosin heavy chain expression) occurred later in the colder embryos. These embryos also exhibited delayed but prolonged expression of myogenin (Fig. 3.5). (For a review of myogenin see Houba and te Pas, Chapter 10, this volume.)

The influence of pre-hatch temperature on post-hatch growth was demonstrated by Nathanailides *et al.* (1995). These authors showed that salmon eggs reared at lower ambient temperature (fluctuating between 5 and 10°C) produced fish that grew significantly better post-hatch, up to 3 weeks, than fish reared at 11°C pre-hatch. All fish were grown at the same post-hatch temperature. Strategies used in embryonic myogenesis can therefore influence at least the initial stages of post-hatch fish growth. Although fish can usually continue to form new fibres throughout life (as already mentioned), the fish from colder incubation regimes have more nuclei and this pre-

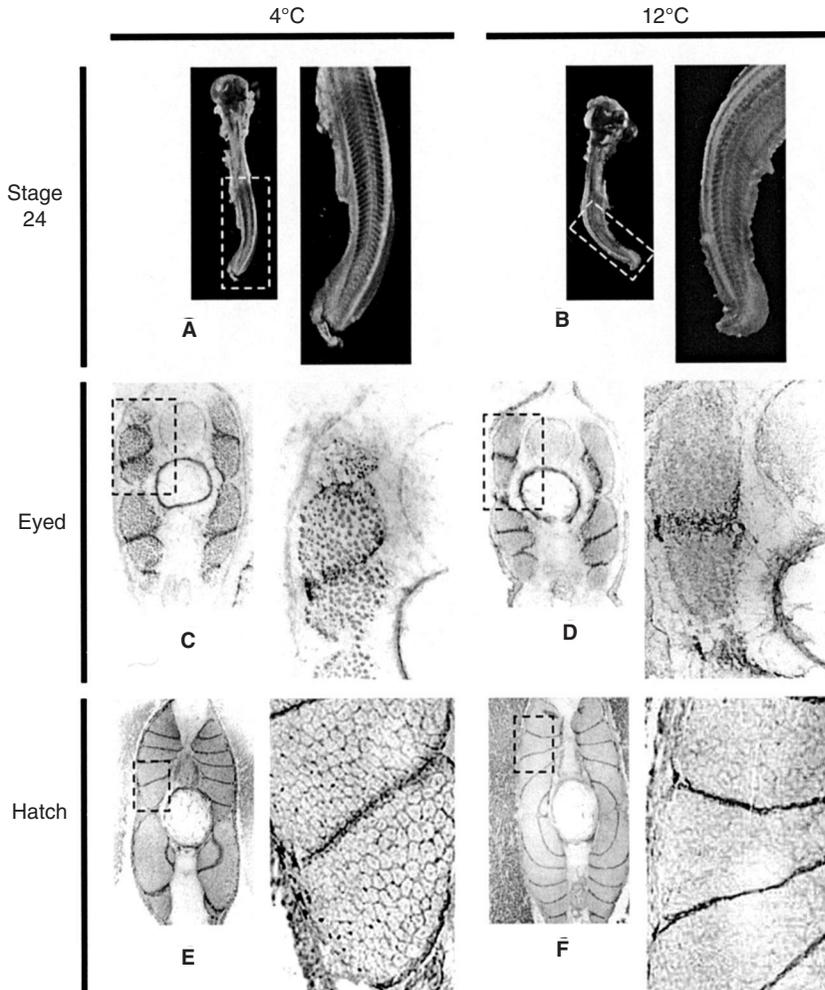


Fig. 3.5. Myogenin expression in rainbow trout embryos reared at 4°C (A, C, E) or 12°C (B, D, F). *In situ* hybridization shows *myogenin* is still expressed at stage 24 in 4°C embryos (A) but there is less at 12°C (B) in the myotomes. Myogenin antibody staining shows more protein at the eyed stage in 4°C embryos (C) than in 12°C embryos (D). At hatching (E and F) *myogenin* expression is seen around the myosepta at both temperatures but only at 4°C are small new fibres expressing *myogenin* seen (E). Photographs taken from Xie *et al.* (2001).

sumably also means more muscle precursor cells for both fibre hyperplasia and hypertrophy.

3.7 Conclusions

Producing animals with high muscle fibre number may be very beneficial for optimizing quantity and quality of meat produced (see Rehfeldt *et al.*, Chapter 1, this volume). Genetic selection can contribute to this aim although heritability estimates

for fibre number are not always high. Developing the various gene mutations which enhance fibre number determination is also possible. It is very clear, however, that one reason for the low heritability estimates is the fact that fibre formation is very susceptible to environmental factors *in utero*. Certain growth promoters, especially somatotrophin, have been shown to be particularly influential factors with respect to fibre number determination. Even the level of maternal nutrition in mammals, and incubation temperature in fish, can have very significant effects on fibre number determination. It is suggested that appropriate genetic selection and optimization of prenatal growth conditions will enable beneficial advances to be made with respect to muscle fibre number. This chapter has emphasized the need to concentrate more on prenatal development in order to better optimize fibre formation. As fibre number is fixed by about the time of birth in most mammals, it might be argued that the meat potential of an animal is determined prenatally.

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4

The Effect of Growth and Exercise on Muscle Characteristics in Relation to Meat Quality

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4.1 Introduction

Optimal meat quality characteristics (e.g. colour stability, tenderness, palatability and water-holding capacity) are important for consumption and economic reasons. These characteristics are the result of metabolic processes in the muscles during the peri- and post-slaughter period. The variation in meat quality is, both within and between animals, very large and likely to be caused by differences in genetic and environmental factors that interact with the peri- and post-mortem biochemical processes. Hereby, muscle fibre type composition, fibre areas and capillarity of muscles seem to be important factors (Henckel, 1995; Klont *et al.*, 1998).

The purpose of this chapter is to summarize the methods for determination of the muscle fibre type composition and capillarity and to review the relationship with meat quality characteristics in livestock animals (cattle and pig). Furthermore, the effects of age and exercise on these muscle characteristics are described in order to discuss the possibilities for modification with the aim of improving meat production and quality.

4.2 Muscle Fibre Types

Skeletal muscle fibre diversity was recognized as early as 1873 when Ranvier distinguished 'white' and 'red' muscles. Only in the early 1960s did it become clear that this distinction is based on the existence of different fibre types (see Pette and Staron, 1990).

4.2.1 Histochemical fibre typing

Initially, in mammalian skeletal muscle, two fibre types were classified: type I and type II fibres (Engel, 1962). It was demonstrated that slow and fast muscles contain myosins with different ATPase activities (Bárány *et al.*, 1965) and that ATPase activity correlates with differences in speed of contraction (Bárány, 1967). Type I fibres were classified as slow contracting, and type II fibres as fast contracting. Investigations dealing with the properties of single fibres demonstrated evidence for this relationship between the myosin composition and contractile properties (Reiser *et al.*, 1985; Eddinger and Moss, 1987).

The observation that slow and fast myosins have different stabilities with alkaline and acid pre-incubations (Sréter *et al.*, 1966; Seidel, 1967) formed the basis for the elaboration of more refined methods for myosin ATPase-based fibre typing. Studies of the pH stability of mammalian myosins revealed the oversimplification of the distinction into only two fibre types and led to the delineation of fast fibre subtypes, i.e. IIA, IIB and IIC fibres (Brooke and Kaiser, 1970). Modifications and additional procedures for classification of fibres based on myosin ATPase activity confirmed the existence of major fast fibre subtypes, but have also identified more than one slow fibre type (I and IC) (Askanas and Engel, 1975). Combining histochemical and biochemical analyses performed on single fibres of rabbit soleus muscle demonstrated that the C fibre population exhibited a continuum of staining intensities between types I and IIA. Microelectrophoretic analyses revealed that type C fibres were characterized by the coexistence of two different kinds of myosin heavy chain (MHC) isoforms in varying ratios: type IC with a predominance of I MHC and type IIC with a predominance of IIA MHC (Staron and Pette, 1986).

Based on the difference in formaldehyde sensitivity of myosin ATPase (Stein and Padykula, 1962), some investigators combined the pH and formaldehyde sensitivities of myosin ATPase. This led to another (now obsolete) classification system, where one slow fibre type (β) and two fast fibre types (α and $\alpha\beta$) were distinguished (Guth and Samaha, 1969; Samaha *et al.*, 1970).

4.2.2 Immunohistochemical fibre typing

The detection of antigenic differences between myosins (Gröschel-Stewart and Doniach, 1969; Masaki, 1974) initiated immunohistochemical analyses of muscle fibres with antibodies (Billeter *et al.*, 1980; Danieli-Betto *et al.*, 1986). The MHC, a major structural component of the thick filaments of the myosin molecules, is responsible for the ATPase activity. With the help of monoclonal antibodies raised against MHC and gene identification (Robbins *et al.*, 1986), it was shown that in mammals MHC occurs in different isoforms, i.e. MHC type I (Weeds and Burridge, 1975; Gauthier and Lowey, 1979) or β (Lompré *et al.*, 1984), IIa and IIb (Dalla Libera *et al.*, 1980; Billeter *et al.*, 1981) and Cardiac- α (Bredman *et al.*, 1991). Note that capitals indicate ATPase-determined fibre types and lower case indicates immunohistochemically determined fibre types.

An additional fast fibre type (IIx) has been identified in muscles of small mammals by using monoclonal antibodies (Schiaffino *et al.*, 1985; Gorza, 1990); another fast myosin heavy chain isoform was (independently) identified by gel electrophoresis in various skeletal muscles of rat (Bär and Pette, 1988). This isoform was named II_d because of its abundance in the diaphragm. Immunoblot analyses demonstrated that IIx and II_d are identical (Schiaffino *et al.*, 1989). In muscle fibres from fetal and newly born individuals, the MHC isoforms 'embryonic' and 'neonatal' (or 'Developmental' or 'fetal') are expressed (Butler-Browne and Whalen, 1984; d'Albis *et al.*, 1986; Butler-Browne *et al.*, 1988; Dingboom *et al.*, 2002). MHC isoform 'Developmental' is also expressed in muscle fibres of regenerating muscles (Sartore *et al.*, 1982).

Multiple expression of MHCs may be the rule in fibres of some muscles (Danieli-Betto *et al.*, 1986; Biral *et al.*, 1988), but occurs particularly under conditions of induced fibre transformation (Staron and Pette, 1987; Maier *et al.*, 1988), for example during growth (Dingboom *et al.*, 2002) or after a change in training regimen. Studies with chronic low-frequency stimulation (Termin *et al.*, 1989) and endurance training (Serrano *et al.*, 2000) showed the induction of fast-to-slow transitions in myofibrillar protein isoforms and fibre types (IIab and IIax or IIad fibres).

4.2.3 Fibre classification based on metabolic enzyme activity

In comparative studies (Bass *et al.*, 1969, 1970; Staudte and Pette, 1972) activities of 'metabolic oxidative enzymes' were shown to be useful for distinguishing different fibre types. Three major fibre types were derived from differences in these enzyme activities: high, intermediate and low oxidative fibres (Ogata and Mori, 1964; Padykula and Gauthier, 1967; Gauthier, 1969, 1974). The combination of metabolic enzyme-based fibre classification and myosin ATPase histochemistry resulted in the classification of three major fibre types in muscles of guinea pig and rabbit: slow-twitch oxidative (SO), fast-twitch oxidative/glycolytic (FOG) and fast-twitch glycolytic (FG) fibres (Barnard *et al.*, 1971; Peter *et al.*, 1972). A method of depleting glycogen in single motor units (Edström and Kugelberg, 1968; Kugelberg and Edström, 1968) made it possible to establish a relationship between metabolic properties, speed of contraction and fatigability of motor units. It was shown that motor units composed of FG fibres are fast contracting but quickly fatigued, while motor

units composed of FOG or SO fibres are fatigue resistant. FOG fibres are fast contracting and SO fibres are slow contracting fibres (Burke *et al.*, 1971, 1973, 1974; Burke, 1981).

4.2.4 Compatibility of the classification methods

Taken together, a variety of fibre types can be distinguished in a given muscle by myosin ATPase-based histochemistry, immunohistochemistry or by metabolic enzyme-based fibre classification, but the existence of these different classification schemes raises the question of compatibility. For example, Green *et al.* (1982) showed complete correspondence between ATPase-determined type I and β fibres, but significant variations between the fast fibre subtypes IIA and IIB on the one hand and $\alpha\beta$ and α on the other hand.

Since the work of Bárány (1967) it is generally accepted that the contraction speed of given muscle fibres relates to the ATPase activity of the MHC part of myosin molecules. The rate of myosin ATPase activity is different in the different MHC isoforms and correlates well with the resistance against acid and alkaline preincubation. Therefore, myosin ATPase-based fibre typing and immunohistochemically based classification produce similar results (Fig. 4.1). However, this correlation is not perfect. Some fibres designated as IIB with ATPase techniques express both IIA and IIB MHCs. On the other hand, fibres characterized as intermediate between IIA and IIB with the ATPase method (type IIA/B fibres) contained only the type IIA MHC isoform (Danieli-Betto *et al.*, 1986; Lopez-Rivero *et al.*, 1996) and a high percentage of fibres classified as IIA with ATPase expressed both fast types of MHC (Dingboom *et al.*, 2002). In adult skeletal muscle of mice, rats, guinea pigs and rabbits all four adult MHC isoforms have been identified (Schiaffino and Reggiani, 1994). In older literature, fibres expressing IId MHC were classified as IIB with the ATPase method. Nowadays, only type I, IIA and IId are believed to be expressed in muscles of large mammals (human: Smerdu *et al.*, 1994; Ennion *et al.*, 1995; horse: Dingboom *et al.*, 2002). However, surprisingly, the expression of IIB MHC isoform is now described for pig longissimus muscle (Lefaucheur *et al.*, 2002), human masseter muscle (Horton *et al.*, 2001) and llama locomotory muscles (Graziotti *et al.*, 2001).

The assumption that metabolic properties should correlate strictly with properties related to different myosin ATPase activities may not be justified. In some mammals, the slow contracting, fatigue-resistant type I fibres are the most oxidative (e.g. human and cat) whereas in other mammals, these fibres are intermediate between IIA (or IIA) and IID (or IID) in their oxidative potential (e.g. rat, guinea pig and horse) (Barnard *et al.*, 1971; Peter *et al.*, 1972; Prince *et al.*, 1976; Reichmann and Pette, 1982; Essén-Gustavsson, 1986). Furthermore, after quantifying the histochemically assessed aerobic oxidative metabolism in the different fibre types, it became clear that no discrete levels exist but an entire spectrum of metabolic activity (Nemeth and Pette, 1981; Reichmann and Pette, 1982, 1984; Pette and Tyler, 1983; White and Snow, 1985), overlapping the fibre types.

For the reasons mentioned above, it can be concluded that classification systems do not appear to be entirely compatible and should therefore not be interchanged.

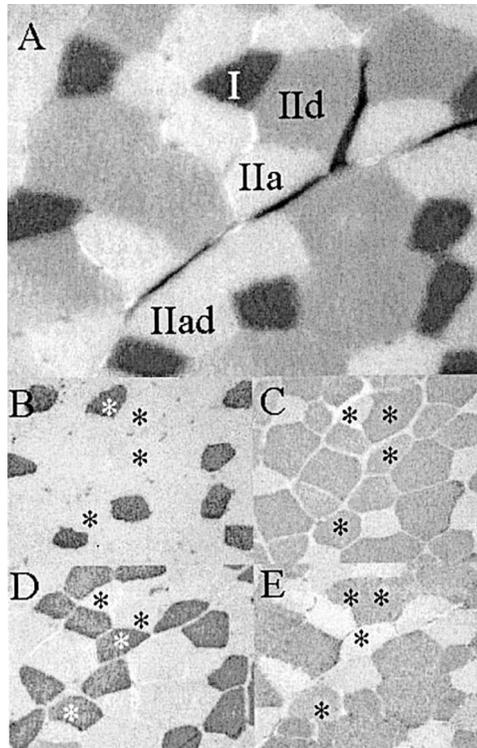


Fig. 4.1. Transverse serial sections from a biopsy taken from the equine gluteus medius muscle. An example of how myosin ATPase-based fibre typing (A) and immunohistochemically based classification (B–E) produce similar results (fibres, marked *). A: ATPase staining pH 4.3; B: Mab 219-1D1; C: Mab 332-3D4; D: Mab 333-7H1; E: Mab 412-R1D5 (Dingboom *et al.*, 2002).

4.3 Capillarity

Muscle fibres need a sufficient blood supply, since oxygen and substrates for energy production (i.e. carbohydrates and fatty acids) must be delivered and metabolites (i.e. lactate and carbon dioxide) must be released by the bloodstream. Capillaries surround the fibres in a way that is shown in Fig. 4.2. In most adult cases the number of capillaries around type I fibres is higher than around type II fibres (Romanul, 1965; Karlström *et al.*, 1991). However this is not always true in all species and muscles.

The capillary supply, or capillarity, can be expressed as the number of capillaries per mm^2 muscle tissue (capillary density) or per muscle fibre (capillary to fibre ratio). However, the value of both parameters varies by the cross-sectional areas of the muscle fibres involved. Therefore, it is more accurate to describe the capillarity by a parameter indicating the muscle fibre area one capillary has to supply (diffusion index) (Andersen and Henriksson, 1977; Rivero *et al.*, 1995; Dingboom, 2002).

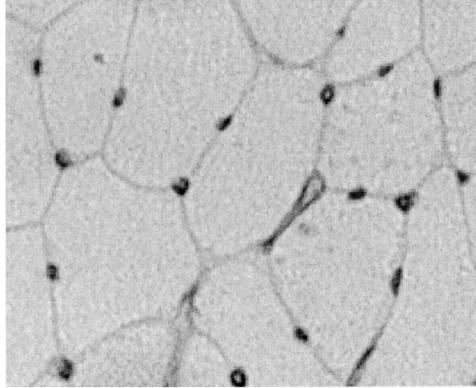


Fig. 4.2. Transverse serial section from a biopsy taken from the equine gluteus medius muscle. Capillaries (dots) that surround the muscle fibres are stained with biotinylated lectin (0.005 mg/ml) (Dingboom *et al.*, unpublished).

4.4 Relationship Between Meat Quality and Muscle Properties

Muscle fibre type composition and capillarity influence meat quality characteristics, but there are considerable differences between and even within species. The following statements are generalized and contradictory results in the literature must be taken into account (Table 4.1; for review see Klont *et al.*, 1998).

Tenderness of the meat is positively related to the cross-sectional areas of the fibres (Seideman and Crouse, 1986). Fibre diameter increases in the order I, IIA(a) and IIB or IID(d) fibres (Rosser *et al.*, 1992). Tenderness is also positively related to the sarcomere length. Within sarcomeres, the contractile filaments actin and myosin act together in the cross-bridge cycle, determining the state of contraction post mortem (= shortening). Shortening is more intense in oxidative type I fibres (Davies *et al.*, 1979; Ceña *et al.*, 1992). Furthermore, particularly the large IIB (Iib) and IID (IId) fibres rely on stored glycogen as a substrate for energy production. The high glycogen content is responsible for a sweet flavour. Summarizing, meat with high percentages of type IIB or IID (IId) fibres is the most tender and sweet. That taste is relative is proven by the fact that other investigators point out that beef with high percentages of red (or oxidative) fibres tastes better than meat with more white (type II) fibres (Seideman and Crouse, 1986). This is most probably the result of the higher lipid content of these red fibre types (Essén-Gustavsson *et al.*, 1994), because there is a weak relation between palatability and intramuscular fat content (Melton *et al.*, 1974).

Colour (i.e. redness) of meat is (partly) determined by the oxygenation of myoglobin (Klont *et al.*, 1998). Type I and IIA fibres have greater myoglobin content (Essén-Gustavsson *et al.*, 1992) as well as a higher concentration of mitochondria compared with type IID fibres. This means that meat with high percentages of oxidative fibres has a red colour, but taking into account that the fibre areas are relatively small and that this fibre type shows a high level of post-mortem shortening and has in general low glycogen content (see above), the meat can be expected to be tough and indeed rather tasteless.

Table 4.1. Relationship between muscle fibre characteristics and meat quality features. The +, 0 and – marks represent a positive, non-existent and a negative relationship, respectively, between muscle fibre characteristics and meat quality features.

Muscle property	Meat quality property	Reference
% slow muscle fibres ^a	– Depth of beef discoloration	Monin and Ouali (1992)
	– Speed of post-mortem beef tenderization	Ouali (1990)
	+ Palatability	Seidman and Crouse (1986)
Glycogen content high	+ Sweet flavour	Klont <i>et al.</i> (1998)
	+ Tendency for PSE pork	Swatland (1984)
		Fernandez and Thornberg (1991)
Depletion	– Ultimate pH, tendency for DFD in pork	
Oxidative capacity	+ Ultimate pH, tendency for DFD in pork	Maltin <i>et al.</i> (1997)
Capillarity	+ Eating quality	Klont <i>et al.</i> (1998)
Haemoglobin content	– Veal colour stability	Klont <i>et al.</i> (1998)
Sarcomere length	+ Tenderness	Cena <i>et al.</i> (1992)
		Davies <i>et al.</i> (1979)
		Seidman and Crouse (1986)
Fibre diameter	0 Beef tenderness	Smulders <i>et al.</i> (1990)
	+ Pork tenderness	Maltin <i>et al.</i> (1997)
	0 Beef tenderness	Crouse <i>et al.</i> (1991)
Intramuscular fat content	+ Pork taste quality	Cameron <i>et al.</i> (1990)
		Fjelkner-Modig and Thornberg (1986)
	+ Palatability	Melton <i>et al.</i> (1974)

^aIn general, slow fibres have a smaller diameter, a lower glycogen content, and a higher oxidative capacity, haemoglobin content and capillarization than fast fibres; slow fibres may or may not have a higher fat content.

Water-holding capacity (WHC) is, especially in pork, an important quality characteristic: 'exudative' (in pale, soft and exudative (PSE) meat) and 'dry' (in dark, firm and dry (DFD) meat) are the extreme types (Lawrie, 1985). Glycogen content is inversely (non-linearly) related to the ultimate pH in the post-mortem maturation of meat (Fernandez and Thornberg, 1991). Low ultimate pH, together with a high temperature, causes protein denaturation, which in turn lowers the WHC. High glycogen content (type II fibres) is a predisposition for PSE meat. Muscle tissue with a high amount of glycogen-depleted fibres is predisposed to become DFD meat. To what extent this happens depends on pre-slaughter behaviour and the muscle involved (Klont *et al.*, 1998).

Type I and IIA(a) fibres are surrounded by more capillaries than IIB or IID(d) fibres (Essén-Gustavsson *et al.*, 1992). Capillarization may be a relevant factor determining the meat quality, since the function of muscles depends to a large extent on the oxygen supply and the potential to remove waste products such as lactate. This way, capillarization can influence the metabolic state of the muscles at time of slaughter and thereby the maturation of the meat during the post-slaughter period.

Table 4.2. Age effect on muscle fibre characteristics of locomotory muscles from different species; +, increase; -, decrease.

Parameter	Age effect	Species	References
% Type I (slow)	+	Rodents	Goldspink and Ward (1979)
		Cattle	Cooper <i>et al.</i> (1970)
	+	Pig	Seideman and Crouse (1986)
		Horse	Suzuki and Cassens (1980)
	-	Rodents	Dingboom <i>et al.</i> (2002)
			Essén <i>et al.</i> (1980)
			Essén-Gustavsson <i>et al.</i> (1983)
			Henckel (1983)
			Roneus <i>et al.</i> (1991)
			Roneus (1993)
-	Sheep	Rivero <i>et al.</i> (1993a)	
		Finkelstein <i>et al.</i> (1992)	
% Type IIB (fast)	+	Cattle	Wegner <i>et al.</i> (2000)
Oxidative capacity	+	Rodents	Dangain <i>et al.</i> (1987)
			Nemeth <i>et al.</i> (1989)
			Ishihara and Inoue (1989)
in IIB fibres	-	Horse	Dingboom <i>et al.</i> (2002)
		Rat	Smith <i>et al.</i> (1988)
Capillary per mm ² (capillary density)	-	Rat	Sillau and Banchemo (1977)
		Pig (a.o.)	Ripoll <i>et al.</i> (1979)
per fibre (capillary rate per fibre ratio)	+	Rat	Kurnoth <i>et al.</i> (1994)
		Rat	Welt <i>et al.</i> (1975)
Mean cross-sectional area of the muscle fibres	+ (growth)		Ripoll <i>et al.</i> (1979)

See comments, Table 4.1. a.o., among others.

4.5 Postnatal Development

After birth, muscles assume their normal function. During growth, muscle fibres change in properties while adapting to their new function. Age effects on muscle characteristics, such as MHC expression, oxidative capacity and blood perfusion, depend on the type of muscle, species and breed (Table 4.2).

4.5.1 MHC expression

Contractile properties of mammalian skeletal muscles change after birth. In the earliest postnatal period changes are a consequence of the development of the innervation pattern from poly- to mono-innervation. For example, Close (1964) observed in postnatal rat muscles that the process of differentiation into fast and slow muscles can be attributed to an increase in the speed of contraction of the fast muscles, while the slow muscles did not change. Other early age effects are the disappearance of juve-

nile MHC isoforms like Developmental MHC (d'Albis *et al.*, 1986 (masseter); Butler-Browne and Whalen, 1984 (locomotory muscles, small mammals); Dingboom *et al.*, 1999 (horses)) and Cardiac- α MHC (Dingboom *et al.*, 1999 (equine locomotory muscles)) in the first weeks after birth.

Postnatal developments in MHC expression that occur later in life are transitional changes between the adult fibre types. They depend on the type of muscle. Most authors describe for different locomotory muscles an increase of fibre type I and/or IIA (IIa) percentages at the expense of the fibre type IIB (IIb) or IId/x fibre population in the early postnatal period and during the period of young adulthood of mammals, both small (Goldspink and Ward, 1979) and large (Cooper *et al.*, 1970; Seideman and Crouse, 1986 (cattle); Suzuki and Cassens, 1980 (pig); Essén *et al.*, 1980; Essén-Gustavsson *et al.*, 1983; Henckel, 1983; Roneus *et al.*, 1991; Roneus, 1993; Rivero *et al.*, 1993a; Dingboom *et al.*, 2002 (horse)). In contrast, it was found that the IIB (IIb) percentage increases at the expense of the fibre type IIA (IIa) in the semitendinosus muscle of cattle (Wegner *et al.*, 2000) and that the percentage of type I fibres decreases in the extensor digitalis longus muscle of sheep (Finkelstein *et al.*, 1992).

4.5.2 Metabolic profile

The metabolic difference between fast and slow muscles in adult mammals was found to be absent in the newborn; it becomes visible during postnatal development (Bass *et al.*, 1970; Margreth *et al.*, 1970; Hudlicka *et al.*, 1973; Dalrymple *et al.*, 1974; Zuurveld *et al.*, 1985). Most likely, this change results from the adaptation to changing usage patterns. In muscle tissue of different mammals it was found that the (biochemically determined) oxidative enzyme activity decreases in the first postnatal weeks (rat: Fratacci *et al.*, 1996; cat: Wada *et al.*, 1995; horse: Dingboom, 2002). In equine muscle the activity of the anaerobic glycolytic enzymes, rather than the aerobic oxidative enzymes, was found to increase during the first year of life (Bechtel and Kline, 1987; Kline and Bechtel, 1990).

Also at the level of the muscle fibres, the metabolic heterogeneity only just becomes clear later during postnatal development (Dangain *et al.*, 1987; Ishihara and Inoue, 1989; Nemeth *et al.*, 1989). However, in contrast to the biochemical studies with homogenized tissue, it was found that the oxidative capacity of the different fibre types increases rather than decreases, suggesting a progressive adaptation to the extra-uterine environment; the different fibre types show different degrees of increase. The discrepancy must be explained by the fact that in biochemical measurements the enzyme activity is expressed as activity per mg muscle protein. While ageing, the protein content of the muscle increases excessively relative to the amount of oxidative enzymes. Some authors describe that the oxidative enzyme activity of IIB (IIb) (Smith *et al.*, 1988) or IId fibres (Dingboom, 2002) decreases in the postnatal period.

4.5.3 Blood supply

Mammals are born with a certain capillary supply, which changes after birth due to growth of the muscle fibres. Growth results in a decrease of fibre density; because of

the larger distance between the surrounding capillaries there is also a decrease in capillary density (Sillau and Banchero, 1977; Ripoll *et al.*, 1979; Kurnoth *et al.*, 1994). Several investigators found in different muscles of small mammals an increase in the number of capillaries around the fibres during growth (Welt *et al.*, 1975; Ripoll *et al.*, 1979). However, since the total area of the fibres in the growing muscle is also increasing, the diffusion index (i.e. the area that one capillary has to supply) was found either to remain constant (Welt *et al.*, 1975) or to decrease (Smith *et al.*, 1989).

4.6 The Influence of Exercise

Human studies have shown that muscle fibre type composition is highly genetically determined (Komi *et al.*, 1977; Komi and Karlsson, 1979; Simoneau and Bouchard, 1995). Studies performed on animals also demonstrate this correlation between genetic background and muscle fibre type composition (horse: Lopez-Rivero *et al.*, 1991, 1996; pig: Szentkuti and Schlegel, 1985). This would imply that fibre type-dependent meat characteristics are partly determined at birth. To change muscle fibre type percentages in a muscle, high intensity exercise must be given for a long time. For example, in a study with human athletes the fibre type composition changed after 3 months of intensive strength and interval training (Anderson *et al.*, 1994). On the other hand, dynamic parameters such as capillarity (Hudlicka *et al.*, 1977) and enzyme activity (Henriksson and Reitman, 1977) can adapt more rapidly to the imposed workload.

4.6.1 Training

Most of the knowledge about the effect of training on muscle properties is derived from studies with human athletes (for review see Wilmore and Costill, 1994). The problem of interpreting training results from studies with animals is that often the training regimen is not described clearly enough. Furthermore, some studies with apparently equivalent training regimens produce contrasting results. Therefore, it is hard to make detailed inferences about the influence of training on the muscle characteristics based on the literature (Table 4.3).

There are many ways to design training protocols but, basically, training can be divided into 'endurance training' and 'sprint training'. Endurance training focuses on the increase of resistance against fatigue of the muscle, by increased aerobic metabolism. In general it can be stated that endurance training enhances the oxidative capacity of all fibre types, stimulates the conversion of type IIB or IID into IIA and increases the cross-sectional area of type I fibres (e.g. horses: Hodgson and Rose, 1987; Lopez-Rivero *et al.*, 1991; Rivero *et al.*, 1993b; Tyler *et al.*, 1998). Sprint training focuses on the increase of capacity for fast and powerful short contractions. Energy will be primarily supplied through anaerobic metabolism, but due to the enhancement of the oxidative capacity of the fast contracting fibres as a result of the training, energy will also be supplied through improved aerobic metabolism. In studies with humans it appeared that sprint (or power) training increases the relative area occupied by the type II fibres (Dawson *et al.*, 1998; Jansson *et al.*, 1990).

Table 4.3. Exercise effects on fibre characteristics of locomotory muscles from different species; +, increase; -, decrease.

	Parameter	Effect	Species	Reference(s)
Endurance	% Type I (slow)	+	Horse	Hodgon and Rose (1987)
	Oxidative capacity			Lopez-Rivero <i>et al.</i> (1991)
	% Type I and IIA(a)			Rivero <i>et al.</i> (1993b)
				Rivero <i>et al.</i> (1995)
Sprint	Type II (fast) fibres	+	Human	Dawson <i>et al.</i> (1998)
	Mean cross-sectional area			Jansson <i>et al.</i> (1990)
	Capillarity per mm ²	+	Horse	Essén-Gustavsson <i>et al.</i> (1989)
			Human	McCall <i>et al.</i> (1996)
			Horse	Essén-Gustavsson <i>et al.</i> (1989)
				Henckel (1983)
Restricted motility	Mean cross-sectional area	-	Human	Tyler <i>et al.</i> (1998)
	% Type I			Haggmark <i>et al.</i> (1986)
De-training	All parameters	Reversal from training	Human	Dingboom <i>et al.</i> (unpublished)
				Mujika and Padilla (2000)
	Capillarity per mm ²	-	Horse	Mujika and Padilla (2001)
				Winters and Snow (2000)
Oxidative capacity				Serrano <i>et al.</i> (2000)
				Guy and Snow (1977)
				Snow and Guy (1979)
				Essén-Gustavsson <i>et al.</i> (1989)
				Foreman <i>et al.</i> (1990)
				Tyler <i>et al.</i> (1998)

See comments, Table 4.1.

4.6.2 Restricted mobility

Most studies concern the effects of de-training, which are generally the reversal of the training effects (human: Mujika and Padilla, 2000, 2001; Winters and Snow, 2000; horse: Guy and Snow, 1977; Snow and Guy, 1979; Essén-Gustavsson *et al.*, 1989; Foreman *et al.*, 1990; Tyler *et al.*, 1998; Serrano *et al.*, 2000). These studies show that the effects of de-training are a decreased capillary density (within 2–3 weeks of inactivity) and a rapid and progressive reduction in oxidative enzyme activities. Although in some cases the muscle characteristics of de-trained individuals remain above sedentary values, usually they return to baseline values in briefly trained individuals. The fibre type composition remains unchanged during the initial weeks of inactivity, but the number of oxidative fibres may decrease in human endurance-trained athletes and increase in sprint-trained athletes within 8 weeks of de-training (Mujika and Padilla, 2001). Muscle fibre cross-sectional area declines rapidly in sprint-trained athletes and in briefly endurance-trained subjects, whereas it may increase slightly in athletes trained for endurance for a longer period.

Effects from immobility are comparable with the effects of de-training but much

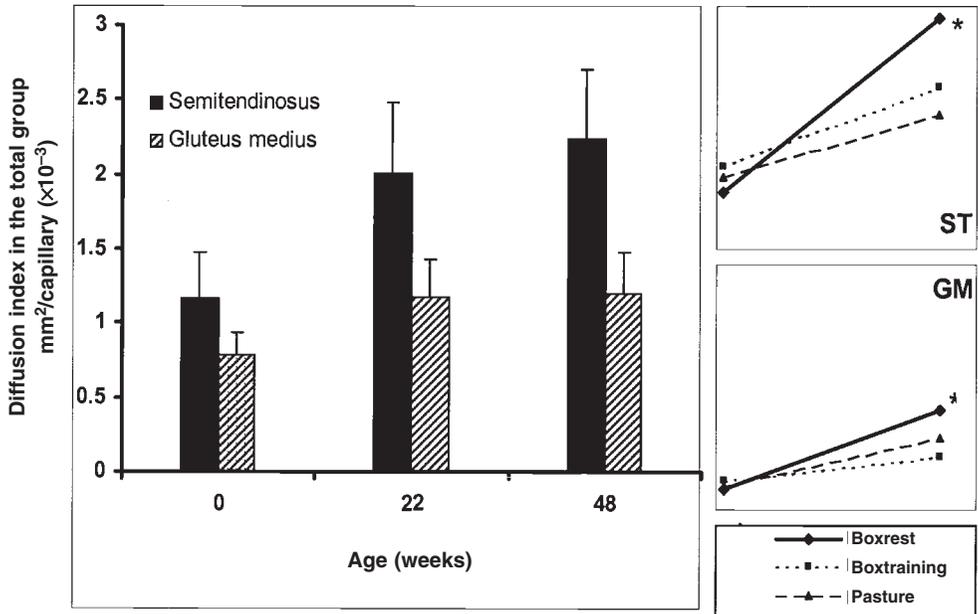


Fig. 4.3. The means (column graphs) and standard deviations (y error bars) of the capillarity in a group of 36 foals at three different ages (0, 22 and 48 weeks) from the semitendinosus (black columns) and the gluteus medius (striped columns). The line graphs at the right demonstrate the means in three different training groups at two different ages (0 and 22 weeks) for the semitendinosus (ST) and the gluteus medius (GM) separately. * Significant exercise effect ($P < 0.01$) (Dingboom *et al.*, unpublished).

more dramatic (Wilmore and Costill, 1994). Forced immobility (for example bed rest) causes muscle atrophy, accompanied by losses in muscle strength and power. Haggmark *et al.* (1986) described a substantial decrease in type I fibre percentages when sudden immobilization occurs after a period of intensive sport training in humans. Dingboom *et al.* (2002) described how and to what extent restricted mobility led to an underdevelopment of the oxidative enzyme activity and capillarity in gluteus medius muscle tissue of young horses (example given in Fig. 4.3).

4.7 Conclusions

The purpose of this chapter was to summarize the methods for determination of muscle fibre type composition and capillarity and to review the relationship with meat quality characteristics in several livestock animals. The effects of age and exercise on the involved muscle characteristics are described, in order to discuss the possibilities for modification with the aim of improving meat production and quality.

Muscle tissue properties are not only influenced by age and exercise. Other factors, for example food (restriction) and pre-slaughter stress level are very important in this light. Nevertheless, to reach optimal meat quality, 'age' and 'exercise' can be considered as possibilities for modification.

In the first place it seems logical that tenderness or juiciness increases while

growing, due to the increase in fibre areas. This was, for example, described by Dikeman *et al.* (1986) who compared steers of 12 with those of 24 months of age. For this reason it seems logical to heighten the slaughter age. However, at some point in time, muscle characteristics (e.g. collagen amount and solubility) and economic reasons (space and feeding costs) become the limiting factors.

Theoretically, exercise can influence muscle characteristics by changing muscle fibre type composition (and consequently changing fibre glycogen and fat content, fibre areas and structural protein arrangement) and capillarity. However, in most cases, livestock animals are kept intensively in small stables and exercise in these circumstances can better be seen as 'more space to move around'. Although some findings indicate, for example, that porcine skeletal muscle adapts to training (McAllister *et al.*, 1997; treadmill exercise: increased oxidative capacity in miniature swine), in general it seems that exercise effects are insignificant. Studies in which pork meat quality of animals housed in the normal circumstances were compared with that of animals kept in a so-called 'enriched environment' demonstrated no differences (Gentry *et al.*, 2002a,b) or only minor ones, e.g. increased water-holding capacity in the 'enriched group' (Klont *et al.*, 2001). Other studies demonstrated minor or contradictory results: for example an increase (Petrov and Tomov, 1985) or decrease (Lewis *et al.*, 1989) in tenderness. Also 'indoor' (Erfält *et al.*, 1993) or treadmill exercising (Hawrysh *et al.*, 1974; Weiss *et al.*, 1975) of pigs showed little or no effect. Already described in earlier studies, exercising in steers seemed to increase the tenderness of their meat, which was due to an increase of the fibre areas (Mitchell and Hamilton, 1933; Bull and Rusk, 1942). Aalhus and Price (1990) also found an increased tenderness in meat derived from sheep that underwent exercise, but these investigators were not able to relate it to muscular hypertrophy.

It can be concluded that fibre type composition may affect a limited number of aspects of meat quality. The benefits of exercise, in terms of meat quality, are minor and ambiguous. The present state of knowledge precludes the possibility of proposing detailed recommendations.

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5

Nutrition, Hormone Receptor Expression and Gene Interactions: Implications for Development and Disease

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5.1 Introduction

Muscle is essential for locomotion, postural maintenance, breathing, cardiovascular function and thermogenesis. Moreover, skeletal muscles also play a key role in determining nutrient oxidation rates and are the main peripheral site of insulin action. Defects in normal muscle development will therefore affect numerous cellular, metabolic and physiological functions. Studies on muscle development of livestock animals are relevant not only to optimization of meat quantity and quality (Wray-Cahen *et al.*, 1998) but also to the health and disease of a wide range of mammals, including companion animals and humans (Dauncey, 1997). The young pig makes a particularly good developmental, nutritional, hormonal and metabolic model for the human infant (Tumbleson and Schook, 1996), and studies on sheep have also provided new insight into the fetal and perinatal development of humans (Fowden, 1995). Studies on muscle development in farm animals thus have important implications for increasing understanding of mechanisms underlying many diseases in humans, including diabetes and cardiovascular disease.

Considerable insight has been gained in recent years into the molecular mechanisms underlying muscle development (see also Houba and te Pas, Chapter 10, and Maltin and Plastow, Chapter 13, this volume). Similarly, there is now detailed understanding of hormone receptor structure and function at the molecular level (see also Goldspink, Chapter 8, this volume). Less clear cut is understanding of mechanisms by which nutrition regulates muscle development, growth and metabolism via genetic and epigenetic events involved in hormone receptor action. Nutritional status and the external environment can profoundly influence the phenotypic expression of a given genotype, and nutrition/environment–gene interactions are especially important during critical stages of development (see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume). Many of these effects are mediated by hormones and their receptors (Ingram and Dauncey, 1986; Dauncey, 1995; Dauncey and Pell, 1998; Dauncey *et al.*, 2001). This chapter focuses on the receptors for thyroid hormones (TH; 3,5,3'-triiodothyronine, T₃, which accounts for most of the biological activity of TH, and thyroxine, T₄) and growth hormone (GH) as mediators of the nutritional regulation of muscle gene expression, during two critical stages of mammalian development: the prenatal and postnatal periods.

5.2 Nutritional Regulation of Muscle Development

During early embryonic life, the commitment of precursor cells to form myoblasts is followed by differentiation into myotubes and maturation into myofibres. The wide range of physiological functions exhibited by striated muscles reflects the many intricate combinations of contractile and metabolic properties occurring in myofibres at the cellular and molecular levels. Contractile activity (slow type I/fast type II) is determined largely by the ATPase activity of the myosin heavy chain (MHC) isoforms expressed within each myofibre (Schiaffino and Reggiani, 1994; Harridge *et al.*, 1996), and metabolic properties (oxidative/glycolytic) are determined by the relative proportions of mitochondria and respiratory enzymes (Rowlerson, 1994; Dauncey and Gilmour, 1996; Harrison *et al.*, 1997). Myofibres can thus be identified according to these properties, and major groups in mature mammalian muscle are:

type I = slow oxidative, type IIA = fast oxidative-glycolytic, type IIX = fast oxidative-glycolytic and type IIB = fast glycolytic.

Undernutrition has long been known to influence muscle hyperplasia and hypertrophy, the effects being dependent on stage of development (Stickland *et al.*, 1975; Ward and Stickland, 1993). However, relatively little was known about the influence of nutrition on muscle development at the molecular level, and especially on myofibre differentiation and MHC isoform expression, until the more recent studies of White *et al.* (2000). The probability is that the effects of nutrition at the molecular level are dependent on stage of development and muscle type. Perinatally, undernutrition delays maturational switching from embryonic–neonatal to mature adult MHC (Brozanski *et al.*, 1991), but in adults the major effect of energy deficiency is a reduction in type II fast fibre size with the size of type I slow fibres being maintained (Schantz *et al.*, 1983). Postnatally, by contrast, undernutrition induces a muscle-specific increase in proportion of type I fibres (Harrison *et al.*, 1996a). Postnatal nutrition has a striking muscle-specific influence on type I MHC expression at both the mRNA and protein levels, and has marked differential effects on the metabolic properties of functionally distinct muscles (White *et al.*, 2000). In young pigs living at thermal neutrality, a low food intake markedly upregulates slow MHC mRNA and protein expression in rhomboideus, whereas effects in longissimus dorsi (longissimus), soleus and diaphragm are not significant. The oxidative capacity of all these muscles increases on a low food intake, and the proportions of slow oxidative fibres increase at the expense of fast glycolytic fibres. These changes in muscle development induced by undernutrition will act to enhance energetic efficiency when energy supplies are restricted.

Environmental temperature modulates the effects of nutrition on muscle development and function at many levels including the expression of MHC isoforms, proportions and size of myofibre types, activities of oxidative and glycolytic enzymes, and abundance of membrane-bound ATPases (Dauncey and Ingram, 1988; Dauncey and Harrison, 1996; Harrison *et al.*, 1996a; Lefaucheur *et al.*, 2001). Mechanisms underlying the nutritional regulation of myosin isoform gene expression and muscle phenotype probably involve complex interactions between nutritionally regulated hormones, and differential effects on their receptors and these are considered in the following sections.

5.3 Hormones, Receptors and Muscle Development

Although contractile activity has a major influence on the transitions between MHC isoforms (Goldspink, 1996), the influence of hormones and growth factors is also significant, and the precise effects are dependent on stage of development (Dauncey and Gilmour, 1996; Bass *et al.*, 1999). Many hormones have been implicated in muscle development and gene expression including TH (Izumo *et al.*, 1986), GH (Ayling *et al.*, 1992), insulin-like growth factors (IGFs) (see also Goldspink, Chapter 8, this volume) (Florini and Ewton, 1992), the transforming growth factor- β (TGF- β) superfamily (Bass *et al.*, 1999), glucocorticoids (GC) (Polla *et al.*, 1994) and insulin (Cotter and Cameron, 1994). Interactions between hormonal systems are also important. TH influence prenatal muscle development via changes in local activity of the somatotrophic axis (Forhead *et al.*, 2002), the perinatal ontogeny of GH recep-

tors (GHR) is modulated by TH status (Duchamp *et al.*, 1996), and cortisol is a major developmental regulator of IGF-I (Li *et al.*, 2002a). This chapter focuses on the roles of TH receptors (TR) and GHR as paradigms of nuclear receptors and plasma-membrane-bound receptors, respectively.

5.3.1 Hormone receptors and regulation of gene expression

Numerous hormones are involved in the complex communication network which operates at intracellular, cell-cell and cell-environment levels to coordinate development. They act as powerful nutritional signals and are in turn subject to modification by nutritional status. Hormonal actions are mediated by receptors and these enable a given circulating level of hormone to have tissue-specific and developmental-stage-specific effects. In general, polypeptide hormones such as GH, IGFs and insulin act on membrane-bound receptors to trigger gene transcription via intracellular signalling pathways (Argetsinger and Carter-Su, 1996; Czech and Corvera, 1999). By contrast, nuclear receptors for lipid-soluble hormones such as TH and GC regulate transcription via DNA binding, multiple distinct histone covalent modifications and chromatin remodelling (Collingwood *et al.*, 1999; McNally *et al.*, 2000; Dauncey *et al.*, 2001; Li *et al.*, 2002b). The classical view of nuclear hormone receptors as having entirely genomic actions has been questioned because some of their actions appear to be non-genomic (Davis *et al.*, 2002; Valverde and Parker, 2002). Moreover, it has been suggested that transmembrane receptors may also internalize and translocate to the nucleus where they act as transcription factors (Raben and Baldassare, 2002). In general, however, most actions of TR are considered to be nuclear whereas GHR operate at the cell membrane.

Nuclear hormone receptors are members of a large superfamily of transcriptional regulators with the ability to activate or repress numerous genes (Wu and Koenig, 2000). Also included in this group are the receptors for retinoids, vitamin D and the sex steroids (Lazar, 1993; Kumar and Thompson, 1999), and the more recently identified orphan receptors (Gustafsson, 1999). The latter group was originally identified without knowledge of their specific ligand and includes peroxisome proliferator-activated receptor gamma (PPAR γ), which is intimately involved in regulating the expression of numerous genes involved in energy metabolism, cell differentiation, apoptosis and inflammation (Desvergne and Wahli, 1999; Houseknecht *et al.*, 2002). In a nutritional context, nuclear receptors are of particular interest because their respective ligands are usually dependent on specific dietary components for their synthesis. For example, iodine and tyrosine are essential for synthesis of TH, cholesterol for GC and vitamin D, and fatty acids for PPAR γ .

5.3.2 Thyroid hormones

5.3.2.1 TH and their actions

TH are key regulators of growth and development, and control numerous cellular, metabolic and physiological functions (Oppenheimer *et al.*, 1987; Dauncey, 1990; Fisher, 1996). They have essential roles in muscle development and function. Prenatally, TH induce myoblasts to exit the cell cycle, differentiate and express a

muscle-specific phenotype. Concurrent with the perinatal increase in circulating TH which occurs in large mammals (Fisher *et al.*, 1977; Berthon *et al.*, 1993), embryonic and neonatal MHC isoforms are repressed and adult MHC isoforms are accumulated (d'Albis and Butler-Browne, 1993), and there are marked muscle-specific changes in cellular metabolism and mitochondrial biogenesis (Herpin *et al.*, 1996). Postnatally, although contractile activity has a major influence on muscle phenotype, TH continue to play an important role, not only by inducing switching from type I slow to type II fast MHC, but also by altering myogenic regulatory factors, membrane-bound ATPases and metabolic enzymes (Izumo *et al.*, 1986; Dauncey and Harrison, 1996; Harrison *et al.*, 1996b).

5.3.2.2 TH receptor structure and regulation of transcription

The multiple actions of TH are exerted predominantly at the genomic level, via TH receptors (TR) acting as DNA-binding transcription factors, which operate as molecular switches in response to ligand (Green and Chambon, 1986; Munoz and Bernal, 1997). TR usually bind to specific DNA response elements as heterodimers with retinoid X receptors (RXR), and activation or repression of numerous genes depends on promoter context and ligand-binding status (Fig. 5.1A and B). In the absence of ligand, the TR–RXR heterodimer usually interacts with a corepressor complex containing histone deacetylase activity, which results in a closed repressed form of chromatin that inhibits transcription. Binding of TH to the receptor triggers a conformational change in the heterodimer that results in replacement of the corepressor complex by a coactivator complex containing histone acetyltransferase activity. Increased histone acetylation in turn results in remodelling of chromatin into an open activated form, enabling access of basal transcription factors and RNA polymerase to DNA, thereby leading to activation of transcription.

In addition to the ability of TR to repress or activate transcription, depending on whether or not ligand is present, a further level of complexity is added because TR occur as a series of isoforms encoded by the proto-oncogenes, *c-erbA- α* and *c-erbA- β* , which each regulate a specific set of target genes (Lazar, 1993). Alternative splicing of the TR α mRNA transcript produces two carboxy-terminal variants, TR α 1 and TR α 2, and similarly the TR β gene produces the two amino-terminal variants, TR β 1 and TR β 2. The TR α 1, TR β 1 and TR β 2 isoforms can all bind TH and activate response elements on target genes. However, structural changes in the hormone-binding domain of TR α 2, and lack of AF-2 transactivation function, prevent this isoform from TH binding and activation of transcription, and hence it is not strictly a receptor for TH (Tagami *et al.*, 1998).

Although the precise functions of TR α 2 are unknown, it probably competes with the other TR isoforms for DNA response elements and hence acts as an inhibitor of transcription. Findings on the tissue-specific and developmental expression of TR isoforms in porcine muscle support this hypothesis (White and Dauncey, 1999; White *et al.*, 2001). A striking muscle-specific pattern of TR α isoform distribution occurs in functionally distinct muscles of 7-week-old pigs kept under controlled conditions of nutrition and temperature. The distribution of TR α isoforms is related to muscle phenotype, with TR α 1 predominating in fast muscle and the non-TH-binding TR α 2 predominating in slow muscle. These differences have important functional consequences: in longissimus, TR α 1 > TR α 2 will result in a high proportion of type II fast myosin for rapid

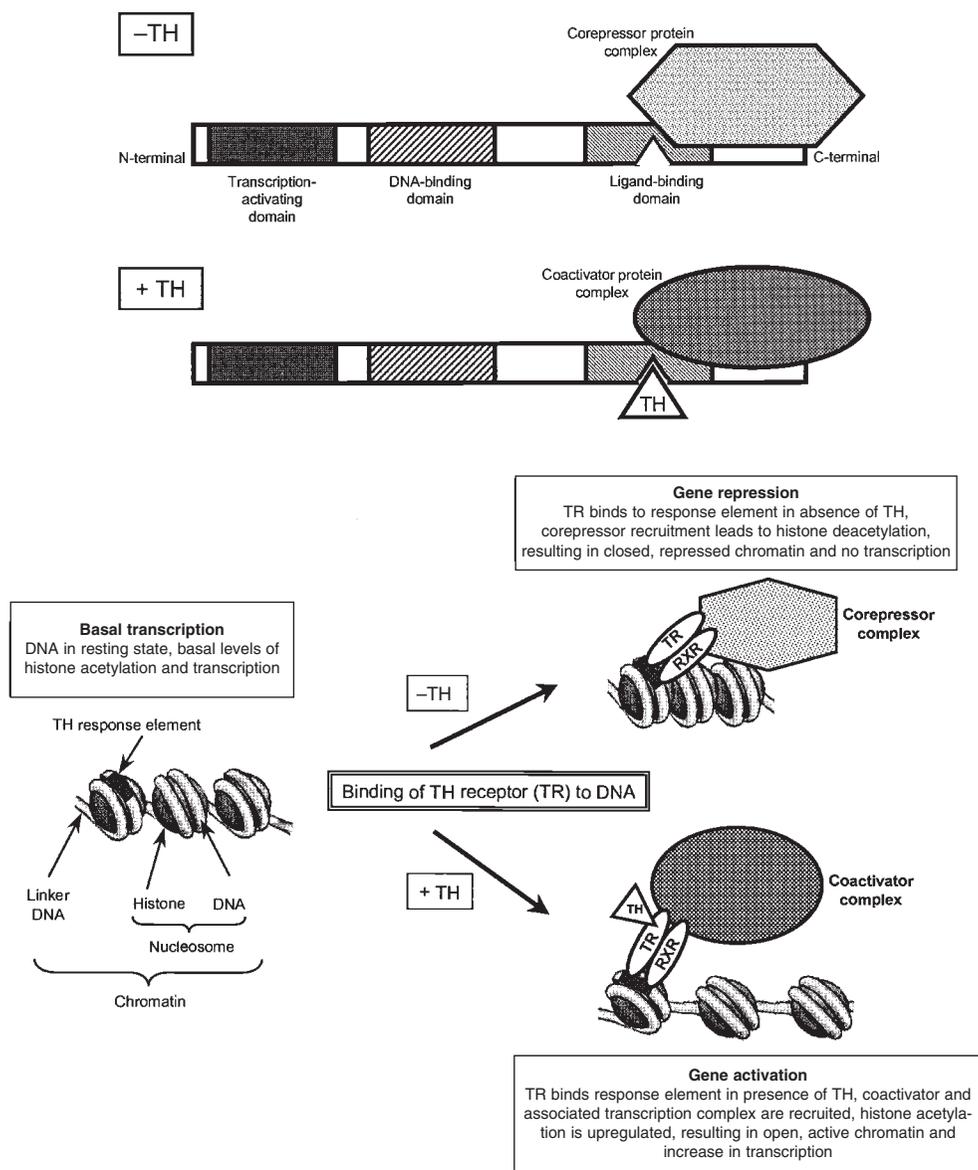


Fig. 5.1. (A) Primary structure of thyroid hormone (TH) receptor demonstrating the recruitment of corepressor or coactivator protein complexes in the absence (-) or presence (+) of TH. (B) Action of nuclear TH receptors (TR) in repression or activation of gene transcription. TR usually bind to specific DNA response elements as heterodimers with retinoid X receptors (RXR). Note that this model operates for TR α 1, TR β 1 and TR β 2 isoforms; structural changes in the C-terminal domain of TR α 2 prevent TH binding, resulting in transcriptional repression, even in the presence of TH (modified from Dauncey *et al.*, 2001, with permission from *Proceedings of the Nutrition Society*).

movement, whereas in cardiac muscle, $TR\alpha1 < TR\alpha2$ will favour the slow myosin needed for sustained contractility.

5.3.3 Growth hormone and insulin-like growth factors

5.3.3.1 GH, IGFs and their actions

One of the most striking effects of GH is to promote bodyweight gain via an increase in lean tissue mass. Administration of GH to farm animals enhances muscle growth, while adipose tissue accretion is reduced (Campbell *et al.*, 1989, 1990; Vasilatos-Younken, 1995). This implies that substrate availability to muscle is increased whereas its disposal and utilization by adipose tissue are reduced by GH, and in dwarf mice GH administration does indeed stimulate muscle protein synthesis (Pell and Bates, 1992). GH is thought to exert its anabolic actions in muscle via locally synthesized IGF-I rather than by direct stimulation of anabolic pathways. In support of this indirect mechanism, exogenous GH increases IGF-I mRNA expression but not that of GHR mRNA or IGF-II mRNA in longissimus of neonatal pigs (Lewis *et al.*, 2000). Expression of GHR and IGF-I is also GH-responsive in semitendinosus muscle of growing pigs (Brameld *et al.*, 1996). The sensitivity and responsiveness of muscle to GH and IGF-I may be regulated by the availability of nutrients such as amino acids. Although infusion of IGF-I alone does not alter muscle protein synthesis in rats, when IGF-I is given in combination with amino acids protein synthesis increases significantly in gastrocnemius, oblique and soleus muscles (Jacob *et al.*, 1996). Additionally, IGF-I may require the IGF binding protein, IGFBP-3, in order to enhance protein synthesis in skeletal muscle (Svanberg *et al.*, 2000).

IGFs play key roles in muscle development, the effects being dependent on stage of development. In general, prenatal effects are concerned with proliferation and differentiation whereas postnatally the major effect is on hypertrophy. Studies on cultured cells have helped to elucidate some of these mechanisms. In cultured myoblasts, IGF-I acts as a potent proliferative and differentiating agent (Florini *et al.*, 1996). Transfection of the IGF-I gene induces differentiation of myotubes and hypertrophy of skeletal muscle, and also attenuates age-related skeletal muscle atrophy in mice by restoring and improving muscle mass and strength (Barton-Davis *et al.*, 1998; Semsarian *et al.*, 1999). A role for IGF-II as an autocrine/paracrine differentiation factor for skeletal myoblasts has also been suggested (Stewart *et al.*, 1996). Indeed, levels of IGF-I and IGF-II mRNA in ovine fetal tissues are uniformly low throughout gestation, except in muscle at the time when secondary fibre formation is taking place (Dauncey and Gilmour, 1996). It has been suggested that IGF-I exerts its effects on muscle development via calcium signalling involving, for example, the calcineurin-mediated pathway (Musaro *et al.*, 1999; Semsarian *et al.*, 1999). Evidence also suggests that the IGF-I-induced increase in proliferative potential of muscle cells is mediated by the phosphoinositide 3-kinase (PI3K)/Akt pathway (Chakravarthy *et al.*, 2000; Tureckova *et al.*, 2001).

5.3.3.2 GH receptor structure and signal transduction

The diverse actions of GH are mediated by the GH receptor (GHR), a member of the cytokine receptor superfamily (Kelly *et al.*, 1994). These receptors are single transmembrane-domain polypeptides, with extracellular ligand-binding domains

and catalytic activity in their intracellular cytoplasmic domains. The human GHR comprises 620 residues. At the amino terminal end, 246 residues represent the extracellular ligand-binding domain and these are followed by a 24-residue hydrophobic sequence which probably codes for the transmembrane domain. These domains are followed by a 350-residue sequence at the carboxyl terminal, representing the intracellular cytoplasmic domain involved in the signal transduction process. In common with other members of the cytokine receptor superfamily, a soluble form of GH-binding protein circulates in plasma. It is identical to the extracellular portion of the GHR, and in rodents it is encoded by a specific mRNA originating from alternative splicing whereas in other mammalian species it is generated by proteolysis of the membrane-bound form of the GHR.

Receptor homodimerization is essential for GHR signal transduction and the homodimer forms a complex with a single molecule of GH (de Vos *et al.*, 1992). In common with other cytokine receptors, the GHR activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, and this is an especially important mediator of GH signal transduction from cell surface to nucleus (Xu and Sonntag, 1996). Other intracellular signalling cascades involved include the mitogen-activated protein kinase (MAPK) pathway and the insulin receptor substrate-1 (IRS-1) and IRS-2 pathways (Maharajan and Maharajan, 1993; Zhu *et al.*, 2001).

5.4 Nutritional Regulation of Hormones, Receptors and Gene Expression

Nutrition has marked effects on the synthesis and metabolism of many hormones involved in development, growth and metabolism (Dauncey, 1995; Brameld, 1997; Holness, 1999; Hornick *et al.*, 2000; Le Dividich and Sève, 2000; Louveau *et al.*, 2000). Regulation is via changes in specific nutrients and also in overall food intake, as occurs during overnutrition, undernutrition and intrauterine growth restriction. Changes in food intake may mediate their effects via differences in energy intake or in specific nutrients such as protein. The finding that the effects of nutrition can be modulated by environmental temperature suggests that energy supply in relation to energy demand is a critical factor in regulating hormone expression. Energy status and energy balance thus play a key role in regulating muscle development via hormones acting as nutritional signals (Dauncey, 1997). Nutrition modulates not only hormone synthesis and metabolism but also hormone receptors. Particularly important is the finding that the response of hormone receptors to nutrition is dependent on stage of development, the specific tissue investigated, and even muscle type (Dauncey *et al.*, 2001). These effects on hormone receptors enable a given circulating level of hormone to have highly specific and diverse developmental and functional actions. This also suggests a mechanism by which early malnutrition may exert long-term or even permanent effects, because changes in cellular development can persist long after optimization of nutrition restores hormone receptors to their optimal level. The following sections discuss the nutritional regulation of TH and GH, and focus on the role of their receptors as mediators of nutrition-gene interactions.

5.4.1 Nutrition, TH and TH receptor

Nutritional status has profound effects on the development and function of muscle. Of the many possible hormonal regulators involved, TH and their nuclear receptors have been strongly implicated in nutritionally induced changes in muscle gene expression. Nutritional quantity and composition markedly affect TH (Ingram and Dauncey, 1986; Dauncey, 1990; Dauncey and Morovat, 1993; Berthon *et al.*, 1996; Harrison *et al.*, 1996a). Energy balance, as affected by either energy intake or energy expenditure, regulates the thyroïdal synthesis, release and metabolism of TH. In the young pig, a high food intake increases thyroid gland activity, circulating levels of T_4 and T_3 , and hepatic deiodinase activity. Conversely, a low food intake reduces thyroid gland activity, plasma TH levels and deiodinase activity. Changes in energy status induced by a cold environment cause a rapid increase in plasma TH levels, via stimulation of the hypothalamic–pituitary–thyroid axis and the thyroid releasing hormone–thyroid stimulating hormone (TRH–TSH) pathway (Evans and Ingram, 1974). However, this increase in circulating TH levels in the cold can be maintained in the long term only if food intake also increases (Macari *et al.*, 1983; Morovat and Dauncey, 1990).

5.4.1.1 Nutritional regulation of total TR

Changes in circulating TH levels alone are unlikely to be responsible for the many actions of nutrition on muscle development; many of these actions are probably regulated by their receptors. Assessment of TR numbers from the maximal T_3 binding capacity (B_{\max}) of isolated nuclei has shown that energy balance plays a key role in regulating total TR. Nuclear TR binding studies on longissimus from young pigs at thermal neutrality (26°C) revealed significantly more TR in animals on a high than a low food intake (Morovat and Dauncey, 1995). Moreover, increasing metabolic demand by lowering the environmental temperature to 10°C down-regulates TR in skeletal muscle (Dauncey *et al.*, 1988). During postnatal development, the effects of food intake on TH and TR are extremely rapid: circulating T_3 increases within 60 min of feeding (Dauncey *et al.*, 1983), and the increase in TR 12–24 h after feeding may play a role in enhancing the thermogenic capacity of the tissues in response to food (Morovat and Dauncey, 1992). Total TR in cardiac muscle are also affected by energy balance: both chronic food deprivation in the rat (Swoap *et al.*, 1994) and low food intake in the young pig (Cotterell and Dauncey, 1991) result in a significant reduction in B_{\max} . These nutritionally induced changes in total TR probably act in addition to changes in TH status to regulate changes in myofibre properties. A decrease in TR protein expression would decrease the ability of TH to regulate TH-responsive genes, making the cell functionally hypothyroid. Thus, differences in receptor binding represent an adaptation that regulates the ability of the tissue to respond to TH.

Intrauterine growth restriction (IUGR) can result from a reduced placental blood supply, which leads to prenatal undernutrition and infants that are born small-for-gestational age (SGA). The SGA piglet provides a naturally occurring form of IUGR which has been used to investigate the influence of prenatal undernutrition on development. At birth, TR numbers are reduced in SGA compared with controls of normal birth-weight, and this may explain their reduced metabolic rate and lower respiratory enzyme activities in skeletal muscle (Dauncey and Geers, 1990; Dauncey, 1995).

A major disadvantage of TH binding studies is that they provide no information on the relative proportions of the TR isoforms. Moreover, TR α 2 cannot be detected by binding assays because this isoform does not bind TH, even though it probably has a vital role as a negative regulator of TH activity (Rentoumis *et al.*, 1990; Yen and Chin, 1994; Saltó *et al.*, 2001; White *et al.*, 2001). Evidence from tissue distribution studies and from the distinctive temporal patterns of appearance of nuclear TR during development suggests that the individual TR isoforms may have distinct functional roles (Oppenheimer *et al.*, 1994; White and Dauncey, 1999; White *et al.*, 2001). The extent to which nutrition regulates TR isoforms in functionally distinct muscles at different stages of development is discussed in the following section.

5.4.1.2 Developmental and nutritional regulation of TR isoform gene expression

Developmental expression profiles of TR isoforms during prenatal and postnatal development reveal new insights into their essential functions in muscle (White *et al.*, 2001). Throughout development, TR α expression is very much greater than that of TR β , and the ratios of TR α 1:TR α 2 and TR β 1:TR β 2 are related to muscle type and stage of development. During cardiac development TR α 2 expression is two to four times greater than that of TR α 1, and expression of both isoforms decreases as development progresses. This developmental expression pattern in cardiac muscle is strikingly different from that occurring in the fast skeletal muscle longissimus (Fig. 5.2). Together with knowledge of MHC isoforms, and the ontogeny of myogenesis and maturation of the TH axis, these findings provide new evidence highlighting central roles for TR α isoforms in myogenesis, and the ratio TR α 1:TR α 2 in determining muscle phenotype and function. This has been confirmed in studies of mice with targeted ablation of TR α 2, in which it was concluded that TR α 1:TR α 2 plays an important widespread regulatory role in mammalian physiology (Saltó *et al.*, 2001). Although TR α are the predominant TR isoforms in muscle, there is probably a role for TR β in maintaining a basal level of responsiveness to TH throughout development and a specific maturational role in the perinatal period (White *et al.*, 2001). These studies suggest that events disrupting the normal developmental profiles of TR isoforms, such as nutritional status, may have an adverse impact on optimal function of skeletal and cardiac muscles.

Both overnutrition and undernutrition influence TR expression in skeletal muscle (Dauncey *et al.*, 2001; Redonnet *et al.*, 2001). The effects of nutrition on TR isoform expression are related to stage of development and muscle type (White and Dauncey, 1998; P. White and M.J. Dauncey, unpublished results). A major effect of prenatal undernutrition on skeletal muscle development is a reduction in secondary myofibre numbers; the effect is greater in predominantly fast muscles (Wilson *et al.*, 1988; Ward and Stickland, 1991; Prakash *et al.*, 1993). Muscle-specific changes in TR isoform expression may therefore be involved in regulating these changes. For example, during IUGR the functional hypothyroidism induced in longissimus by upregulation of TR α 2 would reduce TH-induced formation of secondary myofibres. By contrast, maintenance of TR isoforms in rhomboideus would maintain this tissue's ability to bind TH and secondary myofibre formation would be spared. These differential effects of nutrition on TR and myofibre type would in turn have significant effects on the specific functions of the different muscles.

Postnatally, undernutrition induces a greater upregulation of TR α 2 than TR α 1

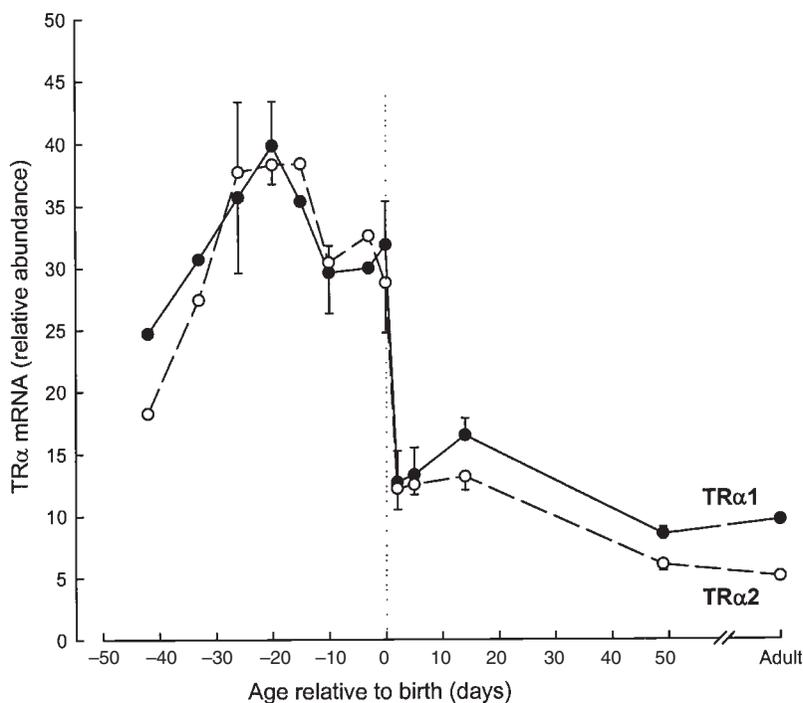


Fig. 5.2. Developmental profile of TH receptor (TR) α isoforms in longissimus dorsi skeletal muscle. Values for relative expression levels of TR α 1 (TH-binding) and TR α 2 (non-TH-binding) are given for pigs aged between -42 days prenatally (i.e. 72 days of gestation; total length of gestation = 114 days) and 2 years postnatally. (From White *et al.*, 2001, with permission from *FASEB Journal*.) Note that: (i) the peak in TR α expression occurs at the time of secondary myofibre formation; and (ii) this developmental expression profile in skeletal muscle is strikingly different from that in cardiac muscle (White *et al.*, 2001).

in skeletal muscle, especially in rhomboideus and soleus (White and Dauncey, 1998; P. White and M.J. Dauncey, unpublished results). Together with a reduction in plasma TH (Dauncey, 1990), these muscles would be rendered functionally hypothyroid, transcription of fast type II MHC would decrease, and slow type I MHC would increase. Indeed, slow type I MHC in rhomboideus is strikingly upregulated by undernutrition, suggesting that muscle-specific changes in TR α isoforms are involved in this response.

The extent to which these differences in nutrition-related TR α isoform expression are related to differences in developmental expression in skeletal muscle (see Fig. 5.2) remains to be established. Nevertheless, nutrition also has major effects on TR isoforms in cardiac muscle, which are again dependent on stage of development (White and Dauncey, 1998; Dauncey *et al.*, 2001). Prenatal undernutrition downregulates cardiac TR isoforms and the decrease is especially marked for TR α 1. Postnatally, by contrast, all cardiac TR isoforms are upregulated by undernutrition and there is a striking 140% preferential increase in TR α 2. Functional hypothyroidism due to this low cardiac TR α 1 expression at birth or the increase in TR α 2 postnatally, combined with reduced plasma TH levels, would have potentially

detrimental consequences for cardiac function and this is discussed in the final section.

5.4.2 Nutrition, GH and GH receptor

It is well established that nutritional status affects the GH–IGF axis. A central dogma of growth regulation is that undernutrition induces growth retardation via the hepatic GHR–IGF axis. Paradoxically, circulating GH levels are increased by undernutrition (Dauncey and Buttle, 1990; Hornick *et al.*, 2000). A lower nutrient influx reduces the release of somatostatin by the hypothalamus and thus reduces negative effects on the synthesis and release of GH. However, GHR mRNA expression in rat liver is reduced by fasting and this is followed by reductions in hepatic IGF-I expression and circulating IGF-I concentration (Straus and Takemoto, 1990). Undernutrition that is mild enough to enable growth also reduces porcine hepatic GHR mRNA, IGF-I mRNA and protein, IGF-I binding protein-3 and circulating IGF-I (Ma *et al.*, 1992; Dauncey *et al.*, 1993, 1994; Weller *et al.*, 1994). Moreover, the effect of nutritional status tends to be greater in a cold environment; the precise effect is dependent on stage of development and relative temperature (Carroll *et al.*, 1999).

5.4.2.1 Regulation of GHR gene expression

The nutritional regulation of GHR expression is tissue-specific and, although GHR mRNA in liver is downregulated by undernutrition, it is upregulated in skeletal muscle (Dauncey *et al.*, 1994; Weller *et al.*, 1994). This tissue-specific regulation of GHR enables a given circulating level of GH to have specific actions in different tissues. An important action of GH in liver is to control growth, via IGF-I production, whereas in muscle it has an essential direct action in regulating metabolism. In general, the functions of GH in regulating metabolism are anti-insulin. GH diverts energy from muscle, and thus upregulation of muscle GHR by undernutrition can be viewed as an adaptation to spare neural and bone growth at the expense of muscle growth, especially during postnatal development. Muscle GHR gene expression increases at a low environmental temperature, suggesting that this adaptation occurs in response to a deficit in energy balance caused by either a low intake or a high energy demand. In the cold, the action of GH in increasing slow type I fibres and the oxidative capacity of muscle will be important because it will enhance metabolic responsiveness but also conserve energy (Ayling *et al.*, 1992).

Tissue-specific regulation of GHR mRNA expression by nutritional status may be related to differential expression of the promoter regions of the GHR gene. Three such promoters have so far been identified: GHR promoter 1 (GHR P1), GHR promoter 2 (GHR P2) and GHR promoter 3 (GHR P3) (O'Mahoney *et al.*, 1994; Adams, 1995; Jiang *et al.*, 1999; Liu *et al.*, 2000). Although GHR P1 is liver-specific, the two other promoters are widely expressed in many tissues, suggesting that GHR mRNA expressed in muscle is transcribed via GHR P2 and GHR P3.

A further level of control over the actions of GH is exerted by specific regulation of GHR within a given tissue type. Muscles with high oxidative capacity have the greatest expression of GHR mRNA, which occurs in descending order of abundance: soleus > heart > rhomboideus = diaphragm > longissimus (Katsumata *et al.*,

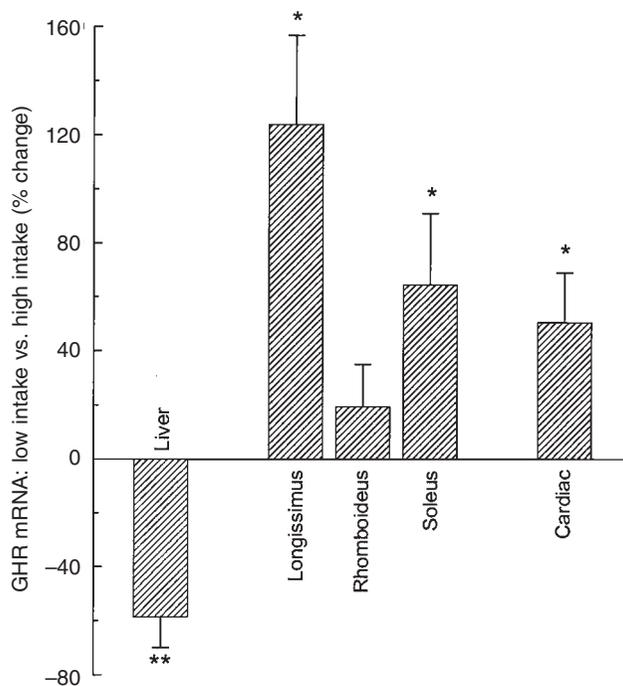


Fig. 5.3. Nutritional regulation of growth hormone receptor (GHR) gene expression in liver and functionally distinct muscles. Percentage change on a low (2% = 20 g/kg/day) compared with a high (6% = 60 g/kg/day) food intake. Means \pm SEM from eight pairs of littermate pigs; ** $P < 0.01$, * $P < 0.05$ (from Katsumata *et al.*, 2000, with permission from *Journal of Nutrition*).

2000), indicating that slow oxidative muscles are more responsive to GH than fast glycolytic muscles. The magnitude of upregulation of GHR mRNA in response to postnatal undernutrition also differs between muscles (Fig. 5.3). The greatest effect occurs in longissimus, which contains mainly fast glycolytic/oxidative-glycolytic fibres and, of the muscles examined, has the lowest expression of GHR mRNA. Of relevance to the muscle-specific responsiveness of GHR to nutrition is the finding that fasting markedly upregulates uncoupling protein (UCP2 and UCP3) mRNA expression in rat muscle (Samec *et al.*, 1998). The magnitude of upregulation is greater in fast glycolytic/oxidative-glycolytic muscles (gastrocnemius and tibialis anterior) than in slow oxidative muscle (soleus). It is probable that muscle adapts to suboptimal energy status by shifting its energy source from glucose to fatty acids via upregulation of GHR, and this upregulation is greatest in longissimus because of its greater capacity to shift substrate from glucose to fatty acids.

More recently it has been found that, in addition to the effects of overall food intake and energy balance, specific nutrients can significantly influence GHR expression in muscle. Reduction in a single amino acid, lysine, in the diet of young growing pigs induces upregulation of GHR mRNA in longissimus and rhomboideus but not in heart (Katsumata *et al.*, 2002). Moreover, a low threonine diet also tends to upregulate GHR in longissimus muscle (M. Katsumata, unpublished data).

5.5 Implications for Health and Disease

5.5.1 Overall consequences of nutrition–hormone receptor–gene interactions

IUGR and postnatal malnutrition can impair both immediate and long-term development, with profound consequences for optimal health and disease (Lucas, 1994; Barker, 1995; Dauncey, 1997; Ozanne and Hales, 1999; Phillips, 2002). Early environment can have long-term effects by programming hormonal systems involved in growth and development, and this in turn leads to cardiovascular risk factors including hypertension, glucose intolerance, dislipidaemia, obesity and type 2 diabetes. Moreover, the risk of later disease is greater when early undernutrition and growth restriction are followed by overnutrition and compensatory growth. There is also considerable concern about the potential adverse effects on domestic animals of exposure to environmental compounds that disrupt normal hormone actions (Sweeney, 2002). The current chapter highlights the critical roles of prenatal and postnatal nutrition in regulating muscle gene expression via nuclear and cell-membrane-bound hormone receptors. The significance of nutrition–nuclear receptor interactions in relation to drug development, and interactions between specific nutrients has been discussed previously (Dauncey *et al.*, 2001). The final sections of this chapter demonstrate the potential effects that nutritionally induced changes in TR and GHR can have on cardiac muscle and insulin sensitivity, respectively.

5.5.2 Nutrition, TR and optimal development of cardiac muscle

Changes in TH status markedly influence the contractile and electrical activity of the heart, and gene inactivation studies have demonstrated a major role for TR α in determining heart rate and contractile performance (Dillmann, 2002). Moreover, the ratio TR α 1:TR α 2 is probably important for regulation of cardiac development and MHC isoform expression (Saltó *et al.*, 2001; White *et al.*, 2001). The promoter region of the rat cardiac α -MHC gene contains at least two positive thyroid response elements (TREs) that increase transcriptional activity when bound to the TH–TR complex (Flink and Morkin, 1990). By contrast, the β -MHC gene has a negative TRE that decreases transcriptional activity when bound to the TH–TR complex (Edwards *et al.*, 1994). Thus, the expression of these muscle-specific genes is regulated by TH in an antithetical manner: TH increases expression of α -MHC while simultaneously decreasing that of β -MHC (Haddad *et al.*, 1997). This strongly suggests that in prenatal undernutrition the decrease in TR α 1 expression and TR α 1:TR α 2, combined with reduced plasma TH (White and Dauncey, 1998; Dauncey *et al.*, 2001), will adversely influence cardiac muscle development by down-regulating α -MHC and contractile performance.

Although TR α 2 cannot actively inhibit transcription (Tagami *et al.*, 1998), its dominant negative activity will protect the tissue from the action of TH by competing with TR α 1 for TRE binding (Yen and Chin, 1994). Postnatal undernutrition markedly upregulates cardiac TR α 2 (White and Dauncey, 1998) and this could have a twofold effect. First, the relatively greater level of TR α 2 will increase competition between TR α 1, TR β 1 and TR β 2 for TRE binding, resulting in reduced α -MHC transcription. Secondly, the preponderance of TR α 2 will increase the probability

that TREs within the β -MHC promoter will be occupied by this non-TH-binding receptor isoform. Consequently, β -MHC transcription will increase. A reduction in food intake in adult rats does indeed result in a reduction of α -MHC and an increase in β -MHC protein expression (Swoap *et al.*, 1994). Functional changes in TH responsiveness as a result of nutritionally induced changes in TR isoforms may therefore profoundly affect myocardial function. Thus, during undernutrition a high level of TR α 2, combined with reductions in circulating TH levels, will reduce cardiac α -MHC transcription and upregulate cardiac β -MHC transcription, leading in turn to a lower intrinsic contractile ability and operational heart rate.

5.5.3 Nutrition, GHR, glucose transporters and insulin sensitivity

The upregulation of muscle GHR gene expression by postnatal undernutrition (Dauncey *et al.*, 1994; Katsumata *et al.*, 2000, 2002) may play a key role in the increase in glucose transporters (GLUTs), which is also induced by undernutrition (Katsumata *et al.*, 1999, 2001). The relevance of these changes to the development of insulin resistance and type 2 diabetes in later life is outlined in Fig. 5.4 and discussed below.

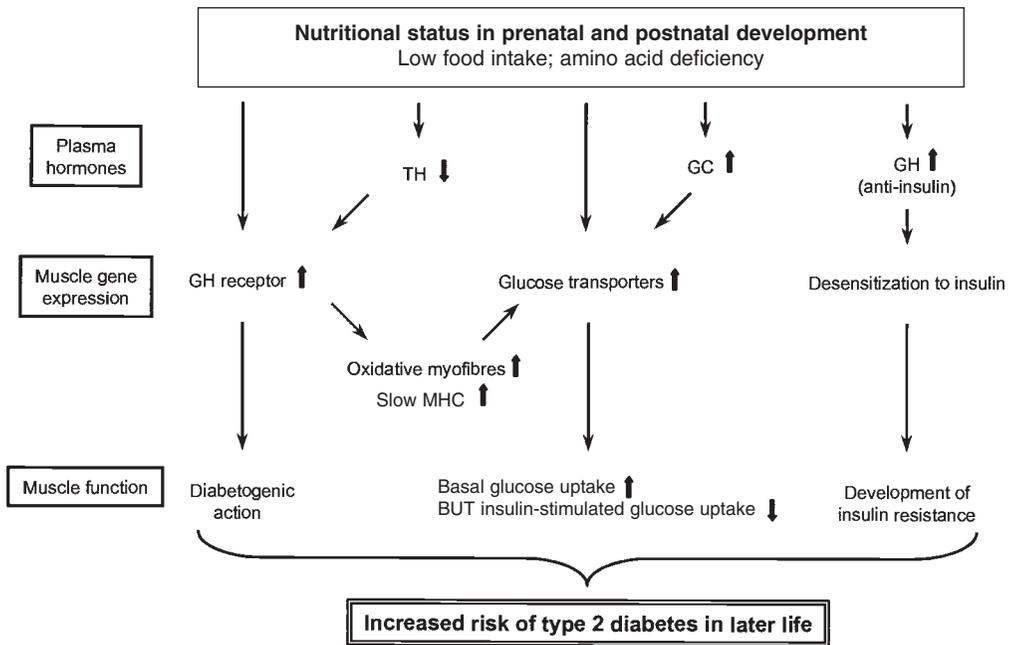


Fig. 5.4. Relevance of nutrition–growth hormone (GH) receptor–gene interactions in early development to insulin sensitivity and risk of type 2 diabetes in later life. Note that: (i) precise details will be dependent on stage of development and muscle type; (ii) actions of thyroid hormones (TH) and glucocorticoids (GC) will be mediated by hormone receptor isoforms, which are themselves subject to nutritional modulation (MHC, myosin heavy chain). Based on data from Duchamp *et al.* (1996), Li *et al.* (1998), Katsumata *et al.* (1999, 2000, 2001) and White *et al.* (2000).

Postnatally, both a low food intake and deficiency of a single amino acid significantly upregulate GLUT1 (basal glucose uptake) and GLUT4 (insulin-stimulated glucose uptake) in skeletal muscle. This upregulation could be due, theoretically, to reductions in blood glucose or muscle glycogen, elevation in glucocorticoids (GC), or an increase in muscle GHR, all of which are induced by undernutrition. Changes in GHR may be especially important in this context. GH has anti-insulin and diabetogenic actions, suggesting that an anti-insulin state is developed by high GHR expression. In order to compensate for this, muscle probably needs to increase GLUT expression. Indeed, there is a higher basal level of insulin-independent glucose uptake and lower insulin-stimulated increment of glucose uptake in isolated muscle of young animals on a low food intake (Katsumata *et al.*, 1999). This suggests that not only the GLUT content of muscle cells but also its subcellular distribution are affected by nutrition. GH stimulates the tyrosine kinase activity of JAK2 and induces tyrosine phosphorylation of insulin receptor substrates (IRS), suggesting that the elevated plasma GH and muscle GHR induced by undernutrition may result in a greater proportion of GLUT remaining on the cell membrane. In protein-deficient rats, insulin secretion from the pancreatic islets is impaired, whereas insulin-induced phosphorylation of the insulin receptor and IRS-1 are increased (Reis *et al.*, 1997). Further, despite low plasma insulin levels during an oral glucose tolerance test, a protein-deficient diet does not affect plasma glucose levels during the test period. This suggests that glucose-stimulated insulin secretion is impaired but that insulin-receptor signalling pathways are stimulated by malnutrition. Age-dependent differences in glucose handling of muscle from rats whose dams were fed a low protein diet during pregnancy also suggest that programming of insulin sensitivity can occur prenatally (Ozanne and Hales, 2002).

An additional factor linking GHR and GLUT4 is the oxidative capacity of muscle. Upregulation of muscle GHR expression by undernutrition probably induces transition from fast glycolytic to slow oxidative myofibres, and this increase in oxidative capacity will in turn lead to higher GLUT4 expression (Katsumata *et al.*, 1999, 2000; White *et al.*, 2000).

In summary, postnatal undernutrition causes upregulation of GHR expression in muscle and this spares neural and bone growth at the expense of muscle growth. However, it may also cause insulin resistance. To compensate for this, GLUT expression in muscle needs to be upregulated. Increased GC may have a role in this upregulation (Li *et al.*, 1998). GC levels increase in undernutrition and they have also been implicated in induction of insulin resistance (Nyirenda *et al.*, 1998; Phillips *et al.*, 1998; Sakoda *et al.*, 2000). This strongly suggests a role for GC in controlling GLUT expression and insulin resistance. Undernutrition will thus result in an anti-insulin/diabetogenic state via modification of GH, GC and insulin signal transduction, leading in turn to changes in GLUT function and insulin sensitivity. In the long term, especially if early undernutrition is followed by overnutrition and compensatory growth in later life, the risk of developing type 2 diabetes will be increased.

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6

The Impact of Minerals and Micronutrients on Growth Control

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6.1 Introduction

Normal growth is dependent on the appropriate administration of nutrients and the presence of growth factors (see also Dauncey *et al.*, Chapter 5, and Goldspink, Chapter 8, this volume). The nutrients are involved, not only as energy sources (amino acids, fats and carbohydrate) or substrates for new protein synthesis (amino acids), but also with a role in the regulation of growth and protein synthesis by interacting with the growth hormone (GH)/insulin-like growth factor (IGF) system, the IGF system (see Dauncey *et al.*, Chapter 5, this volume). Generalized malnutrition is associated not only with growth deficiency and delayed wound healing, but also with impaired function of the IGF system, which is reversed with subsequent nutritional repletion. It is generally accepted that the IGF system mediates many of the cell processes involved in tissue growth and protein synthesis (Thissen *et al.*, 1994).

Impaired growth is a consistent finding with low dietary intake of macro- and/or micronutrients; however, concurrent infections may lead to further anorexia, impaired nutrient absorption and increased gastrointestinal nutrient losses.

Micronutrient deficiency of growing individuals often accompanies shortness of energy and proteins, but may perhaps more often occur with adequate energy intakes. In developing countries, human diets are often unbalanced, principally veg-

etarian with low contents and low bioavailability of micronutrients. In the industrialized countries, the micronutrient contents in agricultural products are steadily decreasing as production is steadily intensifying; the use of growth-stimulating products results in increasing plant yields with higher water content from the same fields. This may reduce the micronutrient contents of human diets and domestic animal diets, with possible development of deficiencies in exposed individuals. Although they are present only in small amounts in the diet, minerals and micronutrients play crucial roles in the regulation of the metabolism of the body, including muscle performance and energy utilization.

This chapter reviews the role of deficiency of micronutrients on growth, protein synthesis and growth factors, with specific focus on the minerals potassium (K), magnesium (Mg) and the micronutrient zinc (Zn). The focus is motivated by the facts that these deficiencies are common and share several characteristics (Dørup, 1994). The review is based on studies of laboratory animals with comments on available data from intervention studies in humans.

6.2 The Effect of Specific Mineral and Micronutrient Deficiencies on Growth and Protein Synthesis

It has been known for decades that nutrients other than carbohydrate, protein and fat are essential for optimum growth (Hopkins, 1906). During the first half of the 20th century a number of essential vitamins, minerals and inorganic micronutrients were identified including iodine, zinc, magnesium, potassium, selenium, copper, molybdenum, chromium, vitamin A and calcium (Golden, 1991; WHO, 1996). The hypothesis of a type I and a type II deficiency was proposed by Michael Golden (Golden, 1988). Type I deficiencies, such as selenium, iodine, vitamin A and vitamin D deficiencies, induce specific signs with initially normal growth: for example, night blindness in vitamin A deficiency or enlarged thyroid gland in iodine deficiency. Type II deficiencies, which are probably much more common, are characterized by primary growth failure and initially no specific signs. He exemplified the types of deficiency with selenium and zinc: 'When a wheat plant is grown in soil low in selenium, the result is a normally sized plant that has a low selenium concentration in its tissues. When a wheat plant is grown on a soil low in zinc, the result is a stunted plant that has a normal concentration of zinc in its tissues' (Golden, 1988).

Impaired growth has been described in dietary deficiency of copper in cattle and pigs (Graham, 1991; Apgar *et al.*, 1995). Recently, manganese deficiency has been shown to reduce growth in rats (Clegg *et al.*, 1998), but in this context, most attention has been drawn towards Zn deficiency and, to a smaller extent, towards Mg and K deficiency.

Deficiencies of K, Mg and Zn are all characterized by primary growth retardation or growth cessation. When animals are fed a diet low in K, but otherwise adequate, a prompt (within a few days) growth inhibition is seen, whereas a pronounced and rapid catch-up growth was seen when switching to a similar but K-sufficient diet (Fig. 6.1) (Dørup, 1994). Also when keeping animals on a Mg-deficient or a Zn-deficient diet, weight gain was rapidly reduced, and growth was resumed when adding the missing element to the diet, a catch-up growth that was even faster than the growth in control animals (Dørup and Clausen, 1991). The young animal is most

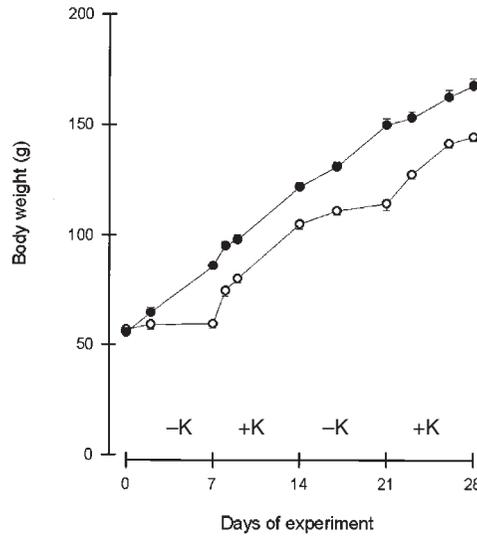


Fig. 6.1. Four-week-old rats were maintained on control food (K-deficient food supplied with K to obtain a content similar to normal food (●-●)) or in periods of 1 week on K-deficient food alternating with periods on the control food (○-○). Periods on K-deficient food are mirrored in growth retardation, whereas the supply of K leads to increased growth rates. Repeated periods of K-deficiency may lead to permanent stunting (figure adapted with permission from Dørup, 1994).

vulnerable, reflecting the higher relative growth rate in young animals. On the other hand, it is difficult to induce K-, Mg- or Zn-deficiency in a non-growing animal unless there is a pathological loss of the nutrient from the body. It should be noted that the reduced weight gain in K-, Mg- and Zn-deficiency is accompanied by reduced bone length (O'Leary *et al.*, 1979; Dørup *et al.*, 1991; Flyvbjerg *et al.*, 1991).

A common feature of the growth retardation in K-, Mg- or Zn-deficiency is that it occurs almost immediately after presenting the animal the deficient diet as a result of reduced food intake. It is not likely that the lack of a specific mineral can be detected by taste, yet there must be another mechanism inducing the pronounced anorexia. Furthermore, the prompt occurrence of anorexia is seen before any tissue depletion of the element can be expected.

As body protein turns over continuously at about 3% per day in adults (Crim and Munro, 1994), estimates of protein synthesis rate represent a sensitive tool for evaluating metabolic changes during dietary restriction. We estimated the rate of protein synthesis in young rats *in vivo* by measuring the amount of [^3H] activity incorporated into the total proteins of skeletal muscle following an intravenous injection of [^3H]leucine. After only 3 days on K-deficient food, [^3H]leucine incorporation into soleus and extensor digitorum muscles was reduced by around 33%, and further reductions (up to 50%) were seen (Dørup and Clausen, 1989). When the animals were acutely K-repleted by an injection of KCl, leading to complete restoration of muscle K within 2 h, the defect in protein synthesis was not restored until 3 days later. Furthermore, in experiments with graded K-content in the food, growth and protein synthesis was reduced in animals with no detectable muscle K-deficit. In con-

trast, experiments with isolated intact muscles showed that the rate of protein synthesis was strongly correlated to the K content of the muscles. Reducing the K content of the muscles *in vitro* by 10% reduced the [³H]leucine incorporation by 7%, whereas a similar reduction in the *in vivo* system inhibited incorporation by 34%. This difference in rate of protein synthesis between measurements obtained *in vitro* and *in vivo* suggests that, in the intact organism, other regulatory factors than the K content in the muscles are more important.

After 14 days on Mg-deficient food or Zn-deficient food, weight gain in young rats was in both cases markedly suppressed, and [³H]leucine incorporation was reduced by 23% and 58%, respectively (Dørup and Clausen, 1991). However, although food intake was reduced, decreased energy intake was not the limiting factor. Pairfeeding experiments clearly showed that Mg- and Zn-deficiency per se had a restrictive effect on growth and protein synthesis independent of energy intake. Furthermore, the pronounced inhibition of growth and protein synthesis during Mg- or Zn-deficiency was seen in spite of no change in the muscle content of the lacking element.

Taken together, the reduction in growth and protein synthesis seen in K-, Mg- or Zn-deficiency cannot easily be related to reduced content of the lacking element in the muscles, and points to some other regulatory mechanisms. Anorexia is another common and early feature that may be an important defence mechanism to delay or prevent more specific clinical signs of deficiency.

The endogenous IGF system plays a central role in regulating and facilitating tissue anabolic processes and normal growth (for systemic effects see Goldspink, Chapter 8, this volume). On the other hand, it is well recognized that energy and protein restriction (see Dauncey *et al.*, Chapter 5, this volume) have profound effects on the IGF system, and it was therefore natural to study the effects of K-, Mg- and Zn-deficiency on this system. Furthermore, it had already been shown that in young Zn-depleted rats, serum IGF-I was markedly reduced (Oner *et al.*, 1984).

6.3 The IGF System and its Regulation

The insulin-like growth factors are present in serum and in most tissues and organs of the body. They are mainly produced by the liver, which is the dominant source of the circulating IGF, but in addition paracrine/autocrine secretion of many organs has been recognized. In muscle tissue, blood-borne IGF-I accounts for about 85% of the total amount (Hodgkinson *et al.*, 1991). Further evidence that blood-borne IGF-I may fulfil specific endocrine functions (for systemic effects see Goldspink, Chapter 8, this volume) is supported by studies showing that exogenous IGF-I stimulates growth in young rats and stimulates protein synthesis in skeletal muscle and other tissues in neonatal pigs (Hizuka *et al.*, 1986; Davis *et al.*, 2002).

The IGF system consists of the IGF factors I and II, the IGF receptors and the IGF binding proteins. In humans and mammals six binding proteins (BPs) have been characterized which, apart from carrying the IGFs in the circulation, act as physiological modulators and regulators of IGF actions (Mohan and Baylink, 2002). The most abundant IGFBP in serum is IGFBP-3, which binds more than 90% of the IGFs in the circulation. Information about the nutritional regulation of IGF-II is sparse, and the following will focus on IGF-I.

The dominant hormonal regulator of IGF-I postnatally is growth hormone (GH), which stimulates the liver synthesis of IGF-I and is central for circulating IGF-I levels. Furthermore, GH regulates the synthesis of the large IGFBP-3. However, IGF-I and IGFBP-3 are also principally regulated by nutritional status and dietary supply of protein and energy, thereby providing an important link between nutrition and growth.

Dietary restriction of energy or protein is associated with a significant decrease in serum IGF-I. Decades ago, it was shown that children with primarily protein depletion (kwashiorkor) as well as those with combined protein-energy malnutrition had reduced bioactivity of somatomedin-C (IGF-I) (Grant *et al.*, 1973; Hintz *et al.*, 1978). In healthy adults, fasting produces a significant decrease in IGF-I reaching hypopituitary levels within 5 days and about 25% of the normal level after 10 days (Clemmons *et al.*, 1981). Refeeding results in normalized values, but adequate intakes of *both* protein and energy are necessary for the restoration of IGF-I after a period of fasting (Isley *et al.*, 1983). The quality of the protein is important, as a diet rich in essential amino acids more readily restores serum IGF-I levels compared with a diet rich in non-essential amino acids (Isley *et al.*, 1984; Clemmons *et al.*, 1985). Conditions other than malnutrition are associated with low IGF-I. In hospitalized patients with anorexia nervosa, inflammatory bowel disease or coeliac disease, IGF-I levels generally correlate well with nutritional status, nitrogen balance and protein intake (Thissen *et al.*, 1994). On this basis, use of IGF-I as a tool for monitoring nutritional support was suggested. Evidence from laboratory animals as well as domestic animals confirms that serum IGF-I decreases with diets low in either protein or energy (Underwood *et al.*, 1986; Spicer *et al.*, 1990; McGuire *et al.*, 1995).

Both fasting and restriction of dietary protein induces increased GH secretion in humans, as well as in pigs, sheep and cows (Breier *et al.*, 1986; Thomas *et al.*, 1990; Buonomo and Baile, 1991; Thissen *et al.*, 1994; see also Dauncey *et al.*, Chapter 5, this volume). Despite the high GH secretion, serum IGF-I is low, indicating that these conditions represent states of GH resistance, which may be associated with either loss of liver IGF-I receptors, reduced hepatic IGF-I mRNA content or reduced hepatic GH-receptor binding (Breier *et al.*, 1988; Thissen *et al.*, 1994). In contrast with human and larger mammals, rats show impaired GH secretion during dietary energy or protein restriction. However, GH treatment causes no stimulation of growth in protein-restricted rats (Thissen *et al.*, 1991). These findings support the concept that protein restriction in general causes decreased IGF-I levels together with GH resistance.

6.4 The Effect of Specific Mineral and Micronutrient Deficiencies on the IGF System

Evidence now exists that the IGF system is impaired not only in states of energy or protein restriction, but also in several other conditions, with growth retardation in relation to specific dietary deficiencies. Most studies have been done with K-, Mg- or Zn-deficient rats, which are also the focus of this review.

Animals fed a K-deficient diet showed a reduction in serum K within a few days, in parallel with a marked decrease in serum IGF-I. When K was supplied again, IGF-I was normalized as were serum K and weight gain (Flyvbjerg *et al.*, 1991).

During moderate graded K-deficiency, a corresponding graded response in serum IGF-I was seen. When stimulating K-deficient rats with GH-releasing factor, a blunted release of GH from the pituitary was seen. K-deficiency leads to a reduction in the concentration of liver GH receptors and GH binding proteins in serum (Hochberg *et al.*, 1995). More recently, it was shown that in K-deficient animals, liver IGF-I mRNA was reduced, whereas no change in liver IGF-I receptor could be detected (van Neck *et al.*, 1997). Administration of GH through mini-osmotic pumps to K-deficient animals resulted in a very small, although significant, increase in weight gain, but growth was far from normalized (Dørup *et al.*, 1992). It could be speculated that normalization of serum IGF-I could induce weight gain in K-deficient animals. However, IGF-I treatment for 14 days restored serum IGF-I in K-deficient rats, but did not result in overall growth of carcass or skeletal muscles (Dørup and Flyvbjerg, 1993).

When rats were switched to a Mg-deficient diet, a rapid drop in plasma Mg and suppression of the normal age-dependent rise in serum IGF-I were seen. Thus, after 1 and 2 days serum Mg was reduced by 34% and 57%, respectively. Mg repletion restored both serum Mg and serum IGF-I (Dørup *et al.*, 1991). In experiments with graded Mg-deficiency, growth inhibition was seen only in groups with reduced IGF-I. In contrast with the findings with K-deficiency, serum GH levels after stimulation with GH-releasing factor were not decreased in Mg-deficient animals (Dørup *et al.*, 1991).

Serum IGF-I has repeatedly been shown to be decreased by Zn-deficiency (Oner *et al.*, 1984; Dørup *et al.*, 1991; McNall *et al.*, 1995). When young rats were kept on a Zn-deficient diet, serum Zn was reduced to 42% of the level in paired controls within 3 days, and to 20% after 14 days, whereas serum Zn was completely restored after 3 days on a Zn-sufficient diet. Serum IGF-I showed a small decrease in the Zn-deficient animals, but the paired animals doubled their IGF-I concentration as a result of the normal age-dependent increase (Dørup *et al.*, 1991). When switching to Zn-sufficient food, there was a rapid rise in serum IGF-I, which closely paralleled the rise in weight gain and serum Zn. In rats maintained on food with graded Zn-content, weight gain was impaired only in the groups with reduced IGF-I, again emphasizing the close relationship between IGF-I and growth (Dørup *et al.*, 1991). As in Mg-deficiency, the GH response to GH-releasing factor was not impaired, suggesting that different mechanisms may be involved in growth control during K-, Mg- and Zn-deficiency. In another study, force-feeding a Zn-deficient diet to rats for 12 days resulted in a 28% decrease in serum IGF-I, together with increased serum GH, when compared with animals receiving an isocaloric Zn-sufficient diet (Roth and Kirchgessner, 1994). In growing lambs kept on a low Zn diet, serum IGF-I levels were reduced, and the GH response to GH-releasing factor tended to be higher (Droke *et al.*, 1993).

In rats, the reduced serum IGF-I levels are accompanied by reduced liver IGF-I mRNA, lower IGF-BPs, a decrease in liver GH receptors and reduced concentrations of serum GH binding proteins (McNall *et al.*, 1995; Ninh *et al.*, 1998). Treatment with GH normalized liver GH receptors and serum GH binding proteins, but did not restore growth or serum IGF-I, indicating that the GH resistance in Zn-deficiency results from defects beyond the liver GH receptor (Ninh *et al.*, 1998). Another study showed that administration of IGF-I to Zn-deficient rats did not induce growth, even when food intake was comparable with control rats (Browning *et al.*, 1998).

Insulin plays a role in the maintenance of serum IGF-I, and is reduced in protein-restricted rats (Thissen *et al.*, 1994). However, in short-term K-deficiency, serum insulin was unchanged, while IGF-I was reduced by 46% compared with pairfed controls (Flyvbjerg *et al.*, 1991). In short-term Mg-deficiency, basal insulin levels were also unchanged, but during Zn-deficiency there was a significant reduction in serum insulin (Dørup *et al.*, 1991). Non-fasted insulin levels show great variability, and it cannot be excluded that part of the reduction in IGF-I and growth in K-, Mg- and Zn-deficiency may be secondary to decreased insulin secretion.

Taken together, K-, Mg- and Zn-deficiency share a number of common characteristics. Reduced growth and protein synthesis is seen without detectable tissue deficits, but with marked decline in the serum concentration of the element as well as of IGF-I. Pairfeeding experiments showed that this was not the result of reduced energy intake. These changes were reversible by the addition of the lacking element. In all three conditions, a relative GH resistance is seen, and for Zn- and K-deficiency, growth retardation is not reversed through administration of IGF-I.

Apart from K, Mg and Zn, a few other micronutrients and specific amino acids have been studied. Rats on a lysine-deficient diet were growth retarded and had reduced serum IGF-I, compared with rats on an isocaloric and isonitrogenous diet (Cree and Schalch, 1985; see also Dauncey *et al.*, Chapter 5, this volume). In another study, in which threonine or tryptophan was omitted from the diet, growth rate was depressed until the missing amino acids were added to the diet, and growth correlated well with serum-IGF-I contents (Noguchi, 2000). In a recent study, young rats fed a manganese-deficient diet were found to reduce their growth rate, had lower circulating concentrations of IGF-I (66% of control level) and had higher circulating GH levels. In addition, circulating insulin levels were reduced (Clegg *et al.*, 1998).

Whether the pattern described above is a general phenomenon, which can be reproduced with other micronutrient deficiencies, remains to be elucidated in future studies. In general, it appears that adequate supplies of specific nutrients and growth factors are both essential for facilitating tissue protein synthesis and growth.

6.5 Human Studies

Studies of malnourished children during rehabilitation may contribute to our understanding of the relationships between nutritional influences, growth and growth factors.

An early study found that total body K was low in malnourished children and normalized with recovery (Alleyene, 1970). Mg-deficiency has been demonstrated in nearly half of marasmic children in India, and there was a significant correlation between height for age and serum Mg (Singla *et al.*, 1998). It has earlier been shown that Mg-supplementation accelerated recovery from malnutrition (Nichols *et al.*, 1978). However, no randomized controlled trials have been performed focusing on the effect of K- or Mg-supplementation, and in this context, most attention has been paid to Zn-deficiency.

Officially, the potential impact of specific nutrient deficiencies was not recognized until recently, although trials with Zn-supplementation have been conducted since the early 1980s (Golden and Golden, 1981). In 1993 it was suggested that Zn may have a role in stunting, especially in developing countries (De Onis *et al.*, 1993),

and in 1997 Zn was included among the micronutrient deficiencies listed as a priority in the World Health Organization's *Third Report on the World Nutrition Situation* (ACC/SCN, 1997).

Recently, several randomized studies on the impact of Zn-supplementation have been conducted in industrialized and developing countries. In a study from Ethiopia, 200 stunted or non-stunted infants received placebo or Zn sulphate for 6 months. Length and weight were markedly increased in the Zn-supplemented infants, averaging 4.32 SD for stunted and 1.88 SD for non-stunted infants (Umeta *et al.*, 2000). Furthermore, Zn supplementation resulted in a lower incidence of anorexia and morbidity from cough, diarrhoea, fever and vomiting in the stunted children. A meta-analysis including 33 randomized controlled intervention trials on the effect of Zn-supplementation showed that Zn produced highly significant positive responses in height and weight increments, with greater growth responses in children with low initial weight-for-age (Brown *et al.*, 2002).

The weight gain seen with Zn-supplementation is likely to be related to increased protein synthesis. Malnourished Jamaican children with chronic marginal Zn-deficiency undergoing nutrition rehabilitation showed greater gains in fat than in muscle, until supplemented with Zn, when proportionately more lean-tissue accretion occurred (Golden and Golden, 1992). In addition to the effect on growth, Zn-supplementation seems to have other substantial clinical effects. A meta-analysis including all randomized trials of the effects of oral Zn-supplementation in children with acute or persistent diarrhoea showed that Zn, given in a daily dose of about twice the recommended daily allowance, significantly reduced the duration of acute or persistent diarrhoea (Bhutta *et al.*, 2000).

There is now evidence that the IGF system is impaired in Zn-deficiency, also in the human setting. In a study of growth-retarded Vietnamese children, Zn-supplementation was associated with increased weight and height after 5 months and, in addition, circulating IGF-I increased significantly, compared with placebo-treated children (Ninh *et al.*, 1996). Recently a detailed study of the effect of Zn-supplementation on IGF-I, IGFBP and various markers of bone and collagen formation was published. The malnourished children had extremely low circulating levels of IGF-I and low IGFBP3; however, with Zn-repletion, weight gain and linear growth were accompanied with increasing circulating levels of IGF-I and IGFBP3 and markers of bone and collagen formation (Doherty *et al.*, 2002).

In general, intervention studies with specific micronutrients in humans have shown smaller effects than predicted from the animal experiments. This may be due to the fact that, in real life, most individuals are only marginally depleted. Furthermore, studies from various geographical areas cannot always be compared, since specific deficiencies may be connected to certain cultures and environments. Most importantly, multiple nutrient deficiencies are likely to coexist, and growth would be expected to be limited by the most deficient nutrient. In accordance, with a dietary supplement which does not contain all the deficient nutrients required for new tissue synthesis, the growth rate will be determined by the most limiting nutrient in the new diet (Golden, 1991).

6.6 Summary and Conclusions

In young individuals, optimum growth and protein synthesis depend on appropriate administration of energy sources and substrates. The quantity and nutritional quality of dietary protein is essential, but specific minerals and micronutrients such as K, Mg and Zn appear to be of independent importance. When animals are fed a diet deficient in K, Mg or Zn rapid decreases in food intake and in serum concentrations occur, with no change (Zn) or a slow decline in concentrations in skeletal muscle (K and Mg). Another common feature is that growth and skeletal muscle protein synthesis are markedly inhibited at normal or near-normal tissue concentrations of the element, which implies that other regulatory factors may be involved.

Insufficient dietary supplies of energy and protein lead to reduced circulating IGF-I and GH resistance. There is now evidence that specific minerals and micronutrients, such as K, Mg and Zn, influence the IGF system. Dietary deficiency of K, Mg and Zn are associated with decreased circulating IGF-I levels and GH resistance independent of energy intake. Many mechanisms are involved, such as down-regulation of liver GH-receptor abundance and GH postreceptor defects, decreased IGF-I gene expression and modulation of IGF actions through changes in IGFBP levels (Thissen *et al.* 1994; Estívariz and Ziegler, 1997). In addition, K- and Zn-deficiency are associated with resistance to IGF-I. The effects cannot simply be explained by anorexia, which is a central and common feature in these deficiency states.

It appears that the supply of energy, protein and specific minerals and micronutrients control IGF-I production and regulate its biological activity. Conditions with insufficient supplies of one or more elements allow the organism to minimize wasteful protein synthesis, preventing unsuitable formation of functionally inadequate tissues.

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7

Na⁺,K⁺-ATPase in Skeletal Muscle: Significance of Exercise and Thyroid Hormones for Development and Performance

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7.1 Introduction

This chapter deals with the Na⁺,K⁺-ATPase of skeletal muscle. Na⁺,K⁺-ATPase is a ubiquitous enzyme in the plasma membrane of animal cells. In skeletal muscle it is essential to maintain the membrane potential and to preserve excitability. Each action potential is associated with loss of K⁺ during repolarization, leading to a rise

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in plasma K^+ . The better Na^+,K^+ -ATPase can keep pace with the loss of K^+ , the longer work can be performed by muscle, suggesting that a rise in Na^+,K^+ -ATPase concentration may improve performance. Because skeletal muscle is one of the largest body compartments, and because of its high Na^+,K^+ -ATPase concentration, it represents the largest single pool of Na^+,K^+ -ATPase, responsible for the acute (minute-to-minute) regulation of plasma K^+ , in particular during exercise. The purpose of this review is to discuss skeletal muscle Na^+,K^+ -ATPase of livestock and domestic species, rodents and human subjects as well as the influence of thyroid hormones and exercise on this enzyme.

7.2 Na^+,K^+ -ATPase in Skeletal Muscle

7.2.1 Working mechanism of Na^+,K^+ -ATPase

In 1941, it was proposed that the plasma membrane of most cells should contain a pump to transport Na^+ out and K^+ into the cell (Dean, 1941; Clausen and Persson, 1998). Sixteen years later, the first article describing Na^+,K^+ -ATPase, sodium and potassium adenosine triphosphatase, was published. In this article, Skou described the presence of an enzyme in cell membranes isolated from shore crab nerves. The enzyme could be activated by Mg^{2+} , Na^+ and K^+ and was thought to be involved in the active transport of Na^+ and K^+ across the cell membrane (Skou, 1957). Because of his discovery of Na^+,K^+ -ATPase, Skou was presented with the Nobel Prize in Chemistry on 10 December 1997 (Clausen and Persson, 1998). Since the first article in 1957, Na^+,K^+ -ATPase has been the subject of numerous studies, mostly performed in rat (Clausen *et al.*, 1998; Overgaard *et al.*, 1999; Clausen, 2000) and human subjects (Medbø *et al.*, 2001; Kjeldsen *et al.*, 2002). Na^+,K^+ -ATPase is situated in the plasma membrane of practically all tissues of all animals (Clausen, 1996). Skeletal muscle contains one of the largest pools of Na^+,K^+ -ATPase in the body as it has a high concentration of Na^+,K^+ -ATPase per gram tissue weight and it represents around 40% of total body weight (Clausen and Everts, 1989; Clausen and Nielsen, 1999).

Na^+,K^+ -ATPase maintains the high internal K^+ and low internal Na^+ concentrations typical of most animal cells. In skeletal muscle, Na^+,K^+ -ATPase is mainly situated in the sarcolemma and in the transverse tubule (T-tubule) system (Williams *et al.*, 2000; Juel *et al.*, 2001) and consists of two α and two β subunits (see Fig. 7.1). Na^+,K^+ -ATPase contains two ATP binding sites, situated on the cytosolic sides of the α subunits. From hydrolysis of the γ -phosphate ester bond in ATP, Na^+,K^+ -ATPase receives enough energy to pump out three Na^+ ions and pump in two K^+ ions in one cycle of activity. The α subunit isoform is a multispanning membrane protein with a molecular mass of about 110 kDa and is necessary for the catalytic and transport properties of the enzyme (Shull *et al.*, 1985; Lingrel and Kuntzweiler, 1994). The subunit is known to exist in three isoforms, α_1 , α_2 and α_3 , which are well conserved among vertebrates (Takeyasu *et al.*, 1990; Pressley, 1992). The α_1 subunit isoform is found in nearly all tissues, the α_2 isoform is expressed primarily in brain, heart and skeletal muscle and α_3 is expressed primarily in brain and heart (Shull *et al.*, 1986; Pressley, 1992; Blanco and Mercer, 1998). A human α -like gene may encode a fourth Na^+,K^+ -ATPase α isoform, expressed in rat testis (Shamraj and Lingrel, 1994). The β subunit isoform of Na^+,K^+ -ATPase spans the membrane once

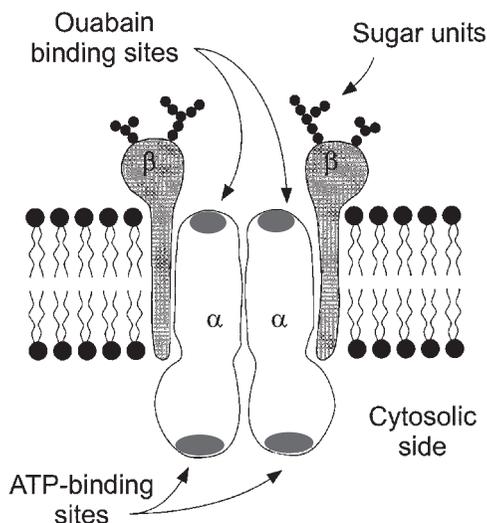


Fig. 7.1. Schematic drawing of the Na⁺,K⁺-ATPase, redrawn from Stryer (1995). The Na⁺,K⁺-ATPase consists of two α and two β subunits, situated in the plasma membrane, with two ATP-binding sites on the cytosolic side and two ouabain binding sites on the extracellular side.

and, depending on the degree of glycosylation, has a molecular weight between 42 and 55 kDa (Ewart and Klip, 1995). The β subunit is needed for normal activity of the enzyme (Blanco and Mercer, 1998) and is found to exist in three isoforms, β_1 , β_2 and β_3 (Gloor *et al.*, 1990; Good *et al.*, 1990; Peng *et al.*, 1997). The β_1 subunit isoform is expressed in most tissues, while isoforms β_2 and β_3 show a more restricted distribution. An increasing number of articles have been published describing a third component of Na⁺,K⁺-ATPase, the γ subunit (Mercer *et al.*, 1993; Béguin *et al.*, 1997; Jones *et al.*, 2001). This small, single-pass membrane protein of about 10 kDa associates with functional $\alpha\beta$ complexes (Béguin *et al.*, 1997; Jones *et al.*, 2001). The exact function of the γ subunit is still unknown.

In skeletal muscle, the α_2 subunit is the predominantly expressed isoform of Na⁺,K⁺-ATPase. The α_1 isoform is expressed as well, but forms only a small fraction of the total amount of α subunits, with an estimated maximum contribution of 25% (Hansen, 2000). The β subunit isoforms present in skeletal muscle include β_1 and β_2 (Martin-Vasallo *et al.*, 1989; Thompson *et al.*, 1999). Together, four $\alpha\beta$ combinations can be formed (Sweadner, 1989), of which $\alpha_2\beta_2$ seems to be the predominant heterodimer in fast glycolytic muscle, while both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers are expressed in slow oxidative muscle (Thompson and McDonough, 1996). These expression profiles of the different isoforms of the Na⁺,K⁺-ATPase subunits in adult skeletal muscle are found both at the mRNA and at the protein level (Orlowski and Lingrel, 1988b; Sweadner, 1989; Thompson and McDonough, 1996; Hansen, 2000).

7.2.2 Quantification

Ouabain, a cardiac glycoside, binds to the extracellular domain of the Na⁺,K⁺-ATPase α subunit. A simple and rapid method, using radioactively labelled ouabain,

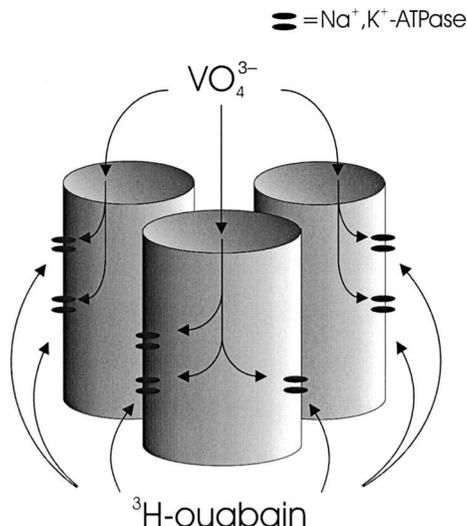


Fig. 7.2. Schematic representation of the binding of ³H-labelled ouabain to a muscle biopsy, redrawn from Clausen and Nielsen (1999). The vanadate (VO₄³⁻) enters the muscle by its open ends, binds to the Na⁺,K⁺-ATPase on the cytosolic side, and locks it in a fixed conformation. ³H-labelled ouabain then binds to the outer surface of the Na⁺,K⁺-ATPase.

was developed to determine the concentration of Na⁺,K⁺-ATPase in skeletal muscle biopsies (Nørgaard *et al.*, 1983). To facilitate the binding of ouabain to the α subunit, the phosphate analogue vanadate (VO₄³⁻), a potent inhibitor of Na⁺,K⁺-ATPase, is used (see Fig. 7.2) (Clausen and Nielsen, 1999). Vanadate binds to the phosphorylation site of Na⁺,K⁺-ATPase on the cytoplasmic side of the sarcolemma, thereby locking the enzyme. Ouabain binds with high affinity to the α_2 and α_3 subunit isoforms, but only with low affinity to the α_1 isoform (Lücking *et al.*, 1996). Using this technique of vanadate-facilitated binding of ³H-ouabain, the total ³H-ouabain binding capacity can be determined with great precision in small biopsies (2–14 mg) of skeletal muscles (Nørgaard *et al.*, 1983). The total number of Na,K pumps in biopsies of mammalian skeletal muscle varies between 75 and 800 pmol/g wet weight (Nørgaard *et al.*, 1983; Clausen *et al.*, 1998; McCutcheon *et al.*, 1999; also see Tables 7.1 and 7.2).

It has been an important issue to establish that Na,K pumps, as quantified using the ³H-ouabain binding technique in intact muscle samples, represent an equal number of active enzyme units. To make a comparison with methods assaying the hydrolytic activity of Na⁺,K⁺-ATPase is difficult, because these methods always imply purification of the preparation resulting in a large variety of recovery of the membrane proteins (Hansen and Clausen, 1988). However, measurements of the K-dependent 3-*O*-methylfluorescein phosphatase activity (Huang and Askari, 1975) in crude homogenates of skeletal muscle and heart revealed values in the same order of magnitude as those obtained with the ³H-ouabain binding assay (Nørgaard *et al.*, 1983; Hansen and Clausen, 1988). Furthermore, measurement of the ouabain-suppressible ⁸⁶Rb uptake in skeletal muscles, under conditions that maximally activate Na⁺,K⁺-ATPase (i.e. Na⁺ loading and high extracellular K⁺), gives values near the

maximum Na,K transport rate calculated from the ³H-ouabain binding capacity. This indicates that the Na,K pumps quantified with this technique are all functional (Clausen *et al.*, 1987; Hansen and Clausen, 1988). The small amount of tissue needed, combined with the great accuracy, makes the method attractive for application in studies in living animals and human subjects, because biopsies can be taken under mild sedation and local anaesthesia. In our department we have measured the ³H-ouabain binding capacity in skeletal muscle from cats, dogs and cattle at different ages, as well as from foals, ponies and adult horses to study the effects of thyroid status, food deprivation and training.

The Na⁺,K⁺-ATPase concentration of control fast, mixed and slow skeletal muscle (for an overview of skeletal muscle types see Reggiani and Mascarello, Chapter 2, this volume) was measured in various animals (see Table 7.1, controls, and Table 7.2, euthyroids). In rat, for example, Na⁺,K⁺-ATPase concentration was measured in slow soleus, fast extensor digitorum longus (EDL) and mixed gastrocnemius muscles (Kjeldsen *et al.*, 1986). No significant difference was found, although it seemed that the faster the muscle, the higher the Na⁺,K⁺-ATPase concentration. Comparable results were found in guinea pig; mixed gastrocnemius muscle showed a Na⁺,K⁺-ATPase concentration of about 380 pmol/g wet weight, while this was about 280 pmol/g wet weight in slow soleus muscle (Leivseth *et al.*, 1992).

In 2-week-old pigs, no difference was found between the slow soleus and rhomboideus muscles and the fast longissimus dorsi muscle (Harrison *et al.*, 1996). Another study in pigs of the same age showed a lower Na⁺,K⁺-ATPase concentration in fast longissimus dorsi muscle, compared with slow soleus muscle (Dauncey and Harrison, 1996). When muscles of pigs of 8 weeks old were compared, a slight difference was also found. Fast longissimus dorsi muscle had a lower Na⁺,K⁺-ATPase concentration than the slow soleus muscle: 320 pmol/g wet weight versus 380 pmol/g wet weight, respectively (Harrison *et al.*, 1994). In horse, similar results were found: faster muscles had a lower Na⁺,K⁺-ATPase concentration (Suwannachot *et al.*, 2001; see also Table 7.1, semitendinosus versus gluteus medius muscle).

7.2.3 Significance of Na⁺,K⁺-ATPase for the regulation of plasma K⁺ and muscle excitability

K⁺ is one of the most abundant cations in organisms and a high intracellular K⁺ concentration is critical for many cell functions, such as growth and division. Extracellular K⁺ is tightly regulated by the combined action of the kidney and skeletal muscle (Palmer, 1999; McDonough *et al.*, 2002). The kidney regulates the extracellular K⁺ concentration in the long term by secreting K⁺ into urine or by actively absorbing K⁺ (Palmer, 1999). Skeletal muscle contains the largest pool of K⁺ and regulates extracellular K⁺ concentration in the short term by activation of Na⁺,K⁺-ATPase (McDonough *et al.*, 2002).

The Na⁺,K⁺-ATPase concentration and activity in skeletal muscle is regulated by multiple factors, as will be discussed in Section 7.2.4. Exercise is one of these factors and it is known to induce a major increase in extracellular K⁺ (Medbø and Sejersted, 1990). During exercise, action potentials are evoked by the influx of Na⁺ from the extracellular fluid into the muscle and followed by the efflux of K⁺. These passive leaks cause a rise in concentration of Na⁺ inside the muscle and K⁺ concen-

tration extracellularly, which activates Na^+, K^+ -ATPase. Na^+, K^+ -ATPase exports three Na^+ ions and imports two K^+ ions in one cycle of activity, resulting in a net export of one positive charge and restoration of the membrane potential (E_m). Without the activity of Na^+, K^+ -ATPase, skeletal muscle would remain exposed to a high extracellular K^+ concentration, which would cause a loss in contractility (Cairns *et al.*, 1995; Verburg *et al.*, 1999). Through restoration of Na^+ and K^+ concentrations and by the electrogenic action of Na^+, K^+ -ATPase, the resting membrane potential of the muscle is protected and the excitability during exercise is retained (Hicks and McComas, 1989; Overgaard *et al.*, 1999; Overgaard and Nielsen, 2001). During maximum activity, the capacity of Na^+, K^+ -ATPase for the K^+ import may be exceeded (Clausen and Everts, 1989). The extracellular K^+ concentration is then increased; excitability is decreased and endurance and force of the muscle are reduced (Clausen and Everts, 1991). During K^+ deprivation, caused by an increase in K^+ secretion by the kidney or by K^+ -deficient food, skeletal muscle loses K^+ and responds with a decrease in the concentration of Na^+, K^+ -ATPase (Nørgaard *et al.*, 1981; Kjeldsen *et al.*, 1984b; Thompson *et al.*, 1999). The K^+ loss increases further and the fall in extracellular K^+ is buffered. The question of whether an increased concentration of Na^+, K^+ -ATPase automatically leads to better performance or endurance is discussed in the next section.

7.2.4 Regulation of Na^+, K^+ -ATPase in skeletal muscle

For the adaptation of cells to changes in Na^+ and K^+ intake, or excitation-induced depolarization, Na^+, K^+ -ATPase has to be under control. This includes acute as well as long-term control. The acute regulation involves the translocation of Na^+, K^+ -ATPase from or to the sarcolemma, or a change in its activity (Ewart and Klip, 1995). The translocation of different Na^+, K^+ -ATPase subunit isoforms might play a role in regulation of Na^+, K^+ -ATPase activity (Blanco and Mercer, 1998). By affecting its synthesis or degradation, Na^+, K^+ -ATPase can be regulated in the long term. Factors that play a role in the acute regulation of the Na^+, K^+ -ATPase activity include excitation, catecholamines, insulin, insulin-like growth factor (IGF-I), amylin and calcitonin-gene-related peptide (CGRP). Those involved in the long-term regulation include thyroid hormone, growth, corticosteroids, training and K^+ supply (see Fig. 7.3) (Clausen and Everts, 1989; Clausen, 1996). Some of the factors that influence Na^+, K^+ -ATPase activity are discussed below.

One of the major factors that causes an increase in Na^+, K^+ -ATPase activity is excitation. This increased activity is necessary to retain excitability during exercise and is caused by the increase in intracellular Na^+ concentration and the increase in extracellular K^+ concentration (Nielsen and Clausen, 1997; Overgaard *et al.*, 1999; Overgaard and Nielsen, 2001).

Adrenaline is one of the catecholamines produced and secreted by the adrenal glands and increases blood pressure and heart rate. Recent studies show that amylin, secreted by the pancreas, and adrenaline both stimulate Na^+, K^+ -ATPase (as measured by its ATP consumption), which is directly associated with a rise in glycolysis and glycogenolysis (James *et al.*, 1999; Clausen, 2000; Therien and Blostein, 2000).

Insulin plays an important role in the maintenance of low intracellular Na^+ concentrations by inducing an increase in activation of Na^+, K^+ -ATPase and by translo-

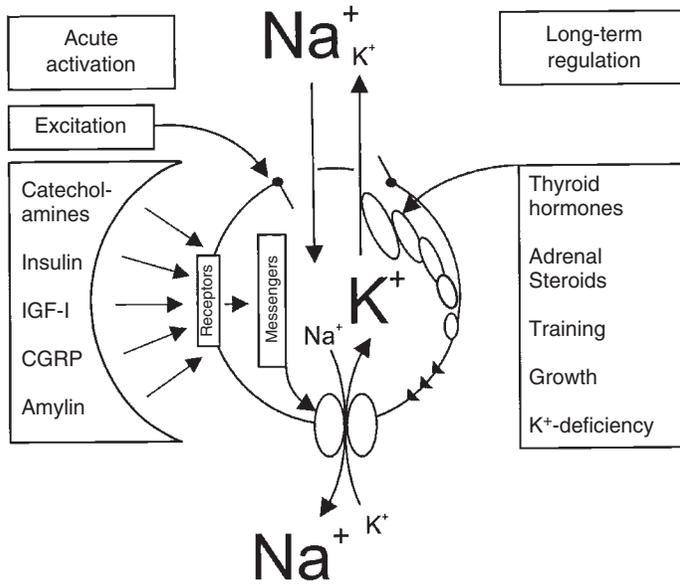


Fig. 7.3. Diagram of the factors that play a role in both acute and long-term regulation of the Na⁺,K⁺-ATPase, redrawn from Clausen (1996). Left: factors eliciting acute stimulation of the Na⁺,K⁺-ATPase. Right: factors known to influence the Na⁺,K⁺-ATPase concentration in the long term.

cation of the subunit isoforms in skeletal muscle (Hundal *et al.*, 1992; Chibalin *et al.*, 2001). Insulin induces the translocation of α_2 and β_1 subunit isoforms from the intracellular compartments to the plasma membrane. The α_1 and β_2 isoforms are not affected.

One of the main factors to exert a long-term effect on Na⁺,K⁺-ATPase is thyroid hormone. T₃, triiodothyronine, is the most important and active thyroid hormone. The thyroid gland mainly produces T₄, thyroxine, which is thought to have little or no activity until it is deiodinated to T₃ (Visser, 1996). Thyroid hormone, also a major regulator of the metabolic rate, increases the concentration of Na⁺,K⁺-ATPase in skeletal muscle (Everts and Clausen, 1988; Harrison and Clausen, 1998). This effect is usually seen throughout the skeletal muscle pool (Clausen and Everts, 1989). Hyperthyroidism increases the amount of α_2 and β_2 subunit isoforms without affecting the amount of α_1 and β_1 isoforms (Haber and Loeb, 1988; Azuma *et al.*, 1993; Hansen, 2000). Hypothyroidism leads to a decrease in Na⁺,K⁺-ATPase concentration in skeletal muscle (Everts *et al.*, 1990; Harrison *et al.*, 1996) due to a selective decrease in the α_2 and β_2 subunit isoforms (Horowitz *et al.*, 1990; Azuma *et al.*, 1993). Again, no effect was measured on the α_1 subunit isoform. In spite of the large rise in Na⁺,K⁺-ATPase concentration in skeletal muscle, hyperthyroidism in rats is associated with increased fatigability and decreased endurance during exercise (Everts *et al.*, 1987; Harrison and Clausen, 1998). The thyroid hormone-induced increase in concentration of Na⁺,K⁺-ATPase is preceded by an increase in passive leaks of Na⁺ and K⁺, indicating that muscle endurance is determined by the leak-to-pump ratio of Na⁺, rather than by the Na⁺,K⁺-ATPase concentration alone (Everts and Clausen, 1988; Harrison and Clausen, 1998).

Growth is another important driving force for regulation of Na^+, K^+ -ATPase in the long term. The Na^+, K^+ -ATPase concentration in skeletal muscle of growing rats shows a sharp rise after birth up to 4 weeks of age, and then declines progressively along with the increase in cell volume (Clausen and Everts, 1989). Recent studies indicate that these age-related changes in rats differ among fast glycolytic and slow oxidative muscle fibres, and may be related to changes in the pattern of expression of the α and β subunit isoforms (Sun *et al.*, 1999). Both the concentration and the activity of Na^+, K^+ -ATPase increase during myogenesis *in vitro* (Benders *et al.*, 1992), a process that is further associated with an increase in the amount of α_2 subunit, while the α_1 isoform remains constant (Higham *et al.*, 1993). During myogenesis *in vivo*, it is also the α_2 subunit isoform that increases substantially (Orlowski and Lingrel, 1988b).

During exercise following a period of training, trained subjects show a smaller rise in plasma K^+ than untrained subjects (Green *et al.*, 1993; McKenna *et al.*, 1993). Since training is known to increase the concentration of Na^+, K^+ -ATPase in skeletal muscle of rat and human, this may explain the blunted rise in plasma K^+ (Green *et al.*, 1993; McKenna *et al.*, 1993; Juel *et al.*, 2001). The excitability during the exercise is retained and fatigue is reduced (Overgaard and Nielsen, 1999, 2001). Trained humans appear to have improved exercise endurance, as evidenced from a decrease in fatigue and increase in work output, when compared with untrained subjects (McKenna *et al.*, 1993; McKenna, 1995). The effect of training on the concentration of Na^+, K^+ -ATPase does not represent a general systemic effect, but is rather related to the muscle groups involved in the training programme (Suwannachot *et al.*, 2001).

7.3 Na^+, K^+ -ATPase in Livestock and Domestic Species

7.3.1 Livestock and domestic species

Although a lot of research has been performed on the Na^+, K^+ -ATPase subunit isoforms in skeletal muscle of rat, not much is known about the isoform distribution in muscle of livestock and domestic animals. On the other hand, Na^+, K^+ -ATPase concentrations have been quantified in skeletal muscle of livestock and domestic animal species and appear to vary considerably between the different species. In the semitendinosus muscle of female draught cattle, for example, a Na^+, K^+ -ATPase concentration of 155 pmol/g wet weight has been measured (Veeneklaas *et al.*, 2002). A value of 2.8 times the concentration in cattle is found in dog skeletal muscle: 440 pmol Na^+, K^+ -ATPase/g wet weight (Schaafsma *et al.*, 2002). Dogs and cattle also differ considerably in body weight (12-fold). When the Na^+, K^+ -ATPase concentration of different animals (in pmol/g wet weight) is plotted against the corresponding body weight, a negative correlation is shown (see Fig. 7.4). Nevertheless, assuming that skeletal muscle accounts for around 40% of body weight, the picture would show a large rise in total Na^+, K^+ -ATPase concentration in the whole muscle pool from small to larger animals. Even if the value of 40% skeletal muscle tissue mass is lower in animals with a much greater body mass than human subjects, the same relationship would still emerge: the greater the body mass, the higher the total Na^+, K^+ -ATPase concentration. This is exactly what has been observed for the metabolic rate: larger animals produce more heat and consume more oxygen than smaller

animals, but per unit body mass the reverse picture is obtained (Porter, 2001). Thus, the oxygen consumption rate per unit body mass is higher in smaller animals when compared with larger ones (Couture and Hulbert, 1995; Porter, 2001; Darveau *et al.*, 2002). This correlation between mass and metabolism can be partly explained by the ratio of whole body surface to volume. When the volume of animals decreases, the body surface (in relation to the volume) increases. The whole body surface of a mouse in relation to its volume is much larger than that of an elephant. Therefore, the mouse loses more heat, has a higher metabolism and consumes more oxygen per gram per minute. A difference in organ size, mitochondrial leakage and ATP turnover between large and small animals may also play a role in the differences in metabolism (Porter, 2001; Darveau *et al.*, 2002). Therefore, it is plausible that Na⁺,K⁺-ATPase concentration differs between animals with varying body weight, in accordance with the difference in metabolic rate. Also, the difference in Na⁺,K⁺-ATPase concentration between large and small animals may reflect that small animals move much more rapidly than larger animals. This observation could in turn imply that the frequency of action potentials is higher in small animals. In fact, in human and dog, oxygen consumption and skeletal muscle Na⁺,K⁺-ATPase concentration both increase with training (Hirche *et al.*, 1980; Green *et al.*, 1993) and it has been estimated that in mammals approximately 19–28% of the oxygen consumption is used by Na⁺,K⁺-ATPase (Rolfe and Brown, 1997). In sheep and calves, up to 29 and 40%, respectively, of the oxygen consumption was suppressible by ouabain, which represents the amount of oxygen used by Na⁺,K⁺-ATPase (Gregg and Milligan, 1982; McBride and Early, 1989).

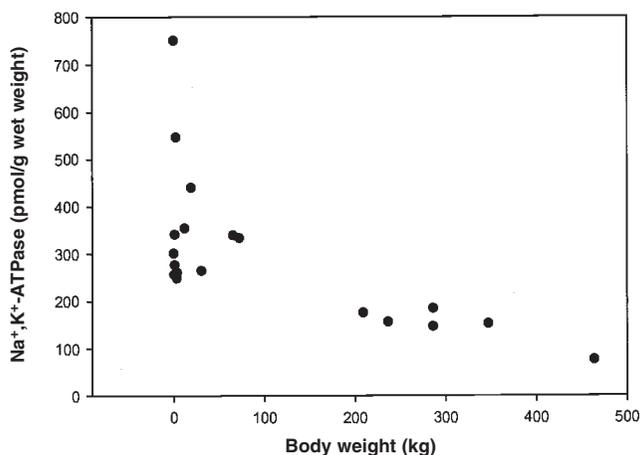


Fig. 7.4. The concentration of Na⁺,K⁺-ATPase in skeletal muscle of different animals, as a function of the corresponding body weights. Animals presented in this graph are: 4-week-old and adult rat (Nørgaard *et al.*, 1983; Harrison and Clausen, 1998), rabbit (Green *et al.*, 1992), guinea pig (Leivseth *et al.*, 1992), cat (I.A. Schaafsma, Utrecht University, personal communication), dog (Schaafsma *et al.*, 2002; B.A. Schotanus, Utrecht, personal communication), neonatal and 8-week-old pig (Harrison *et al.*, 1994, 1996), cattle (Veeneklaas *et al.*, 2002), Shetland pony (Suwannachot *et al.*, 2000), foals and adult horses (McCutcheon *et al.*, 1999; Suwannachot *et al.*, 2001). Human subjects are also included (Green *et al.*, 1993; McKenna *et al.*, 1993).

The correlation between skeletal muscle Na^+, K^+ -ATPase concentration and weight cannot be fitted to a straight line. Between 0 and 10 kg body weight, Na^+, K^+ -ATPase concentrations are measured that are much higher than those at body weights between 10 and 500 kg. The two highest values, 750 and 546 pmol Na^+, K^+ -ATPase/g wet weight have been measured in younger animals: in 4-week-old rat and neonatal pig, respectively (Harrison *et al.*, 1996; Harrison and Clausen, 1998). This directly explains why these values are so high when compared with adult animals of the same weight group. After birth, Na^+, K^+ -ATPase concentration is high; it decreases directly after birth because of hypertrophy of the skeletal muscle; the diameter of muscles increases, while the number of Na^+, K^+ -ATPase units remains equal. The Na^+, K^+ -ATPase concentration, expressed in pmol/g wet weight is then decreased.

7.3.2 Exercise

As already discussed in Section 7.2.4, exercise is one of the factors involved in the long-term regulation of Na^+, K^+ -ATPase. Although most research investigating the role of exercise in Na^+, K^+ -ATPase regulation has been performed in rat and human subjects (Green *et al.*, 1993; McKenna *et al.*, 1993; McKenna, 1995; Overgaard and Nielsen, 1999, 2001; Juel *et al.*, 2001), livestock and domestic species have been investigated as well. In this section, the effect of exercise on Na^+, K^+ -ATPase of guinea pig, rabbit, dog, sheep, cattle, Shetland ponies and horse will be discussed (see Table 7.1).

Both female and male draught cattle showed significant increases in skeletal muscle Na^+, K^+ -ATPase concentration after a 2 h training programme performed for only 8 days (Veeneklaas *et al.*, 2002). No further increase in Na^+, K^+ -ATPase concentration was found when training was continued up to 15 days (Veeneklaas *et al.*, 2002). In trained Hereford steers, a significant reduction of the plasma K^+ peak concentration was observed during a short exercise bout (Fosha-Dolezal and Fedde, 1988). Compared with untrained steers, trained steers could run faster, indicating an increased performance capability.

During exercise, plasma K^+ increases substantially in horses and decreases again after the exercise (Harris and Snow, 1992; McKeever *et al.*, 1993; Gottlieb-Vedi *et al.*, 1996; Jahn *et al.*, 1996; McCutcheon *et al.*, 1999). At higher intensity exercise, plasma K^+ increases even more and endurance appears to decrease with exercise intensity (Harris and Snow, 1992). Trained horses have a blunted increase in plasma K^+ during exercise and an increased skeletal muscle Na^+, K^+ -ATPase concentration compared with untrained horses (McCutcheon *et al.*, 1999; Suwannachot *et al.*, 2001). This increased Na^+, K^+ -ATPase concentration was maintained during a 6 month period of detraining subsequent to the 5 month sprint-training period (Suwannachot *et al.*, 2001). Low intensity training, on the other hand, had no significant effect on plasma K^+ and Na^+, K^+ -ATPase concentration of the gluteus medius of Shetland ponies (Suwannachot *et al.*, 2000). Horses of varying ages also expose differences in response to exercise. In 13-year-old horses, plasma K^+ values during and after exercise were higher than those of 3–5-year-old horses, accompanied by decreased endurance (Harris and Snow, 1992).

After 18 weeks of immobilization of the hind limb of sheep, a decrease in the

Table 7.1. Effects of training on the concentration of Na⁺,K⁺-ATPase in skeletal muscle biopsies of human subjects, guinea pigs, rabbits, cattle, Shetland ponies and horses. EDL, extensor digitorum longus.

Species	Muscle	³ H-ouabain binding sites (pmol/g wet weight)			Reference
		Control	Trained	Effect (%)	
Human	Vastus lateralis	339 ± 16	385 ± 19	14	Green <i>et al.</i> (1993)
Human	Vastus lateralis	333 ± 19	387 ± 15	16	McKenna <i>et al.</i> (1993)
Guinea pig	Soleus	276 ± 18	345 ± 14	25	Leivseth <i>et al.</i> (1992)
Guinea pig	Gastrocnemius	382 ± 21	500 ± 25	31	Leivseth <i>et al.</i> (1992)
Rabbit	EDL	248 ± 8	461 ± 67	86	Green <i>et al.</i> (1992)
Cattle (female)	Semitendinosus	155 ± 6	201 ± 13	30	Veeneklaas <i>et al.</i> (2002)
Cattle (male)	Semitendinosus	196 ± 9	228 ± 12	16	Veeneklaas <i>et al.</i> (2002)
Shetland ponies	Gluteus medius	174 ± 19	180 ± 26	3	Suwannachot <i>et al.</i> (2000)
Horse (5 months)	Semitendinosus	146 ± 5	192 ± 4	32	Suwannachot <i>et al.</i> (2001)
Horse (5 months)	Gluteus medius	184 ± 6	224 ± 9	22	Suwannachot <i>et al.</i> (2001)
Horse (4–8 years)	Gluteus medius	75 ± 3	100 ± 5	33	McCutcheon <i>et al.</i> (1999)

concentration of Na⁺,K⁺-ATPase in the vastus lateralis muscle of 39% was measured (Jebens *et al.*, 1995). At the same time, plasma K⁺ was elevated by 70%. During a period of remobilization, Na⁺,K⁺-ATPase concentration returned to control values (Jebens *et al.*, 1995). Immobilization of the hind limb of guinea pig also reduced the Na⁺,K⁺-ATPase concentration. Within 3 weeks, Na⁺,K⁺-ATPase concentration in gastrocnemius muscle was decreased by 23% (Leivseth *et al.*, 1992). However, during the fourth week of immobilization, levels returned to control values. Three weeks of running exercise increases the Na⁺,K⁺-ATPase concentration by 31% in fast gastrocnemius muscle and 25% in slow soleus muscle (Leivseth *et al.*, 1992).

Chronic, low frequency stimulation of the extensor digitorum longus (EDL) muscle of rabbit increases the Na⁺,K⁺-ATPase concentration, an effect that was significant after 4 days (Green *et al.*, 1992). The rise in Na⁺,K⁺-ATPase continued until a maximum of 461 pmol Na⁺,K⁺-ATPase/g wet weight was reached after 10 days of stimulation (Green *et al.*, 1992). This increase of 86% is much higher than all other values of trained animals (see Table 7.1). This is probably due to the use of electrical stimulation instead of physical stimulation and therefore a higher intensity of stimulation of the muscle.

When dog gastrocnemius muscle was stimulated electrically, a rapid rise in plasma K⁺ was detected followed by a gradual decrease. After a short recovery, plasma K⁺ became even lower than the resting control value (Hirche *et al.*, 1980). After a period of 6 weeks of training on a treadmill, dogs show a resting plasma K⁺ concentration that is lower than that of untrained dogs (Knochel *et al.*, 1985). Furthermore, the Na⁺,K⁺-ATPase activity was increased in trained dogs and peak plasma K⁺ during exercise was blunted (Knochel *et al.*, 1985).

7.3.3 Thyroid hormones

Thyroid hormone is the major endocrine factor that exerts a long-term effect on Na^+, K^+ -ATPase in skeletal muscle, by increasing its concentration (Everts and Clausen, 1988; Harrison and Clausen, 1998). Most of the research on the effect of thyroid hormone on Na^+, K^+ -ATPase has been performed in hypo- and hyperthyroid rats. In addition, a number of studies have been performed in domestic and live-stock animals (see Table 7.2).

Hypothyroidism is the most frequent thyroid disorder encountered in dogs, while hyperthyroidism is observed more often in cats. Hypothyroid dogs exhibit a skeletal muscle Na^+, K^+ -ATPase concentration that is decreased by 41% compared with euthyroid dogs (Schaafsma *et al.*, 2002). Plasma K^+ concentration was elevated by 14% in resting hypothyroid dogs and during exercise plasma K^+ increased significantly, while in euthyroid dogs, no rise in K^+ was observed (Schaafsma *et al.*, 2002). In hyperthyroid cats, plasma total thyroxine (T_4) concentration was increased approximately fourfold and Na^+, K^+ -ATPase concentration was 73% higher, compared with euthyroid cats (I.A. Schaafsma, Utrecht University, personal communication).

Hypothyroid pigs 14 days old show a decrease in Na^+, K^+ -ATPase concentration in both slow and fast muscles; in longissimus dorsi, soleus and rhomboideus muscle, Na^+, K^+ -ATPase was decreased by 16% (Harrison *et al.*, 1996). Ouabain-sensitive respiration is augmented in hyperthyroid sheep, compared with euthyroid sheep (McBride and Early, 1989), an indication that the Na^+, K^+ -ATPase concentration is indeed increased.

Table 7.2. Comparison of the concentration of Na^+, K^+ -ATPase in skeletal muscle biopsies of rats, dogs, cats and pigs in different thyroid states. Abbreviations: hypoth., hypothyroid; hyperth., hyperthyroid; ND, not determined.

Species	^3H -ouabain binding sites (pmol/g wet weight)				References
	Muscle	Hypoth.	Euthyroid	Hyperth.	
Rats (4 weeks)	Soleus	ND	577 ± 19	1172 ± 33	Everts and Clausen (1988)
Rat (10 weeks)	Soleus	ND	256 ± 11	625 ± 18	Harrison and Clausen (1998) Schaafsma <i>et al.</i> (2002)
Dog	Sternothyroid	264 ± 27	440 ± 27	ND	Schaafsma (Utrecht, personal communication); Harrison <i>et al.</i> (1996)
Cat	Sternothyroid	ND	260 ± 18	450 ± 46	Harrison <i>et al.</i> (1996)
Pig (14 days)	Longissimus dorsi	477 ± 29	554 ± 14	ND	Harrison <i>et al.</i> (1996)
Pig (14 days)	Soleus	462 ± 28	554 ± 32	ND	Harrison <i>et al.</i> (1996)
Pig (14 days)	Rhomboideus	446 ± 28	538 ± 29	ND	Harrison <i>et al.</i> (1996)

7.4 Na⁺,K⁺-ATPase During Development

7.4.1 *In vivo*

During growth and development, muscles are formed in a process called myogenesis (see also Rehfeldt *et al.*, Chapter 1, Houba and te Pas, Chapter 10, and Maltin and Plastow, Chapter 13, this volume). During maturation, the Na⁺,K⁺-ATPase concentration changes: it is high after birth and decreases thereafter because of hypertrophy of the skeletal muscle. The diameter of skeletal muscle increases while the number of Na⁺,K⁺-ATPase units remains equal, causing a decrease in Na⁺,K⁺-ATPase concentration. Therefore, a difference in the concentration of Na⁺,K⁺-ATPase is found between animals of different age. This is also shown in Fig. 7.4; the highest values have been measured in younger animals, 4-week-old rat and neonatal pig (Harrison *et al.*, 1996; Harrison and Clausen, 1998). When these highest Na⁺,K⁺-ATPase values are compared with values of older animals of the same species, a decrease in Na⁺,K⁺-ATPase concentration of 66 and 35% can be measured, respectively (Harrison *et al.*, 1994, 1996; Harrison and Clausen, 1998).

In rat, the [³H]ouabain binding site concentration in soleus muscle showed a sharp rise up to 4 weeks of age, and then slowly decreased (Kjeldsen *et al.*, 1984a). This was paralleled by changes in the mRNA expression of the Na⁺,K⁺-ATPase isoforms; from birth to 2 weeks of age, the Na⁺,K⁺-ATPase α_1 subunit isoform remained constant while the α_2 isoform increased 89-fold. The α_3 isoform increased to a lesser extent (Orlowski and Lingrel, 1988b). At the protein level, the Na⁺,K⁺-ATPase α_1 subunit isoform steadily decreased from 2 to 4 weeks of age, while the α_2 isoform increased (Sweadner *et al.*, 1992). From 6 to 30 months of age, on the other hand, the α_1 subunit isoform increased in hind limb muscle and the α_2 isoform decreased in soleus and gastrocnemius muscle (Sun *et al.*, 1999). The Na⁺,K⁺-ATPase β_1 subunit isoform increased and the β_2 isoform decreased in gastrocnemius muscle. The β_1 isoform remained constant in soleus muscle (Sun *et al.*, 1999). The Na⁺,K⁺-ATPase concentration and activity increased again in senescent rats (Sun *et al.*, 1999). In mice, the skeletal muscle Na⁺,K⁺-ATPase α_1 subunit isoform was also expressed at constant levels, while the α_2 isoform increased from embryonic day 18.5 to 19 days of age (Cougnon *et al.*, 2002). The β_1 subunit isoform could be detected at birth and increased during development. From day 5, the β_2 isoform was also expressed and it increased until day 19 (Cougnon *et al.*, 2002). In both rats and mice, Na⁺,K⁺-ATPase concentration increased after birth until it reached a plateau at maturity.

In female draught cattle a slight age-dependent decrease in Na⁺,K⁺-ATPase concentration was observed between 1 and 7 years of age, while this phenomenon was less clear for the bulls, probably due to their higher activity in the herd (Veeneklaas *et al.*, 2002). The Na⁺,K⁺-ATPase dependent respiration was 26% higher in skeletal muscle from younger calves compared with older calves (Gregg and Milligan, 1982).

Also in horses, a difference in the skeletal muscle Na⁺,K⁺-ATPase concentration between younger and older animals has been found (McCutcheon *et al.*, 1999; Suwannachot *et al.*, 2001). In foals of 5 months, a concentration of 184 pmol Na⁺,K⁺-ATPase/g wet weight was measured in the gluteus medius (Suwannachot *et al.*, 2001), while in adult horses a concentration of 75 pmol/g wet weight was found

(McCutcheon *et al.*, 1999) (see Table 7.1). After sprint training these values rose to 224 and 100 pmol/g wet weight, i.e. increases of 22 and 33%, respectively (McCutcheon *et al.*, 1999; Suwannachot *et al.*, 2001).

In pigs, skeletal muscle Na⁺,K⁺-ATPase concentration started to increase before birth and continued to increase until a peak was reached 2 days after birth (Dauncey and Harrison, 1996). Skeletal muscle of chicken showed an increase in Na⁺,K⁺-ATPase concentration from 0.3 pmol/mg protein to 8.8 pmol from embryonic day 11 to 21, a 29-fold increase (Vigne *et al.*, 1982).

7.4.2 *In vitro*

Compared with *in vivo*, less research is performed *in vitro* on the developmental changes in the concentration of skeletal muscle Na⁺,K⁺-ATPase or its different subunit isoforms. For *in vitro* research, cells can be isolated from skeletal muscle of animals or humans, creating a primary cell culture, or various skeletal muscle cell lines can be used. The cells are cultured as myoblasts that can, under specific circumstances, form myotubes. These processes of proliferation and differentiation mimic myogenesis, which takes place during growth and development.

In the C₂C₁₂ cell line, originating from mouse skeletal muscle, the Na⁺,K⁺-ATPase α_1 and α_2 , but not the α_3 subunit isoforms are expressed (Orlowski and Lingrel, 1988a; Higham *et al.*, 1993). During myogenesis, α_2 isoform mRNA increased 12-fold, while the α_1 subunit isoform, at both the protein and the mRNA level, remained constant (Orlowski and Lingrel, 1988a; Higham *et al.*, 1993; Ladka and Ng, 2000). This resembles the *in vivo* situation described in Section 7.4.1 (Cougnon *et al.*, 2002).

In primary cultured rat skeletal muscle cells, the Na⁺,K⁺-ATPase α_1 subunit isoform was expressed to a higher extent in myotubes than in myoblasts. Both the α_2 and α_3 isoforms were not expressed *in vitro*, although the α_2 isoform was detected in freshly isolated rat skeletal muscle. The β_1 and β_2 subunit isoforms were expressed in the primary cell culture and the concentrations did not change during myogenesis (Sharabani-Yosef *et al.*, 1999). At the mRNA level, the Na⁺,K⁺-ATPase α_1 subunit isoform increased during differentiation of the myotubes and the α_2 isoform could be detected in myotubes as well. The β_1 and β_2 mRNA peaked at the onset of fusion of the myoblasts to myotubes (Sharabani-Yosef *et al.*, 1999). In the L8 rat cell line, Na⁺,K⁺-ATPase activity peaks when myoblasts start to fuse. Both protein and mRNA of the Na⁺,K⁺-ATPase α_1 and β_1 subunit isoform were measured, while the α_2 , α_3 , and β_2 subunit isoforms were not detectable (Sharabani-Yosef *et al.*, 2001). These *in vitro* measurements of the various Na⁺,K⁺-ATPase subunit isoforms do not closely resemble the situation in rats *in vivo*, as described in Section 7.4.1. Results from *in vitro* research can differ from *in vivo* results, known to be a possible disadvantage of *in vitro* research.

The Na⁺,K⁺-ATPase activity of human skeletal muscle in primary cell culture increased three- to fivefold during myogenesis (Benders *et al.*, 1992). Surprisingly, the Na⁺,K⁺-ATPase concentration and activity were significantly higher *in vitro* than *in vivo* (biopsies) (Benders *et al.*, 1992).

When cells were isolated and cultured from chicken skeletal muscle, Na⁺,K⁺-ATPase concentration increased from day 0 to day 3–6 of differentiation (Wolitzky

and Fambrough, 1986). When compared with *in vivo* research, the Na⁺,K⁺-ATPase concentration on day 0 *in vitro* is equal to the concentration measured *in vivo* (biopsies), and is about four times higher *in vivo* than *in vitro* after differentiation (Vigne *et al.*, 1982).

7.4.3 Thyroid hormones

Thyroid hormones are known to play an important role during myogenesis. During early stages of development, maternal thyroid hormones are known to be present in the fetus, until the fetus produces thyroid hormones itself (Prati *et al.*, 1992; Burrow *et al.*, 1994). After birth, thyroid hormone levels continue to increase; triiodothyronine (T₃) increases until 21 days and thyroxine (T₄) until 28 days of age in neonatal rats (Chanoine *et al.*, 1993) and both T₃ and T₄ increase at least until 28 days of age in foals (Murray and Luba, 1993). Results from our department showed that in C₂C₁₂ cells, originating from mouse skeletal muscle, thyroid hormones (T₃ and T₄) stimulated myogenesis (S. Slob, Utrecht, personal observation). These same results were found in a chicken skeletal muscle primary cell culture (Nakashima *et al.*, 1998). Hypothyroidism inhibited myogenesis in rat both *in vivo* and *in vitro* (Jacobs *et al.*, 1996).

The postnatal peak of thyroid hormones, detected in various animals, may be one of the causes of the postnatal peak in Na⁺,K⁺-ATPase, described in Section 7.3.1. This can also be seen in the reduction in skeletal muscle Na⁺,K⁺-ATPase, measured during postnatal development of hypothyroid pigs. The Na⁺,K⁺-ATPase concentration in both fast and slow skeletal muscle decreased by 15–16% in hypothyroid pigs after 14 days of age, compared with control pigs (Harrison *et al.*, 1996). On the other hand, in rat, hypothyroidism had no effect on the expression of skeletal muscle Na⁺,K⁺-ATPase α_1 and α_2 subunit isoforms from birth to 4 weeks of age (Sweadner *et al.*, 1992).

When T₄ was given to rat skeletal muscle cells in culture, the activity of the Na⁺,K⁺-ATPase was increased (Bannett *et al.*, 1984). In skeletal muscle primary cells of rat, thyroid hormone (T₃) increased the mRNA concentration of the Na⁺,K⁺-ATPase α_1 , α_2 , β_1 and β_2 subunit isoforms (Sharabani-Yosef *et al.*, 2002). The protein contents of the α_1 , β_1 and β_2 subunit isoforms were increased by T₃ as well. However, the expression of the α_2 isoform protein was not detectable, even after treatment with thyroid hormone (Sharabani-Yosef *et al.*, 2002). Apparently, this type of primary muscle cell culture contains the α_2 mRNA, but does not express the protein at a detectable level (Sharabani-Yosef *et al.*, 1999, 2001).

7.5 Conclusion

Skeletal muscle Na⁺,K⁺-ATPase is essential for maintenance of the membrane potential and to preserve muscle excitability. Its concentration decreases from around 800 to 75 pmol/g wet weight when comparing immature rats with adult horses, i.e. it decreases proportionally to the increase in body mass, comparable with the decrease in metabolic rate per unit body mass. Training of foals, adult horses and draught cattle leads to an increase in skeletal muscle Na⁺,K⁺-ATPase concentration,

associated with a blunted rise in plasma K^+ during an exercise test. Hypothyroidism leads to a large reduction in Na^+,K^+ -ATPase in skeletal muscle, and is associated with a higher peak in plasma K^+ during an exercise test. An important difference is that the effect of thyroid hormone is seen throughout the skeletal muscle pool, while the effect of training seems to be restricted to the muscle groups involved in the exercise programme. Thus, studies in livestock and domestic animals confirm the observations in small rodents and human subjects. These studies may help to develop effective training programmes for better performance of racehorses or higher power output in draught cattle in developing countries. Finally, studies with dogs and cats may serve as animal models for studies on K^+ homeostasis and performance that require larger animals than small rodents.

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8

Local and Systemic Regulation of Muscle Growth

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8.1 Summary

Muscle is the most abundant tissue in vertebrate species and with only a few exceptions it represents a main source of quality protein in the diet of *Homo sapiens*. In wild species the energy cost of carrying a large muscle mass has to be balanced against the survival value of that muscle mass for providing power to escape or to capture prey. In domestic species these constraints do not exist and selective breeding has resulted in livestock that produce muscle very rapidly. However, further improvement in quality as well as quantity requires knowledge of the molecular and cellular events involved in muscle growth regulation. During the postnatal growth period muscle fibres increase in length as well as girth. The latter is associated with a large increase in the number of myofibrils within each fibre. This occurs because of longitudinal splitting of existing myofibrils when they reach a critical size. Increase in fibre length involves the serial addition of sarcomeres on to the ends of the existing myofibrils in response to traction by the elongating bones; a force that is both local and mechanical in nature. The additional sarcomeres are preceded by the synthesis

of additional proteins and these events are locally as well as systemically regulated. The growth hormone/insulin-like growth factor (IGF)-I axis has been regarded as an important main regulator of tissue growth in general. However, locally produced forms of IGF-I are important and it is now appreciated that they may have different forms with different functions. We cloned two different locally produced IGF-Is that are expressed by skeletal muscle. One of these was apparently expressed in response to physical activity, which now has been called 'mechano growth factor' (MGF). The other is similar to the systemic or liver type (IGF-IEa) and is important as the provider of mature IGF-I required for upregulating protein synthesis. MGF differs from the systemic IGF-I as it has a different peptide sequence that is responsible for activating satellite cells. Therefore, it appears that these two forms of local IGF-I have different actions that are important regulators of muscle growth.

8.2 Introduction

Human beings are omnivorous animals and, unlike herbivores, are unable to metabolically synthesize certain amino acids termed 'essential amino acids'. Meat, which mainly consists of skeletal muscle, is a rich dietary source of these essential amino acids as well as minerals such as iron. Skeletal muscle is a very abundant tissue which makes up over half the body mass in most mammals and over 75% in some species of fish. Just after birth, muscle mass increases at a very rapid rate and agricultural animals have been selectively bred to extend this rapid growth phase. The question remains as to what limits this period of rapid growth and why in later life there is a progressive decline in the rate of accretion of muscle mass. The evidence indicates that this is not due to an age-related increase in protein degradation but to a slowing in the synthetic rate; in other words decreased gene expression. Therefore, an important question is, how can the complex processes of muscle growth including gene transcription, translation into proteins and their assembly into structures such as myofibrils be achieved at such a rapid rate and at what molecular level is the process controlled? In order to understand growth potential it is important to study these cellular and molecular events and to distinguish how they are regulated locally and systemically.

8.2.1 Muscle accretion during development and growth

During the early postnatal growth phase, muscle mass may double every few days and requires the synthesis of large amounts of specialized proteins such as actin and myosin. By using stretch combined with electrical stimulation it was possible to induce the tibialis anterior muscle in the mature rabbit to increase in mass by over 35% in 7 days (Goldspink *et al.*, 1992). This involves an increase in muscle length with the addition of numerous new sarcomeres in series with no diminution in girth. This rate of sarcomere assembly can be calculated given that, as there is only one gene copy per nucleus for a given myosin type, the number of new myosin molecules synthesized in each muscle fibre per nucleus can be estimated from structural cellular measurements. In these circumstances approximately 2000 new sarcomeres each containing about 500 myosin filaments are added to each of about 1000 myofibrils

per fibre. The number of myosin molecules per myosin filament is 300. Assuming that only one type of myosin heavy chain is being produced by each fibre, this means that about 30,000 myosin molecules are produced per nucleus per minute (Goldspink, 1985). Even if the parallel and antiparallel of the predominant myosin heavy chain gene are transcribed, this would still be 15,000 myosin molecules produced per nucleus per minute. In the young male laboratory mouse, muscle mass doubles every 4 to 5 days during the first month after birth (Griffin and Goldspink, 1973). This is also true of some agricultural animals such as pigs and carp. Therefore, the synthetic machinery when induced is truly impressive. This serial adaptation of sarcomeres on to the end of existing myofibrils (Griffin *et al.*, 1971) occurs in response to stretch imposed by the bones (Williams and Goldspink, 1973) undergoing elongation and can be regarded as a local effect. This is physiologically important as it is the mechanism via which sarcomere length is adjusted to produce the optimum overlap of actin and myosin filaments for that functional length (Goldspink *et al.*, 1992). However, there has to be upregulation of protein synthesis for the continuing adaptation to a new functional muscle length.

Muscle mass accretion is a balance between the rate of protein synthesis and the rate of protein degradation. Protein breakdown, as well as synthesis, is higher during the early growth phase (Goldspink and Goldspink, 1976, 1986). This is important for the tissue adaptation and remodelling that occurs in early life although during this phase synthesis exceeds breakdown rates. In the normal hamster diaphragm the rate of protein synthesis was five times higher in the 30-day-old animal than the 100-day-old animal and 7.5 times higher than the 230-day-old animal, whilst protein degradation rates were only about 2.5 to three times higher in the younger animals (Goldspink and Goldspink, 1976). It must be borne in mind that the amino acids liberated by protein turnover are re-utilized and that enzymatic degradation and re-synthesis of protein is expensive in terms of energy. Hence, there is limited scope for increasing muscle growth rates by merely suppressing protein degradation.

8.2.2 Myofibrillogenesis and assembly of sarcomeres

During the last two decades we have obtained insight into how myofibrils and sarcomeres are assembled. Although *de novo* assembly of sarcomeres into myofibrils is a somewhat different process from their proliferation during post-embryonic development, some of the mechanisms appear to be common, including the intracellular transport of RNA. The evidence indicates that RNA of sarcomeric proteins is transported to the site of synthesis and assembly (Larson *et al.*, 1969; Russell *et al.*, 1992) and that this is achieved because of a signal sequence or 'zip code' in the 3' untranslated region (3'UTR) of the RNA transcripts (Lawrence and Singer, 1986). The accumulation of specific RNAs that code for structural proteins at the ends of the muscle fibres, which are the regions of longitudinal growth, has been shown in growing mammalian muscle (Russell *et al.*, 1992) and fish muscle (Ennion *et al.*, 1999). For the production of more sarcomeres, moving mRNA, which can be translated over and over again, is a better option than moving large proteins through the viscous cytoplasm with organelles including the myofibrils as physical obstacles. As described below, the lengthening of myotubes and muscle fibres involves serial addition on to the ends of existing myofibrils and in order to translocate RNA transcripts

to the ends there is apparently a microtubular system. Recent work by Kiri and Goldspink (2002) using gel retardation has indicated that attachment between mRNA 3'UTR and the transport system may involve muscle type aldolase. Binding of aldolase to the 3'UTR of myosin isoform genes may also reduce the degradation of that particular myosin type and increase the number of times it is translated. Thus there appears to be a link between the metabolic differentiation and the expression of a given myosin type. Each myosin isoform gene is a single copy gene and, for the myofibrillar system in skeletal muscle to develop so rapidly during the growth spurt, all the nuclei presumably have to be activated and the mRNA then translocated to the sites of synthesis and assembly.

It is interesting that initially embryonic and then neonatal myosin is used to assemble the sarcomeres in skeletal muscle in mammals (McKoy *et al.*, 1998) and in fish (Ennion *et al.*, 1999) but not in cardiac muscle. The myocardium starts very early in embryogenesis and develops gradually so there appears to be no need for embryonic cardiac myosin isoforms. Skeletal muscle develops much more rapidly before and just after birth, and the embryonic myosin genes may have different activation signals which result in them being expressed at high levels in a different cellular environment from that of a mature animal. Also the embryonic myosins may assemble more readily. Once the myosin monomers are synthesized it appears that they self assemble. Work by Fischman's laboratory using cell-free systems to generate myosin showed that if myosin filaments were included in the system then the nascent myosin monomers exchanged with those in the myosin filaments (Baker *et al.*, 1982). Brenda Russell's laboratory used the fact that in small mammals the prevailing cardiac α myosin is rapidly switched to β myosin by thyroid hormone. Using specific antibodies they were able to detect where the new myosin monomers were localized and found that there was free exchange particularly at the ends of the myosin filaments (Russell *et al.*, 1992). Therefore, assembly into filaments appears to follow the law of mass action. However, a scaffolding system is required to organize the assembly of the myosin, actin and other sarcomeric protein monomers, and titin and certain other cytoskeletal proteins such as desmin and vimentin appear to play this role. Although the filaments may simply polymerize out, the determination of their length is an intriguing problem. Sequence information suggests that titin may act as the scaffold for polymerization and self-assembly of actin and myosin monomers. However, the question is still: what controls the synthesis of these structural proteins and is it the same mechanism that operates during postnatal growth as during embryonic differentiation of the tissues?

8.2.3 Myogenic factors

Myogenic factors have received considerable attention as they are undoubtedly important in muscle differentiation, but there is little evidence that they are involved in postnatal muscle growth (see also Houba and te Pas, Chapter 10, this volume). Developmental biologists have been attracted to studying muscle because mesodermal cells receive signals that induce them to undergo terminal differentiation to form myoblasts. In skeletal muscle these fuse to form muscle straps that develop into myotubes by dissolution of the cross membrane. Following this, the myotubes become innervated and attached by tendons to form proper muscle fibres. Although

myofibrillar proteins can be detected at earlier stages at low levels in mononucleated myoblasts, the synthetic machinery for producing myofibrillar and other muscle-specific proteins such as creatine kinase becomes fully switched on after myoblast fusion. Over the last decade, great progress has been made unravelling the signals involved in initiating each one of these steps. The first myogenic factors to be discovered were MyoD (Weintraub *et al.*, 1989), in which targeted mutations result in neonatal death as it is required for the myogenic lineage (Megency *et al.*, 1996), and myogenin, which in transgenic knockout experiments shows decreased levels of many muscle-specific transcripts (Hasty *et al.*, 1993). These myogenic regulatory factors (MRFs) are helix–loop–helix transcriptional proteins (Lassar *et al.*, 1991) that bind to a regulatory element called the E-box or CANNNTG motif (Murre *et al.*, 1989) in the 5' flanking sequence of genes such as the myosin family. One or more E-boxes are present in muscle-specific genes, including those of myosin light chains 1 and 3 (Wentworth *et al.*, 1991), troponin (Lin *et al.*, 1991), cardiac actin (Sartorelli *et al.*, 1990) and creatine kinase (Lassar *et al.*, 1989; Braun *et al.*, 1990). MRFs form heterodimers (Murre *et al.*, 1989; Benezra *et al.*, 1990; Sun and Baltimore, 1991; Olson, 1992) with other transcriptional factors such as E12 or E47. The activation of expression of muscle genes involves the simultaneous binding of complexes of several transcriptional factors to the upstream regulatory elements. These transcriptional factors may be inhibitory such as Id (Benezra *et al.*, 1990) or, as is the case with the LIM proteins (Kong *et al.*, 1997), they may induce muscle gene transcription. Once the individual muscle gene has been activated, the level of its expression may then be regulated by growth factors such as IGF-I (see also Houba and te Pas, Chapter 10, this volume).

There has been a lot of interest in a negative muscle regulatory factor discovered recently that has been named myostatin and which is expressed early in development (see also Kambadur *et al.*, Chapter 14, this volume). This belongs to the transforming growth factor beta (TGF- β) family but its expression is restricted to muscle tissue (McPherron *et al.*, 1997). During early muscle differentiation the proliferation of myoblasts and myoblast fusion are in effect two competing processes. Factors such as fibroblast growth factor (FGF) and TGF- β retard the latter process. One of the results from the myostatin knockout experiments is an increase in muscle mass. This is associated primarily with a greater number of myocytes during embryological development. This gene, when mutated, is responsible for 'double-muscling' in breeds of cattle, such as the Belgian blue, which exhibit muscle hypertrophy (McPherron and Lee, 1997), and seems to be associated with upregulation of MyoD expression (Oldham *et al.*, 2001). It is not clear what role myostatin and MRFs in general play after embryonic differentiation and to what extent they are influenced by local and systemic growth factors. However, recent research by Ravi Kambadur's group in New Zealand using an MGF knockout mouse indicates that myostatin suppresses muscle growth by maintaining the satellite cells in a quiescent state. This is interesting as MGF, as described below, apparently activates these muscle satellite (stem) cells. Chris McMahon's group also working at AgResearch in New Zealand have shown that male mice express less myostatin than female mice, which provides an explanation why the males produce more skeletal muscle (McMahon *et al.*, 2003). The relationship between growth factors and myostatin has yet to be determined.

Even with the discovery of MRFs, the activation and repression of specific muscle genes is more complicated than we first thought as this usually involves the cooperation of several transcriptional factors. Once muscle has been formed there

are other systemic and local factors that determine the differentiation of the fibres into different phenotypes. Recently, Spielgeman's group at Harvard University (Lin *et al.*, 2002) over expressed a co-activation factor in transgenic mice, PGC-1 α , that is involved in mitochondriogenesis and found that the fibres in these animals were much redder in colour and there was a higher ratio of slow type myosin I to fast type myosin 'fibres'. While there was a 10% increase in type I fibres in these transgenic mice, this cannot be regarded as a mechanism of muscle fibre type determination. Some light now has been shed on the regulation of myosin type using cultured muscle cells. The fast to slow transition seems to be associated with Ca²⁺ transients (Meissner *et al.*, 2000) and involves the calcineurin pathway (Meissner *et al.*, 2001). This again is a local effect as during development it will be those fibres that are activated frequently in which calcium fluxes will be the greatest. This is in accord with the data for muscles *in vivo* in which continuous electrical stimulation (Pette and Staron, 1997) and stretch with or without electrical stimulation (Goldspink *et al.*, 1992) switch on the slow type genes. In the adult muscles it is also known that MyoD is highest in the fast fibres and myogenin highest in the slow fibres and that overexpression of the latter in transgenic mice increased the levels of oxidative enzymes but did not induce a switch in myosin heavy chain types (Hughes *et al.*, 1999). Therefore, the MRFs may play a role in maintaining fibre phenotype (for the MRF gene family see Houba and te Pas, Chapter 10, this volume). However, from the point of view of muscle mass regulation we need to determine what regulates the regulators and how the MRFs cooperate with autocrine/paracrine as well as systemic growth factors in determining the levels of expression of specific genes?

8.2.4 Muscle stem cells

Although muscle is regarded as a post-mitotic tissue the muscle fibres require more nuclei for growth and local repair. Once embryonic differentiation is complete no further cell division takes place in the muscle fibres of skeletal muscle. The additional nuclei required for the increase in muscle fibre diameter and length are derived from satellite cells, which are essentially muscle stem cells. Satellite cells in skeletal muscle were first described by Mauro (1961) and it is now realized that these cells provide the extra nuclei for postnatal growth (Schultz, 1996). They are also involved in repair and regeneration following local injury of muscle fibres (Grounds, 1998; Grounds *et al.*, 2002). In mice at birth they account for about 30% of the muscle nuclei and the percentage drops to about 5% in the adult (Bischoff, 1994). In non-growing, undamaged tissue the satellite cells are quiescent and are usually detected just beneath the basal lamina expressing M-cadherin. When activated they express c-met as well as MyoD and myf5, and later myogenin (Cornelison and Wold, 1997). The origins of satellite cells are still uncertain. They were thought to be residual myoblasts but there is accumulating evidence that they may also originate from pluripotent stem cells (reviewed by Seale and Rudnicki, 2000); bone marrow cells (Ferrari *et al.*, 1998) as well as epidermal cells (Pye and Watt, 2001). These cell types have been shown to fuse and adopt the muscle phenotype when introduced into dystrophic muscle. However, they represent a very small percentage of potential satellite cells as compared to the residual myoblasts. More investigations have indicated that at least some of the satellite cells are derived from progenitor cells of the vasculature.

The number of satellite cells that can be activated to divide and fuse with the existing muscle fibres is probably one of the limiting aspects of muscle growth. The muscle (stem) satellite cells represent additional packages of genes and they have received considerable attention not only for the reasons given above but because they represent a way of transferring genes. Myoblasts/satellite cells (McGeachie *et al.*, 1993; Partridge, 1993, 2000) have been used to transfer the dystrophin gene. However, the realization that pluripotent stem cells can develop into muscle, liver or neuronal tissue presents the possibility of not just transferring genes but of rebuilding tissue *in situ*. This, therefore, brings us into the era of tissue engineering and it is highly probable that within the next decade or so muscle tissue will be reconstituted following physical trauma and in children with congenital and hereditary problems of muscle growth. The finding that muscle tissue can be induced to de-differentiate in mammals (Odelberg *et al.*, 2000) as well as in amphibia may have an application in increasing the number of nuclei in agricultural animals. Increasing the number of copies of structural as well as regulatory genes would presumably increase the growth potential. However, rather than transferring cells or causing muscle to de-differentiate it would seem that increasing the levels of factors (such as MGF) that activate satellite cells to divide and fuse is a better strategy for increasing growth potential.

8.2.5 Muscle mass, phenotype and energetic considerations

In this era of molecular biology many people think in terms of genetic programming. However, a prerequisite for the survival of animal species is adaptability. Muscle is one of the best examples of a tissue that has an inherent ability to adapt. It provides the power not only for locomotion but for a number of life-sustaining processes. Therefore, its ability to function efficiently and economically over a range of conditions is crucial to survival. Although genetic programming is important during embryological development, after parturition tissue mass and phenotype are to a large extent determined by environmental signals. It has been shown that changes in contractile function can be brought about quite rapidly by switching on one subset and repressing another subset of genes (Goldspink *et al.*, 1992). In this way the tissue can be optimized for power output, rapid movement or fatigue resistance. These contractile characteristics are determined by the type of myosin cross bridge, i.e. the type of molecular motor that produces force. Different molecular motors are coded for by different sarcomeric myosin heavy chain (hc) isogenes, which in the vertebrates comprise a family of separate genes. The type I myosin gene encodes a slow molecular motor which produces slow movement very economically and this is clearly important in herbivores. However, for speed and power the fast molecular motors are encoded by type II myosins. This also requires muscle mass and for wild species there has to be a trade-off between maximum muscle power output and the energy cost of carrying the mass around. Unlike wild species, agricultural animals have been bred for maximum muscle mass production and therefore the energetic considerations are different. However, it must be borne in mind that muscle fibre phenotype is one of the main factors that determines meat quality, particularly because the connective tissue differs in these different types of muscles. As the horse may also be included as 'live stock' it has to be viewed differently from animals that are bred primarily for meat production.

8.3 Systemic Control of Muscle Growth

Until recently it was not appreciated that IGF-I was not just a source of mature IGF-I, but exists in different forms with different biological actions. Circulating IGF-I has been associated with general tissue growth and is produced by the liver under the influence of growth hormone (GH). The IGF-I produced by liver is bound to IGF-I binding proteins of which there are several. IGF-IBP3 is one of the main ways of carrying IGF-I in the circulation. Experiments in sheep show that it is affected by level of nutrition and that this is induced by GH (Hodgkinson *et al.*, 1991; see also Dørup, Chapter 6, this volume). However, the systemic type of IGF-IEa is expressed in several tissues studied and in the Cre Lox knockout experiments in which the IGF-I gene was specifically inactivated in liver, the mice grew almost normally (Yakar *et al.*, 1999). Also Bass *et al.* (1994) found that systemic administration of IGF-I failed to alter normal or GH-enhanced growth, indicating a somewhat different role for blood-borne IGF-I in stimulating growth. However, local infusion of anti-IGF-I into specific sites did have a growth retarding effect. Our recent findings, however, do show that in human volunteers (Hameed *et al.*, 2002) GH does upregulate muscle IGF-I and if this is combined with exercise training it upregulates MGF expression. This is interpreted as GH administration resulting in a higher level of the primary transcript, which can then be spliced in the direction of MGF in active muscles. Growth hormone, however, has long been known to stimulate growth of tissues such as bone but one of its main effects on muscle is to decrease the fat content. This has been shown in sheep (Bass *et al.*, 1991; Spencer *et al.*, 1994). Hypophysectomy in rats is known to result in decreased growth but even in these animals muscles were capable of hypertrophy when mechanically overloaded by tenotomy of a synergistic muscle (Goldberg, 1967). This can now be explained by the local expression of IGF-I splice variants by the overloaded muscle (Owino *et al.*, 2001). Gene transfer experiments by other groups have also been carried out using a viral construct containing the liver type of IGF-I. This resulted in a 25% increase in muscle mass, but this took over 4 months to develop (Musaro *et al.*, 2001). Hence MGF is apparently much more potent than the liver type of IGF-I. Transgenic mice have been generated that overexpress systemic IGF-I (Mathews *et al.*, 1988), and IGF-I has been locally overexpressed in mouse muscle by viral-mediated gene transfer (Barton-Davis *et al.*, 1998; Barton *et al.*, 2002). Although these authors describe the IGF-I as muscle-specific IGF-I, it is important to realize that the IGF-I used in these cases was only muscle specific because it was under the control of muscle regulatory elements that had been spliced into the vector (Musaro *et al.*, 2001). Therefore, in these gene transfer experiments it is difficult to determine the role of systemic versus local IGF-I in muscle growth. The importance of distinguishing between local and systemic forms of IGF-I is covered in a recent review by Peter Rotwein (2003).

It is probable that GH, as well as inducing the expression of circulating IGF-I, will in any case have an indirect effect on muscle growth via bone elongation and increased body mass. In this way the mechanical signals that are involved in the local production of IGF-Is are indirect but result in the upregulation of local IGF-Is. Although nutrition and diet have been extensively investigated and are known to affect circulating IGF-I levels (Hua *et al.*, 1995), the optimum types of exercise for livestock have not been determined. It is clear that in farmed salmon the muscle

tissue is less firm than that of wild salmon and of poorer quality particularly as smoked salmon.

This review does not include a discussion of the systemic factors that affect production of the sex hormones or the cytokines produced in sub-clinical infections, although they will influence muscle growth. Likewise, it does not include introduced growth-promoting substances such as β agonists and antimicrobial agents, which may be regarded as systemic factors, albeit not naturally occurring.

8.4 Cloning of Local Insulin-like Growth Factors

Our group has for a good number of years carried out studies on the cellular aspects of muscle fibre growth, hypertrophy and atrophy. During the last decade we have used molecular biology methods to determine the molecular regulation involved. We have shown that muscle fibres increase in girth by hypertrophy in response to exercise and overload and that this is due almost entirely to an increase in the number of myofibrils within the fibres, although other organelles also increase in number. This is a local mechanical effect as the myofibrils have been shown to build up to a certain size and then split into two or more daughter myofibrils (reviewed by Goldspink, 1996; Goldspink and Harridge, 2002). This is preceded by an increase in synthesis of muscle proteins.

In order to elucidate the molecular regulation of muscle growth, a technique known as differential display was used to compare gene expression in non-growing with rapidly growing muscles. To detect any muscle growth factor that would be expressed at a low copy number we used the rabbit tibialis muscle, which was subjected to stretch and/or electrical stimulation as this had been shown to result in a 35% increase in muscle mass within a week (Goldspink *et al.*, 1992). This work detected a messenger RNA that was only detectable when the muscle was stretched and/or electrically stimulated. This was cloned and sequenced and was found to be a splice variant of the IGF-I gene (Yang *et al.*, 1996). This local growth factor, which is expressed in response to stretch and increased load (Yang *et al.*, 1996; McKoy *et al.*, 1999; Hill and Goldspink, 2003), was called mechano growth factor (MGF) as it has different exons from the systemic liver type of IGF-I. There is a 49 base-pair insert of exon 5 in the human mRNA (Fig. 8.1) and a 52 base-pair insert in the rat. This causes a reading frame shift so the downstream sequence differs from the liver systemic-type IGF-IEa. Also, unlike the liver type IGF-I, MGF is not glycosylated and hence is smaller. MGF has the same insulin-like ligand binding domain (mature IGF-I) as the other forms of IGF-I but the difference in the C-terminal sequence is important as this determines its binding protein affinity. These growth factors have a short half-life but this is extended when they bind to an IGF-I binding protein. The tissue distribution of IGF-I and MGF binding proteins is therefore important as they target the action and act as a time-release mechanism for these growth factors.

Another consequence of the fact that the C-terminal peptide is different is that MGF acts as two different growth factors. The evidence indicates that it not only upregulates local protein synthesis but induces the muscle satellite (stem) cells to multiply. As mentioned above, the latter are now known to be very important in muscle hypertrophy and repair of local damage.

The IGF-IEa splice variant interacts with different binding proteins, some of

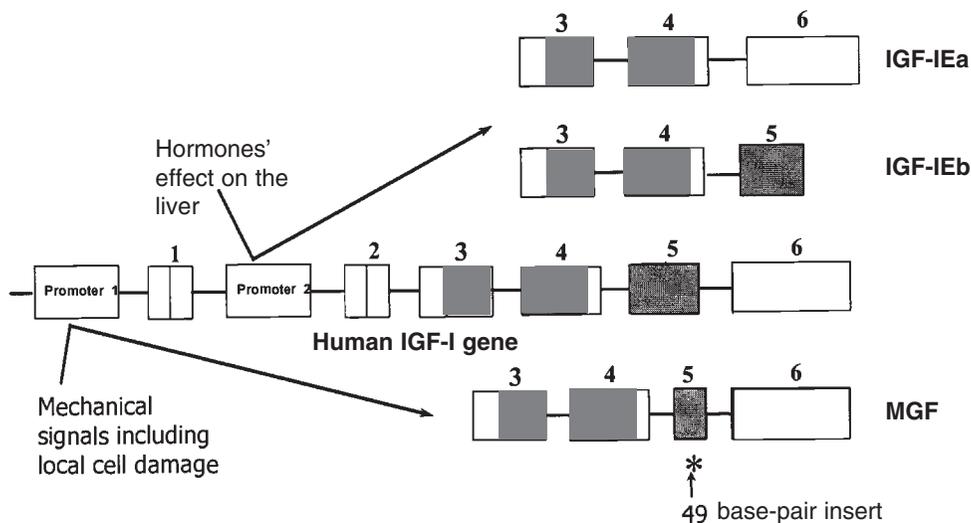


Fig. 8.1. The different exons of the insulin-like growth factor gene and the way in which they are spliced in response to hormones, such as growth hormone (GH), and to mechanical signals. The latter encodes MGF, which has a 49 base-pair insert between exons 4 and 6 that introduces a reading frame shift, resulting in a different E domain and downstream peptide sequence. This means that MGF is effectively two growth factors as the downstream sequence alone activates mononucleated myoblasts (satellite cells) to divide whilst preventing myotube formation. The splicing to IGF-IEa also occurs in muscle but the main source of systemic IGF-IEa is the liver. Recent work indicates that there is some overlap between the induction of both forms of IGF-I, as growth hormone administration combined with exercise results in higher levels of both IGF-IEa and MGF, but particularly in MGF. Evidence indicates that, although the MGF and IGF-IEa have different start sites, both are upregulated by growth hormone. However, the position of the GH response element(s) apart from promoter 2 is not known. IGF-IEb is detectable in human muscle but its function has not been investigated. This, however, is not equivalent to the IGF-IEb in rodents.

which exist in the interstitial tissue spaces of muscle. Therefore, IGF-IEa will have a paracrine effect as it will tend to be retained in the muscle tissue in which it is expressed. Interestingly, muscles of the pig, which is one of the fastest-growing mammals, have considerable amounts of total IGF-I as detected by antibody staining (S. Gilmour, personal communication) and it is expected that much of this is bound to one of the specific binding proteins. The production of additional sarcomeres at the end of existing myofibrils during longitudinal growth is associated with upregulation of IGF-I gene expression, as seen in *in situ* hybridization studies. In this capacity, its function is presumably to upregulate protein synthesis in this region of longitudinal growth.

Also, in recent studies, our London group has shown that the injection of a mammalian expression vector containing MGF cDNA into muscles in the mouse resulted in a 25% increase in muscle fibre cross-sectional area within 2 weeks (Goldspink, 2001). Hence this local growth factor is very potent in inducing growth in girth and length of muscle fibres during postnatal growth.

Recent *in vivo* experiments in which IGF-I expression was induced by stretch

combined with electrical stimulation have shown that MGF expression, unlike IGF-IEa expression, precedes muscle satellite cell activation (Hill *et al.*, 2003). Although IGF-I has been previously implicated in satellite cell activation it seems that at least their initial activation is via MGF. This is supported by *in vitro* experiments, which show that MGF, as well as increasing protein synthesis, also initiates multiplication of mononucleated myoblasts and prevents differentiation (Yang and Goldspink, 2002). This can be induced using only the carboxy end of the MGF peptide (the E domain) and this action is not blocked by using an antibody to the IGF-I receptor. Therefore, MGF apparently has a dual function. Although muscle is regarded as a post-mitotic tissue, the extra nuclei required for growth are provided by the satellite cells fusing with the existing muscle fibres. The role of MGF in determining satellite cell number during early muscle growth is being studied in collaboration with Professor Stickland's group at the Royal Veterinary College, London University. A decline in muscle stem cells has also been associated with disease states such as Duchenne's muscular dystrophy as well as age-related muscle loss. For these reasons the London group has developed methods to quantify muscle stem cell activity using known markers in relation to MGF and IGF-IEa expression (see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume).

The amount of cytoplasm that each myonucleus can control is limited and it has been suggested that one of the limiting steps in this growth process is determined by the number of satellite cells. This is in accord with the finding that the number of cells that can be activated declines with age. Recent findings in rats (Owino *et al.*, 2001) as well as in humans (Hameed *et al.*, 2003) show that older muscles are less able to produce MGF when overloaded, which may provide part of the answer to why the high postnatal growth rate cannot be maintained as well as the possibility that the muscle may run out of satellite cells.

Old age is not strictly relevant to livestock production. However, it is an extension of postnatal growth and this helps with the understanding of growth processes. This is also true in certain medical conditions such as muscular dystrophy. It was found that dystrophic muscle cannot respond to mechanical strain by producing MGF (Goldspink *et al.*, 1996), which is in accord with the suggestion that these muscles are deficient in satellite cells (Barton *et al.*, 2002). Recently, it was shown that the dystrophic changes can be partly ameliorated by blocking myostatin (Bogdanovich *et al.*, 2002) and this has also been shown using IGF-I gene therapy (Barton *et al.*, 2002). It is unlikely that these, and particularly the MGF splice variant, share some of the same signalling pathways. Recent work by Hameed *et al.* (2004) using elderly human volunteers shows that growth hormone administration upregulates IGF-IEa, but when combined with resistance training it results in markedly elevated MGF expression. Presumably the effect of growth hormone is to produce more primary transcript, which can be spliced towards MGF when the muscles are active. This provides an example of how systemic factors can moderate the production of local factors that influence muscle growth.

8.5 Conclusions

Muscle growth, however, cannot be manipulated in isolation from that of the rest of the musculo-skeletal system as the skeletal system must be able to support the animal.

This appears to be a problem with the transgenic pigs and other animals that have been pushed to the limits by selective breeding, such as broiler chickens. The main aim in the future should perhaps be to use our emerging knowledge of molecular genetics to increase quality as well as quantity of meat. As mentioned above, this may involve changing the muscle fibre type composition. Muscle growth in relation to disease prevention is also very important as infected animals do not grow well because of the systemic factors that cause muscle cachexia, even though this may be at sub-clinical level. Knowledge of the expression of these factors, which are locally produced and enter the circulation, is important because disease resistance and, therefore, the way these cytokines interact with growth factors needs investigation. The way systemic factors such as growth hormone and testosterone interact to influence the expression of local factors also needs to be studied. The emerging methods of scanning the genome and using proteomics to quantify these at the protein/peptide levels can be studied in conjunction with selective breeding (see also Maltin and Plastow, Chapter 13, this volume). These would seem to offer an alternative and perhaps a more effective way of improving livestock quality than resorting to the transgenic biology approach.

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9

Proteolytic Systems and the Regulation of Muscle Remodelling and Breakdown

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9.1 Introduction

The continuous degradation and re-synthesis of proteins is of essential importance in all cells. This protein turnover allows cells to adapt to changing external requirements. Although protein degradation of intact proteins appears wasteful, it allows cells to accurately and rapidly regulate protein concentrations. The same rapid and accurate regulation cannot be attained by adjusting the rate of protein synthesis. Protein synthesis is a complex process that requires RNA synthesis, followed by translation, modification and folding. In contrast to protein synthesis, protein degradation only involves one irreversible step. In living cells protein degradation has to be strictly controlled. Skeletal muscle is an interesting organ tissue in which to study protein degradation and its regulation because muscle tissue is highly adaptable and more adaptable than most other tissues in several ways:

1. In response to strength training a muscle can increase in size and strength. This adaptation does not occur by an increase in the number of muscle cells (muscle fibres) but by a size increase of existing muscle cells (see also Rehfeldt *et al.*, Chapter 1, this volume).
2. Muscle metabolism can also change in response to training. Endurance training increases the mitochondrial activity and induces a replacement of contractile proteins with slower contracting proteins (Pette *et al.*, 1975). The combination of increased oxidative capacity and reduced energy consumption makes the muscle more fatigue resistant (see also Reggiani and Mascarello, Chapter 2, and Slob *et al.*, Chapter 7, this volume).
3. Muscle cells are multinuclear and the number of nuclei in muscle cells varies. The average volume per nucleus (myonuclear domain) varies much less (Allen *et al.*, 1999). With training, dividing muscle precursor cells fuse with existing muscle fibres increasing the number of nuclei (Moss and Leblond, 1970).
4. The changes mentioned above are reversible: if a muscle is not used its size is reduced, the number of mitochondria and nuclei per fibre are reduced and the slow-contracting proteins are degraded at a higher rate than faster types (Hikida *et al.*, 1997).
5. Muscle size is also reduced during starvation and in many diseases. In disease and starvation muscle can function as a protein store releasing amino acids from muscle to be used elsewhere in the body, most importantly the liver and immune system (Karinch *et al.*, 2001).

All of these properties combined make muscle one of the most adaptable tissues in the body. As mentioned above proteolytic enzymes make all these adaptations possible. However, proteolytic enzymes must be strictly regulated. Skeletal muscle comprises 40–50% of the total body mass. If protein degradation and synthesis in such a massive tissue are not strictly regulated, the amino acid levels would increase or decrease out of control. A second mechanism that prevents this is the relatively slow turnover of the skeletal muscle contractile (myofibrillar) proteins. For example, myosin heavy chain, the major myofibrillar protein in muscle, has an estimated half-life of 54 days in rats (Papageorgopoulos *et al.*, 2002). It is surprising to see how fast skeletal muscle can adapt in spite of this low basal turnover rate. A dramatic loss of skeletal muscle mass can be observed within days during sepsis (Cooney *et al.*, 1997).

The effects of training, disuse, starvation or chronic disease on skeletal muscle mass and composition can usually be observed within a few weeks. This means that the proteolytic enzymes in muscle must be both strictly regulated and highly specific to allow rapid change in activity and replacement of contractile proteins in spite of a low protein turnover.

Another important feature of proteolysis in muscle is that degradation can occur without a temporary loss of function. The contractile elements (myofibrils) in the muscle are so tightly packed that they are inaccessible for most proteolytic enzymes. In addition, any proteolytic enzyme capable of degrading myofibrillar proteins would quickly destroy this structure, even if it degraded only one of the myofibrillar proteins. This means that proteolysis in muscle cannot be fully explained by just considering temporal regulation of proteolytic activities or even substrate specificities of proteolytic enzymes. An explanation of proteolysis in muscle must also include spatial regulation to explain how proteolysis can occur without loss of function. In recent years the simple view of the myofilament system (considering only the major constituents) has become more and more complex through the identification of new sarcomeric and sarcomer-associated proteins (Clark *et al.*, 2002). The roles these newly identified proteins play in myofibril assembly and degradation are largely unknown. Most studies on myofibril degradation have focused on the expression and activity of proteolytic enzymes and whole muscle protein balance.

Muscle cells or muscle fibres can be classified according to their fibre type (for a review see Reggiani and Mascarello, Chapter 2, this volume). The fibre type was originally defined by staining for myosin ATPase activity but can also be distinguished by contractile speed, the presence of specific myosin heavy chain isoforms, or the activities of glycolytic or oxidative enzymes (Table 9.1). When myosin heavy chain isoforms are used to define the fibre type, four types can be distinguished (I, IIa, II_d/x and IIb). Fibres with mixed isoforms are also observed but usually only three combinations are found: I+IIa, IIa+II_d/x and II_d/x+IIb. Each fibre type contains its own set of enzymes and (contractile) proteins. During remodelling changes in fibre composition are observed (Table 9.2). Fibre type transition follows a reversible fixed pattern: I ↔ IIa ↔ II_d ↔ IIb. This remodelling is not the result of degradation or formation of fibres. The number of fibres is constant and fibre formation or degradation is only rarely observed. Thus the change can only be accounted for by the remodelling of individual fibres. Remodelling from fast- to slow-contracting type can be induced by overloading or chronic low frequency stimulation. Remodelling from slow to fast is observed in unloaded or denervated muscle (Pette and Staron, 2000). This slow to fast remodelling is associated with breakdown (atrophy) of the muscle. Increased muscle breakdown also occurs during disease or starvation. In these conditions muscle breakdown provides amino acids for protein synthesis in the liver (Breuille *et al.*, 1998). During remodelling and breakdown the number of fibres remains constant. Changes in muscle size occur through changes in fibre diameter. Strength training increases average fibre diameter while disuse reduces diameter. Table 9.2 summarizes the changes that were observed in skeletal muscles from several frequently used animal models for muscle atrophy or remodelling.

Both in breakdown and remodelling proteolysis is essential. This chapter will focus on the regulation of proteolysis in skeletal muscle. First the models and methods for study of muscle protein metabolism will be summarized. This will be fol-

Table 9.1. Muscle fibre types and characteristics. The table shows the correlation between the different muscle fibre properties: contractile velocity, myosin heavy chain isoforms, succinate dehydrogenase (SDH) activity, α -glycerophosphate dehydrogenase (GPD) activity, and myosin ATPase typing.

Myosin heavy chain isoform	Maximum shortening velocity ($\mu\text{m/s}$ per half sarcomer) (means \pm SEM)	SDH mean activity and range	GPD mean activity and range	Myosin ATPase typing
IIb (only expressed in small mammals)	Not found in human muscle ^a Rabbit 5.5 ± 0.3 Rat 5.0 ± 0.2 Mouse 6.6 ± 0.6	0.31 0.19–0.46	0.17 0.10–0.25	Type IIb
II d/x ^a	Human 3.0 ± 0.9 Rabbit 3.9 ± 0.1 Rat 4.1 ± 0.2 Mouse 4.3 ± 0.4	0.40 0.28–0.57	0.15 0.04–0.27	Type II d/x ^a
IIa	Human 1.4 ± 0.1 Rabbit 2.7 ± 0.3 Rat 3.1 ± 0.1 Mouse 3.6 ± 0.3	0.50 0.28–0.68	0.10 0.04–0.23	Type IIa
I/IIa	Not determined	0.44 0.36–0.54	0.09 0.12–0.22	Ic/IIac/IIc
I (cardiac β)	Human 0.33 ± 0.02 Rabbit 0.67 ± 0.05 Rat 1.4 ± 0.1 Mouse 1.7 ± 0.2	0.35 0.26–0.57	0.07 0.03–0.25	Type I
(Pette and Staron, 2000)	(Pellegrino <i>et al.</i> , 2003)	(Rivero <i>et al.</i> , 1999)		(Staron, 1997)

^aMyosin and fibre types II d and II x are the same; both names are used in the literature (Pette and Staron, 1990). In human muscle only two type II myosin heavy chain isoforms are expressed. The original name for human fibre type II d/x and myosin heavy chain type II d/x was type II b. Because of the resemblance of this fibre type with rat II d fibres it has been renamed (Pette and Staron, 2000). Although the gene for the type II b is present in humans, this isoform is not detectable in human limb muscle (Staron, 1997; Horton *et al.*, 2001).

lowed by a review of regulatory mechanisms of the major proteolytic systems present in muscle. Regulatory mechanisms will be discussed in two parts: unspecific and specific regulation of proteolytic systems. Unspecific regulation can occur through changes in activity or expression of proteolytic enzymes. Specific regulation involves regulation of the degradation of substrates. In this section the substrate targeting mechanisms for the major proteolytic systems and the implications for muscle remodelling and breakdown will be discussed.

Table 9.2. Changes in fibre types and muscle size in various animal models for remodelling or breakdown of skeletal muscle. CLFS, chronic low frequency stimulation.

	CLFS, stretch or loading	Denervation or unloading	Cachexia	Sepsis
Fibre type shifts	I II →I Id →I a →I	I→I a →I Id ←I b	I→I a →I Id →I b in mouse soleus	Preferential degradation of fast type muscle
Muscle mass, fibre diameter	Increase	Decrease	Decrease	Rapid decrease
References	(Roy <i>et al.</i> , 1982; Goldspink <i>et al.</i> , 1991; Pette and Staron, 2000)	(Musacchia <i>et al.</i> , 1990; Fauteck and Kandarian, 1995; Huey and Bodine, 1998; Castro <i>et al.</i> , 1999)	(Diffie <i>et al.</i> , 2002)	(Cooney <i>et al.</i> , 1997)

9.2 Models and Methods Used to Study Proteolysis

9.2.1 Animal models

Skeletal muscle protein metabolism can be studied *in vitro* or *in vivo* each with their specific advantages and disadvantages. Animal models have been developed to study muscle breakdown and remodelling. Frequently used models are treadmill running or chronic low frequency (10 Hz) stimulation (CLFS) as models for endurance training, hind limb unloading/suspension as a model for disuse, caecal ligation and puncture as a model for sepsis, and cancer, denervation and burn injury models. Many experiments have been done with these models to evaluate the effects on skeletal muscle protein degradation and synthesis. The models also provide a good system for controlled tests of the effectiveness of treatments to improve muscle protein balance. Disadvantages of these animal models are animal welfare considerations, the influence of other tissues (especially the immune system) on the results, contamination of muscle samples with other (infiltrating) cell types and the limited number of time points for measurements. In spite of their limitations these models are frequently used because they still provide the only means to study long-term effects on skeletal muscle.

Small, thin muscles can also be incubated *ex vivo* for a few hours. The incubation time has to be limited because protein degradation is increased in incubated muscles. A second limitation for muscle maintenance *ex vivo* is oxygen diffusion. The muscle must be sufficiently small or thin to allow the diffusion of oxygen to the muscle interior. The incubation of muscle *ex vivo* enables studies that require the application of high (toxic) concentrations of inhibitors of protein synthesis or degradation. These combinations of inhibitors with monitoring of amino acid release have been used to distinguish four major proteolytic systems responsible for protein degradation in muscle (Table 9.3): (i) the lysosomal system; (ii) the calcium-dependent proteolytic system or calpains; (iii) the ATP-dependent non-lysosomal proteolytic system

Table 9.3. Proteolytic systems responsible for release of amino acids from incubated muscle and regulation in various animal models. Symbols used: =, not significantly different from control; ↑, significant increase; ND, not determined.

Model	Lysosomal	Calcium-dependent (mainly calpains)	Non-lysosomal calcium-independent ATP-dependent (mainly proteasomes)	Non-lysosomal calcium-independent ATP-independent (e.g. caspases, serine proteases)	References
Sepsis	=	=	↑	ND	(Tiao <i>et al.</i> , 1994)
Cancer	↑	=	↑	=	(Baracos <i>et al.</i> , 1995)
Fasting	=	=	↑	=	(Medina <i>et al.</i> , 1991; van den Hemel-Grooten <i>et al.</i> , 1997)
Denervation	=	=	↑	=	(Furuno <i>et al.</i> , 1990; Medina <i>et al.</i> , 1991)
Unloading (combined measurements)		↑		↑	(Taillandier <i>et al.</i> , 1996)

or the ubiquitin-proteasome system; and (iv) other proteases or ATP-independent, calcium-independent, non-lysosomal proteolysis.

Inhibitor experiments have also been performed on incubated muscle from the various animal models mentioned above. This revealed the contributions of the four proteolytic systems to the increases in muscle protein degradation observed in these models (Table 9.3).

9.2.2 *In vitro* (myotube) models

Muscle precursor cells can be isolated from muscle and maintained in culture for long periods. These precursor cells and cell lines derived from them can be stimulated to differentiate and fuse, forming multinucleated myotubes capable of contraction (Burton *et al.*, 2000). Myotubes are complementary to the *in vivo* models mentioned above. Most importantly, individual substances can be tested for effects on myotubes without the influence of other tissues. Another benefit of using myotube is the ability to compare protein and enzyme concentrations, activities and locations in different conditions in a reproducible manner. Myotubes can be maintained in culture for longer periods than incubated muscle and oxygen diffusion is not a problem. This combination opens the possibility for a more detailed study of the regulation of protein metabolism in skeletal muscle under different conditions. However, a major challenge is to establish conditions that simulate remodelling or breakdown as it occurs in animal models in myotubes. A second limitation is that myotubes can only be maintained in culture for up to 1 week. For these reasons the main use of these models has been the study of muscle differentiation. However, several recent publications have demonstrated the usefulness of these models to study remodelling and breakdown mechanisms (Guttridge *et al.*, 2000; Swoap *et al.*, 2000).

A problem with *in vitro* models remains that the high level of structural organization and differentiation found in skeletal muscle cannot (yet) be rivalled.

9.2.3 Methods

Many of the methods mentioned below are also discussed in Rooyackers and Nair (1997). However, their review was focused on human *in vivo* studies of muscle protein metabolism. Many of the limitations mentioned by Rooyackers and Nair are not valid in animals from which muscle tissue can be readily taken to perform *in vitro* or *ex vivo* studies.

9.2.3.1 Lean body mass measurements

Fat-free or lean body mass is frequently used in animal models for disease and in patients to monitor muscle atrophy in muscle wasting diseases such as sepsis, cancer and diabetes. Because skeletal muscle is the main component of lean body mass it can be used to obtain a rough estimate of total body muscle. This method involves the estimation of total body fat for which different methods are available (Jensen *et al.*, 1993). The major benefit of this method is that repeated measurements can be made in the same animal or patient. The main problems with the method are its inaccuracy and the rough estimation it provides for total muscle mass.

9.2.3.2 Balance studies

Phenylalanine, tyrosine and lysine are not degraded or synthesized in skeletal muscle. These amino acids can be used to monitor net muscle protein loss (or gain) by measurement of the blood flow and analysis of the arteriovenous concentrations. This method is used *in vivo* to monitor net protein synthesis or degradation. The main disadvantage of this method is that it is not possible to measure synthesis or degradation rates and possible contributions from tissues other than skeletal muscle, most importantly skin (Biolo *et al.*, 1994).

9.2.3.3 Expression and activity of proteolytic enzymes

Determination of mRNA levels in muscle biopsies can be used to obtain an indication of the relative importance of the proteolytic systems in muscle (Mansoor *et al.*, 1996). This may result in errors if cells other than muscle cells are present in the biopsies. In addition, increased mRNA levels may not be correlated with increased protein expression (Lecker *et al.*, 1999). A way to circumvent these problems is to use immunohistochemistry to stain muscle sections for different proteolytic enzymes (Sultan *et al.*, 2001). Another possibility is to combine analysis of expression with inhibitor studies. However, the problem always remains that expression and activities are not necessarily correlated (Voisin *et al.*, 1996). Activity measurements comprise the assessment of enzyme activity or proteolytic capacity in homogenized muscle fractions under chosen (optimal) conditions. These methods can be performed under similar conditions and effects of enzyme modifications and increasing or decreasing endogenous inhibitor concentration and inhibitor binding may be preserved. Two disadvantages still remain: (i) activity may result from other cell types (immune cells, vascular cells, or connective tissue cells) present in muscle; and (ii) the method cannot be used to estimate the breakdown of specific substrates *in vivo*.

Substrate degradation *in vivo* also depends on the accessibility of the substrate to the protease. The first problem could be solved by measuring proteolytic activity inside the cell *in vivo* with fluorescent substrates but measurement of intracellular transport of substrates and proteases and substrate–enzyme interactions remains a difficult task. For myofibrillar proteins the release of proteins from the myofibril is probably the rate-limiting step in degradation (Solomon and Goldberg, 1996).

9.2.3.4 Labelling studies

Labelling studies are required for the measurement of substrate. In these studies the incorporation of (stable or radioactive) isotope-labelled amino acids is determined. If amino acids are used that are not degraded in muscle this allows the calculation of the fractional synthesis rate. This rate is the fraction of protein that is replaced within a specific period. This rate can be determined for individual proteins. The labelled amino acid can be delivered to the muscle in two ways: at a high concentration for a short period, or at a lower concentration for longer periods.

The high concentration method or flooding dose technique has the benefit that every amino acid incorporated within the labelling period is labelled. This method has its limitations, especially when applied *in vivo* because the high concentration of amino acid itself can influence protein metabolism and the short labelling period limits its application to short-lived substrates. To measure long-lived proteins without influencing protein metabolism the low dose or continuous infusion method has to be used. With this method only a fraction of the amino acids incorporated in proteins is labelled. To calculate the turnover rate this fraction needs to be known. Two possibilities are available to measure this fraction (Papageorgopoulos *et al.*, 2002): (i) determination of the fraction of tRNA-bound labelled amino acids; or (ii) determination of the fraction of labelled amino acids in a short-lived protein. The first method has the disadvantage that it requires difficult aminoacyl tRNA isolation. The second method has the disadvantage that the protein used has to be short-lived relative to the infusion period. Most abundant proteins in skeletal muscle are long-lived.

The major benefit of these methods is that it is possible to measure turnover rates of specific substrates. A modification of this method can be used to measure substrate degradation rates. This can be accomplished by pre-labelling proteins and monitoring the disappearance of labelled proteins in the absence of labelling (pulse–chase experiment). In the pulse–chase set-up re-utilization is not measured but it can be inhibited (*in vitro* or *ex vivo*) with inhibitors of protein synthesis. Muscle labelling studies can be performed *in vitro* or combined with dissection, fractionation, purification and/or separation of the protein(s) of interest. It is also possible to use labelling in combination with inhibitors to identify the proteases responsible for degradation.

9.2.3.5 Methyl-histidine measurement

Methyl-histidine (MeHis) is a constituent of actin and myosin. After release from protein breakdown it cannot be reincorporated in proteins. Most MeHis is located in skeletal muscle. These properties allow MeHis in the urine to be used to estimate muscle breakdown (e.g. Mansoor *et al.*, 1996). However some urinary MeHis can also be released from newly synthesized myosin and actin or other tissues in the body (mainly intestinal smooth muscle (Millward and Bates, 1983)). In *in vitro* studies

MeHis concentrations in the incubation medium have been similarly used to measure protein degradation (Thompson *et al.*, 1999). For sheep and swine MeHis secretion cannot be used to estimate muscle protein breakdown because it is partially retained in muscle as the dipeptide balenine (Rathmacher and Nissen, 1998).

9.2.3.6 Inhibitor studies

All measurements of muscle degradation can be combined with more or less specific inhibitors to determine the relative importance of the different proteolytic systems (lysosome, proteasome and calpain systems). However, inhibitor studies have to be treated with caution for several reasons: (i) proteolytic systems are essential for the animal, therefore high concentrations of inhibitors are toxic and can only be used *ex vivo* or *in vitro*; (ii) the inhibitors of proteolysis are often not completely specific or inhibition is incomplete; and (iii) a major drawback of using inhibitors is that proteolytic enzymes play important roles in signalling pathways. An important example for skeletal muscle is that the proteasome is essential for activating the transcription factor NF- κ B. NF- κ B is a candidate transcription factor involved in the activation of muscle degradation (Li *et al.*, 1998; Guttridge *et al.*, 2000). Thus, an important alternative explanation can be offered for the success of proteasome inhibitors for slowing protein breakdown in muscle (Tawa *et al.*, 1997; Hasselgren *et al.*, 2002). Similarly, the proteolytic enzyme calpain 3 appears to be required for stabilizing muscle indicating that this protease is also involved in a regulatory pathway (Ono *et al.*, 1998). The combined effect of stabilizing calpain 3 and destabilizing calpains 1 and 2 could explain why inhibition of calcium-dependent proteolysis has no effect on muscle proteolysis in the models shown in Table 9.3.

9.3 Regulation of Protease Expression

Most of the current scientific knowledge on muscle wasting is based on balance studies combined with inhibitor studies to determine the relative involvement of the lysosomal, calpain and proteasomal enzymes (Table 9.2). Knowledge of the regulation of protein degradation in muscle is still limited. It appears that at least some regulation of the proteolytic enzymes occurs at the level of transcription. Expression levels are easily determined by isolating RNA from muscle biopsies, or whole muscles. In various models of remodelling and breakdown, expression and activity levels in response to exercise have been determined. However, some of these studies only measured RNA levels without determination of the enzyme activity. The relationship between expression and activity for proteolytic enzymes is not straightforward. Many proteases are expressed as inactive precursors (e.g. procathepsins) or are regulated by endogenous inhibitors (e.g. calpastatin).

9.3.1 Lysosomal proteases cathepsin B, D, H and L (see also Hopkins and Taylor, Chapter 17, this volume)

Lysosomal proteolysis in incubated muscle was not involved in the increase in proteolysis induced by 48 h of fasting in rats (Busquets *et al.*, 2002). Activities of the lysosomal proteases cathepsin B, H and L in skeletal muscle homogenates were also

unchanged between 24, 48 and 72 h of fasting (Andreu *et al.*, 1995). Upregulation of lysosomal protease activity is observed during muscle remodelling. This includes remodelling induced by exercise (cathepsin B+L) (Sohar *et al.*, 1989; Feasson *et al.*, 2002), unloading (cathepsin L) (Ikemoto *et al.*, 2001) and denervation (cathepsin D) (Tagerud and Libelius, 1984; Mansoor *et al.*, 1996). Upregulation of cathepsin B and L is also observed during sepsis (Voisin *et al.*, 1996; Deval *et al.*, 2001) and chronic disease (Deval *et al.*, 2001; Bosutti *et al.*, 2002). It is surprising to see that in all conditions except fasting lysosomal protease activities are upregulated while experiments with inhibitors indicated that lysosomes are not important for muscle degradation (Table 9.3). There are different possible explanations: (i) only proteolytic capacity is upregulated in these conditions and not protein degradation; (ii) lysosomal protein degradation is upregulated but provides only an insignificant contribution to total muscle proteolysis; or (iii) the activity of the lysosomal proteases is readily taken over by another proteolytic system when lysosomal proteolysis is inhibited.

9.3.2 Proteasomal pathway

Experiments with inhibitors have shown that inhibition of the ubiquitin proteasome pathway leads to a significant inhibition of proteolytic degradation in skeletal muscle. The ubiquitin proteasome pathway thus appears to be responsible for the majority of the protein degradation in muscle (Table 9.3). The importance of the proteasomal pathway is reflected in the upregulation of its constituents by exercise (Sultan *et al.*, 2001), fasting (Medina *et al.*, 1991), disuse (Mansoor *et al.*, 1996; Ikemoto *et al.*, 2001), and during sepsis (Voisin *et al.*, 1996) and cancer (Baracos *et al.*, 1995). Substrate specificity of the ubiquitin proteasome pathway is determined by the E3 or ubiquitin ligase enzyme (Fig. 9.1). Three ubiquitin ligase enzymes have been implicated in muscle proteolysis: E3 α (Solomon *et al.*, 1998b), muscle ring finger protein 1 (MuRF1) and MAFbx (Bodine *et al.*, 2001) (also called atrogin-1 (Gomes *et al.*, 2001)). The E3 α ligase is the best known of these ligases. It has been studied for its involvement in the rapid degradation of a subset of proteins with destabilizing N-termini. This proteolytic pathway is called the N-end rule pathway (Fig. 9.2). E3 α cooperates with two ubiquitin-conjugating E2 enzymes, E2_{14k} HR6A and E2_{14k} HR6B.

The E2 enzymes that cooperate with MAFbx and MuRF1 are not known and neither are the substrates that are ubiquitinated by these E3 enzymes. Both MAFbx and MuRF1 mRNA are upregulated during muscle atrophy (Bodine *et al.*, 2001; Gomes *et al.*, 2001). Activity of these enzymes cannot be measured because of the lack of specific inhibitors and substrates. However, in mouse knockouts muscle atrophy was reduced when either MAFbx or MuRF1 expression was abolished.

The major ubiquitin ligase responsible for skeletal muscle proteolysis was long thought to be E3 α or Ubr1 (Solomon *et al.*, 1998b). More recent experiments with knockout mice lacking E3 expression confirm the importance of E3 α and found a 25–30% decrease of ubiquitinylation of endogenous skeletal muscle protein compared with wild type. The E3 α knockouts had reduced muscle and adipose tissue masses (Kwon *et al.*, 2001). These abnormalities hinder a direct comparison of these mice with normal mice to check the involvement of the N-end rule pathway in muscle breakdown and remodelling. Tissue masses in a knockout of one of the N-end rule E2 (ubiquitin-conjugating) enzymes, HR6B/E2_{14k}, were normal. A com-

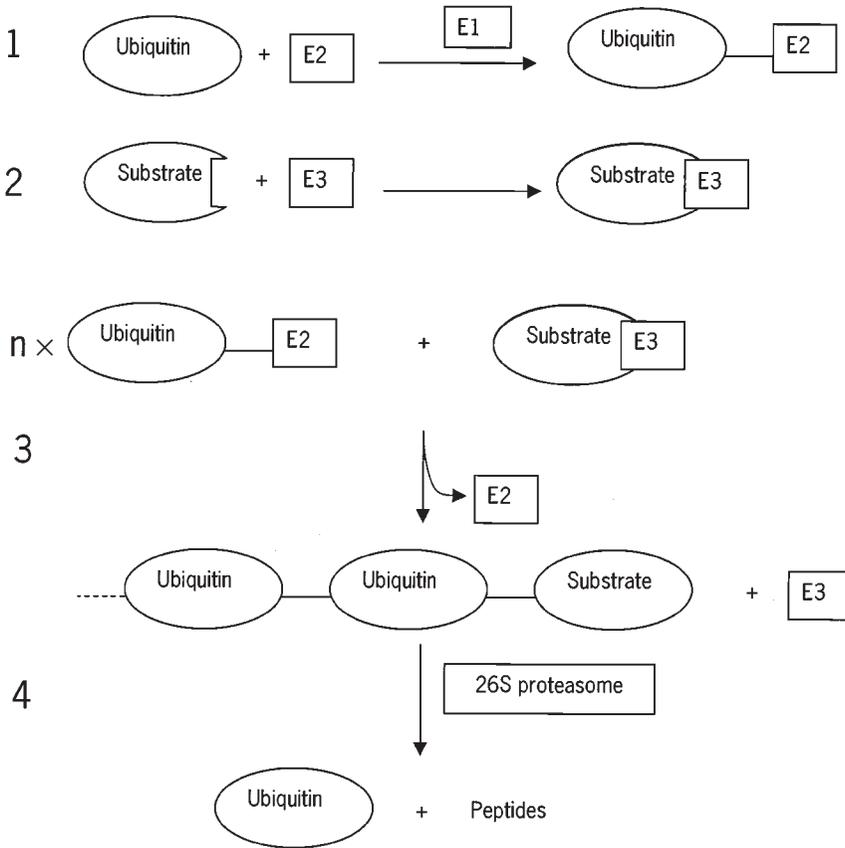


Fig. 9.1. Proteasomal degradation (derived from Glickman and Ciechanover, 2002). Proteasomal degradation involves four steps: (1) the ubiquitin-activating enzyme E1 attaches ubiquitin to one of the ubiquitin carrier proteins also called ubiquitin-conjugating or E2 enzymes; (2) substrate is recognized and bound by an E3 (ubiquitin ligase) enzyme; (3) after E3 binding, multiple activated ubiquitin molecules are transferred to the substrate forming a polyubiquitin chain on the substrate; (4) the polyubiquitinated substrate is deubiquitinated and degraded by the 26S proteasome.

parison of knockouts with wild type mice showed that the lack of HR6B had no effect on the decrease in muscle mass induced by fasting. Messenger RNA of this enzyme is normally upregulated during fasting. The other N-end rule E2 enzyme (HR6A) did not compensate for the loss of HR6B and it was confirmed that the combined enzymes were downregulated by 60% (Adegoke *et al.*, 2002). This suggests that, although the N-end rule pathway is responsible for a large percentage of the normal muscle protein turnover, it is not involved in the increase of protein breakdown during fasting. It will be interesting to compare proteolysis in the HR6B, MAFbx and MuRF1 knockouts with wild type mice to investigate the contribution of these enzymes to the increased protein degradation in various other models for skeletal muscle breakdown and remodelling.

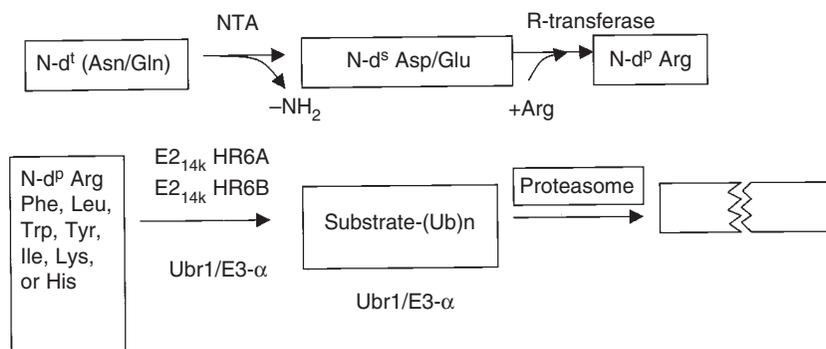


Fig. 9.2. The N-end rule pathway (derived from Varshavsky, 1996). Three types of substrates are degraded by the N-end rule pathway. The N-terminal amino acid determines the substrate type. N-end rule substrates have tertiary, secondary or primary destabilizing residues. The tertiary destabilizing (N-d^t) residues glutamine and asparagine are converted to secondary destabilizing residues by N-terminal amidase (NTA). Substrates with secondary destabilizing residues (N-d^s) aspartate and glutamate are converted to substrates with the primary destabilizing (N-d^p) arginine by R-transferase. Substrates with primary destabilizing residues are ubiquitinated by E2_{14k} and E3 α and degraded by the proteasome.

9.3.3 Calcium-dependent proteolysis

Three calcium-dependent proteases are present in skeletal muscle: (i) m-calpain (calpain 1); (ii) μ -calpain (calpain 2); and (iii) p94 (calpain 3). Of these three enzymes only p94 is specifically expressed in skeletal muscle; m-calpain and μ -calpain are also called the ubiquitous calpains because they are expressed in all tissues. Activity of the ubiquitous calpains is suppressed by calpastatin. The activity thus depends not only on calpain expression but also on the relative level of calpastatin. Increased mRNA expression of μ -calpain was found in sepsis (Voisin *et al.*, 1996) and head trauma patients (Mansoor *et al.*, 1996). In many other models no difference was found in calpain expression even though calpain activity was increased; increased protein expression without changes in mRNA was observed during fasting (Ilian and Forsberg, 1992). In models for heart disease and cancer the calpastatin to calpain ratio was increased in the absence of changes in calpain levels (Costelli *et al.*, 2001). Thus, calpain activity is increased in conditions of increased muscle degradation. Remodelling induced by chronic stimulation also induces calpain 1 and 2 (Sultan *et al.*, 2001).

The muscle-specific calpain p94 appears to be regulated in exactly the opposite way although less literature is available. Decreases of p94 were observed after exercise (Feasson *et al.*, 2002), chronic stimulation (Sultan *et al.*, 2001) and denervation (Stockholm *et al.*, 2001). The opposite role of p94 in muscle proteolysis can be explained by the stabilizing function of calpain 3 in skeletal muscle. A lack of p94 is the cause for limb girdle muscular dystrophy type 2A (LGMD2A) (Richard *et al.*, 2000). Thus while the two ubiquitous calpains increase muscle proteolysis, p94 expression protects the muscle from proteolysis. These opposite functions of the calcium-dependent proteases in muscle may explain the discrepancy between calpain activity and calcium-dependent proteolysis in incubated muscle (Table 9.3).

Recent experiments with transgenic mice overexpressing calpastatin confirm the importance of μ - and m-calpains for muscle proteolysis (Tidball and Spencer, 2002). Calpastatin overexpression inhibited both the proteolysis and the slow to fast remodelling in immobilized muscle.

9.4 Regulation of Substrate Degradation

The mechanisms of the different proteolytic systems vary among the different cellular proteases. Proteases that are present in a separate compartment from their substrates can be continuously active without causing damage to the cell. This is the case for proteasomes because only unfolded proteins or peptides can gain access to the active sites. To a lesser extent this also applies to the lysosomes, where proteins have to be transported over the lysosomal membrane before they can be degraded in the acidic environment inside the lysosome. Degradation by lysosomes and proteasomes can be controlled by regulating the transport of the substrates to the cellular compartment with the protease activity.

A completely different and stricter regulatory mechanism must control proteases that can freely move through the sarcoplasm. Several mechanisms are known that prevent damage to cellular proteins by these proteases: (i) limited substrate specificity; (ii) endogenous inhibitors; and (iii) the protease is expressed as an inactive precursor that requires activation. The regulatory mechanisms of the lysosomal, proteasomal, calcium-dependent and energy/calcium-independent proteolysis are discussed below.

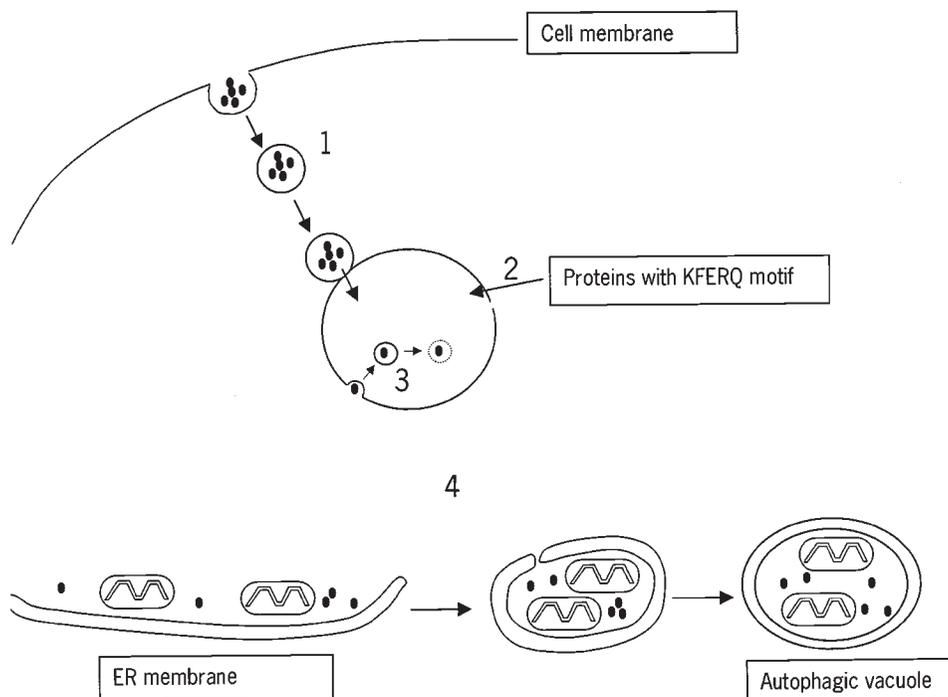
9.4.1 Regulation of lysosomal degradation

Lysosomes form a specialized cellular compartment for protein degradation. Lysosomal proteolysis fulfils a central function in the degradation of extracellular and membrane proteins that enter the cell through endocytosis. However, the lysosomal system can also degrade intracellular proteins. Lysosomal proteolysis can degrade proteins that enter the lysosomes through four different pathways (Fig. 9.3) (Cuervo and Dice, 1998):

1. Extracellular and membrane proteins can enter the cell and finally the lysosomes (endocytosis) (Kaisto *et al.*, 1999).
2. Cytoplasmic proteins can be directly taken up by the lysosomes (micro- or basal autophagy) (Mortimore and Poso, 1987).
3. Cytoplasmic proteins can be transported across the lysosomal membrane (the chaperone-mediated pathway) (Agarraberes and Dice, 2001).
4. Organelles and cytoplasm can be enclosed in a membrane and form autophagic vesicles (macroautophagy) (Marino *et al.*, 2003).

9.4.1.1 Phagocytosis/endocytosis

The first pathway can contribute to degradation of muscle during muscle inflammation that occurs after muscle damage when macrophages enter the muscle and degrade damaged cells (Farges *et al.*, 2002). Muscle damage followed by inflamma-



Four pathways for lysosomal degradation:

1. Endocytosis
2. Chaperone mediated-transport
3. Microautophagy
4. Macroautophagy

Fig. 9.3. Lysosomal degradation (derived from Cuervo and Dice, 1998). The four pathways for protein translocation to the lysosomes are shown.

tion can be induced by blunt trauma or by exercise. Interestingly, structural adaptation of muscle after eccentric exercise does not occur if inflammation is prevented with anti-inflammatory drugs (Lapointe *et al.*, 2002).

9.4.1.2 Microautophagy

Microautophagy is the uptake of cytoplasm by the lysosome. This process provides a contribution to the continuous, slow turnover of cytoplasmic proteins. Microautophagy can import ubiquitinated proteins into the lysosomes (Laszlo *et al.*, 1990). The function and importance of this pathway in skeletal muscle is unknown. In contrast to macroautophagy discussed below, microautophagy does not appear to be increased during fasting (Mortimore *et al.*, 1983).

9.4.1.3 Direct chaperone-mediated transmembrane transport

Lysosomes selectively degrade proteins that bind to the heat shock cognate protein hsc70. The hsc70 protein recognizes proteins with a KFERQ peptide motif (Terlecky and Dice, 1993). An antibody to the KFERQ sequence substrates demonstrated that proteins containing this sequence are conserved in skeletal muscle during starvation while they are degraded in heart and liver (Wing *et al.*, 1991). This and the absence of KFERQ sequences in the major myofibrillar protein indicate that this pathway is not important for the degradation of myofibrillar proteins.

9.4.1.4 Macroautophagy

Macroautophagy has been extensively studied in yeast. Autophagy in yeast involves a protein conjugation system (Mizushima *et al.*, 1998). The same system has also been found in human (Marino *et al.*, 2003) and mouse (Mizushima *et al.*, 2002) tissues. The protein conjugation system is required for the formation of autophagic vacuoles. Autophagic vacuoles form from Golgi or endoplasmic reticulum membranes. The membrane encloses a volume of cytosol including organelles. After fusion of the autophagic vacuole with a lysosome the contents are completely degraded. No evidence has been found for the occurrence of macroautophagy in skeletal muscle during normal remodelling and breakdown. The myofibrillar structures may be too large for degradation by this pathway. However, it could contribute to the degradation of other intracellular proteins and the removal of mitochondria. Macroautophagy does contribute to proteolysis in some myopathies where vacuoles are observed in skeletal muscle fibres (reviewed in Nishino, 2003).

9.4.2 Regulation of the proteasome

Regulation of the proteasome activity was reviewed recently (Glickman and Ciechanover, 2002). Proteasomal proteolysis involves four enzymes (Fig. 9.1): E1 (ubiquitin-activating enzyme) activates ubiquitin by generating a high-energy thiol-ester bond; E2 (ubiquitin-conjugating enzyme) carries the activated ubiquitin to the substrate; E3 (ubiquitin ligase) recognizes the substrate and catalyses the transfer of ubiquitin to the substrate; finally the 26S proteasome (a large cylinder-shaped multi-enzyme complex) removes ubiquitin from the substrate (Verma *et al.*, 2002; Yao and Cohen, 2002), unfolds the substrate and translocates it past the active sites (Benaroudj *et al.*, 2003) generating short peptides.

Substrate degradation involves two separate ATP-dependent steps. First, multiple ubiquitin molecules are activated and attached to the substrate. Then polyubiquitinated substrate is degraded by the proteasome and ubiquitin is released. Ubiquitinylation occurs after substrate and E2 enzyme are both bound to ubiquitin ligase or E3 (Glickman and Ciechanover, 2002). The proteasome pathway has a large number of E3 enzymes for the recognition of many different substrates. The E2 enzymes have a more limited diversity and often one E2 enzyme can interact with several E3 enzymes although the reverse is also possible (e.g. E3 α can interact with E2_{14k}, HR6A or HR6B (Adegoke *et al.*, 2002)). By contrast, the first and last steps of the pathway are carried out by only one enzyme (E1) or enzyme complex (the proteasome). The logical site for specific regulation of proteasomal degradation is therefore at the level of ubiquitinylation. As mentioned above three ubiquitin

ligase enzymes relevant for skeletal muscle proteolysis have been identified. The N-end rule pathway ligase E3 α , and the atrophy related ligases MuRF1 and MAFbx.

9.4.2.1 The N-end rule pathway

The N-end rule pathway is responsible for the degradation of proteins with destabilizing N-terminal residues (Bachmair *et al.*, 1986). Three different types of substrates can be distinguished by their N-termini. (i) Tertiary destabilizing residues asparagines and glutamine can be converted to (ii) the secondary destabilizing residues aspartic and glutamic acid by N-terminal amidase. An arginine residue from arginyl-tRNA can be attached to the secondary destabilizing residues. This generates a protein with (iii) the primary destabilizing residue arginine. The other primary destabilizing residues are Phe, Leu, Trp, Tyr, Ile, Lys and His. Proteins with primary destabilizing residues are bound by the E3 α enzyme that catalyses polyubiquitinylation of the substrate (Fig. 9.2).

The importance of the N-end rule pathway has been evaluated in skeletal muscle extracts by Solomon and co-workers (Solomon *et al.*, 1998b). These investigations demonstrated the importance of this pathway for skeletal muscle proteolysis:

1. E3 α inhibitors decrease ubiquitinylation by 40–45% in skeletal muscle extracts while E3 α increases it.
2. Depletion of arginyl-tRNA with RNase reduced ubiquitinylation by 20–25%.
3. DN E2_{14k} that binds ubiquitin irreversibly reduces ubiquitinylation while the addition of E2_{14k} increases it.

Experiments with E3 α inhibitors were also used to evaluate the importance of this pathway in the increase in ubiquitinylation during muscle atrophy caused by sepsis or cancer (Solomon *et al.*, 1998a). The inhibitors significantly reduced the increased proteolysis in these models. It was concluded that the N-end rule pathway is largely responsible for the increase in muscle proteolysis in these models. The important question that arises with the more recent discovery of MAFbx and MuRF1 enzymes is whether they are also inhibited by the simple dipeptide inhibitors that were used.

9.4.2.2 MAFbx and MuRF1

The substrates for these E3 enzymes are as yet unknown. Mouse knockouts clearly demonstrated their importance for protein breakdown in skeletal muscle during immobilization, denervation and unloading (Bodine *et al.*, 2001). MuRF1 has been demonstrated to bind to titin near the m-line where it may be required to maintain m-line structure (Centner *et al.*, 2001). Localization studies for MAFbx are not yet available. MAFbx could be co-precipitated with both cullin1 and skp1 (Bodine *et al.*, 2001). This indicates MAFbx functions as an SCF-type ubiquitin ligase. SCF-type ubiquitin ligases usually target phosphorylated substrates (Glickman and Ciechanover, 2002). A lot of questions on MuRF1 and MAFbx remain open, most importantly: what are their substrates (myofibrillar/structural proteins or regulatory/signalling proteins) and do they also contribute to proteolysis during sepsis and chronic stimulation?

9.4.3 Regulation of calpain (see also Hopkins and Taylor, Chapter 17, this volume)

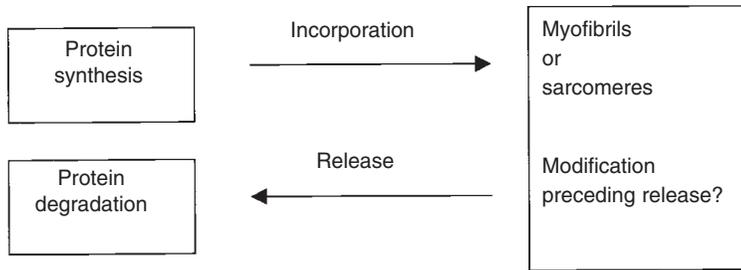
The major difference between m-calpain and μ -calpain lies in their calcium sensitivity *in vitro*. While m-calpain requires millimolar amounts of calcium for activation, μ -calpain can be activated by micromolar amounts. m-Calpain and μ -calpain were discovered because of their ability to remove the Z-discs from myofibrils without causing other large ultrastructural changes. This led to a new model of sepsis-induced muscle breakdown where breakdown is initiated by a calpain-induced release of myofilaments (Hasselgren and Fischer, 2001). This hypothesis provides an attractive explanation for how the proteasome can be responsible for the majority of protein degradation (Table 9.3) while the same proteasome is unable to degrade intact myofibrils (Solomon and Goldberg, 1996). Other mechanisms may be responsible for the release of myofibrillar proteins (Fig. 9.4) but only this theory is supported by experimental evidence. Lack of specific inhibitors has long prevented confirmation of the importance of m-calpain and μ -calpain for muscle proteolysis, but calpastatin overexpression in transgenic mice can reduce muscle atrophy after unloading by 30% (Tidball and Spencer, 2002). The inhibition of slow to fast remodelling in the same model suggests that the calpains are also involved in the degradation of myofibrils during remodelling.

Calpain, mainly μ -calpain, is partly associated with myofibrils. Myofibril-bound calpain degrades desmin, nebulin, titin and troponin T when activated with calcium *in vitro* (Delgado *et al.*, 2001). In addition α -actinin is released and calpain undergoes autolysis. The mechanism of calpain activation *in vivo* is still unconfirmed. The main problem is that the intracellular calcium levels never become as high as the concentrations required to activate calpain *in vitro*, although it cannot be excluded that locally high intracellular calcium concentrations may exist. The mechanism of activation probably involves not only release from the calpain inhibitor and calcium binding, but also another activating step. Possible other activators are membrane binding, unknown activating proteins and partial autolysis (Mellgren, 1987).

In comparison with the well-studied ubiquitous calpains, knowledge on the skeletal muscle-specific calpain, p94, is very limited. The major problem in studies of p94 is its inherent instability. All known types of LGMD2A are caused by a loss of p94 expression or activity (Ono *et al.*, 1998). This stabilizing role demonstrates that the action of p94 on skeletal muscle is indirect (e.g. structural or signalling). The muscle-specific calpain localizes to the m-line and I-band regions where it binds to titin. The titin kinase domain is also located next to the m-line as is MuRF1. This collection of a protein kinase, ubiquitin ligase and protease suggests that this region has an important regulatory function. How this regulation works and what it regulates (assembly, degradation, signalling) remain subjects for future study.

9.4.4 Regulation of caspases in skeletal muscle

Caspases are essential proteases for programmed cell death or apoptosis in many tissues. Whether programmed cell death occurs in muscle is still a matter of debate (Primeau *et al.*, 2002). However, many characteristic features of apoptosis have been observed in skeletal muscle. Chromatin condensation, DNA fragmentation (Smith *et*



Possible assembly/disassembly (incorporation/release) mechanisms

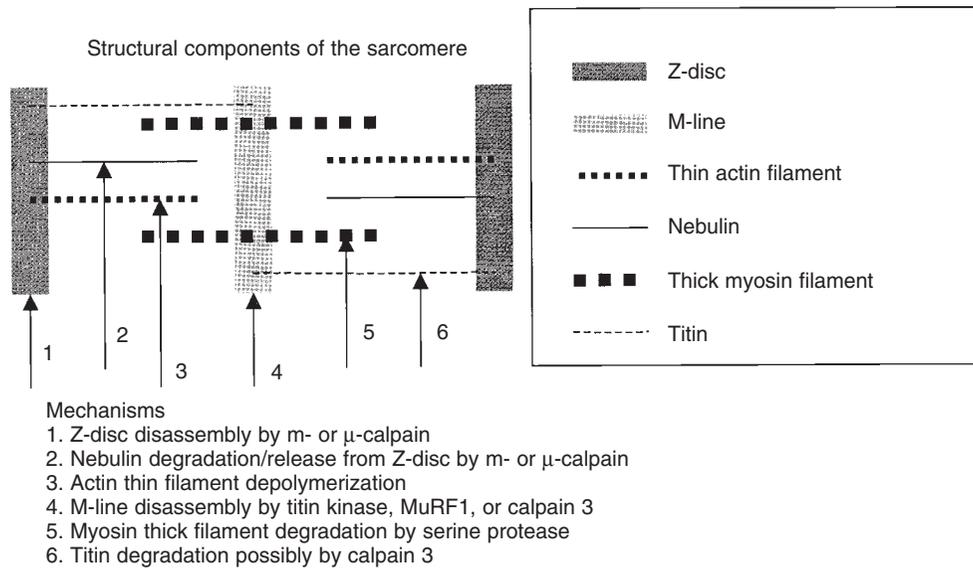


Fig. 9.4. Myofilament release. Myofilament proteins are inaccessible for most proteases once incorporated into myofibrils. Release of myofilament proteins from the myofibril is probably the rate-limiting step in their degradation (Solomon and Goldberg, 1996). Different mechanisms have been proposed to allow release of myofibrillar protein: (i) calpain-mediated Z-disc disruption and release of myofilaments (Williams *et al.*, 1999); (ii) nebulin degradation; (iii) actin filament depolymerization resulting in short thin filaments and loss of structural integrity (Riley *et al.*, 2000); (iv) disassembly initiated by signalling complex consisting of titin kinase, MuRF1 (McElhinny *et al.*, 2002) and possibly calpain 3 (Hackman *et al.*, 2002); (v) myosin thick filament degradation by serine protease bound to the myofibril (Hori *et al.*, 1998; Sangorin *et al.*, 2000); (vi) titin degradation, possibly by calpain 3 bound to titin at two sites (Sorimachi *et al.*, 1995).

et al., 2000), caspase activity (Belizario *et al.*, 2001) and phosphatidylserine redistribution (Van den Eijnde *et al.*, 2001) have been observed. However, it is not clear if these processes in skeletal muscle are associated with complete or partial degradation of the muscle fibres. In addition, experiments showed that phosphatidylserine surface exposure (Van den Eijnde *et al.*, 2001) and caspase activation (Fernando *et al.*, 2002) occur during, and may even be required for, fusion of myoblasts.

9.4.4.1 Caspase during hypertrophy and atrophy

Fusion of precursor cells to muscle fibres has many similarities to apoptosis. Besides the activation of caspases, calpains are also activated and phosphatidylserine is exposed on the cell surface. The remodelling and hypertrophy that occur after muscle loading or training involve proliferation of satellite cells followed by fusion of satellite cells with existing muscle cells (Moss and Leblond, 1970). This causes an increase in the number of nuclei in the muscle cells. At the same time the number of mitochondria increases, fibre diameter increases, and fibre type changes towards more fatigue-resistant types. During atrophy exactly the opposite happens. It is still a matter of debate whether the change in nuclear number during atrophy occurs at all and if it occurs by fibre necrosis or by nuclear apoptosis with similarity to programmed cell death in mononucleated cells (Wada *et al.*, 2002).

Muscle atrophy has many similarities to programmed cell death. The sarcoplasm shrinks, nuclear morphology changes occur, DNA fragmentation can be observed, and nuclei and mitochondria are degraded. However, there are also important differences: muscle cells do not break up into vesicles and many nuclei and mitochondria remain intact. Knowledge about apoptosis in muscle is very limited and was mainly investigated in association with muscular dystrophies. Muscular dystrophies are usually characterized by a combination of muscle atrophy and simultaneous regeneration. Therefore, some of the apoptotic features associated with cell fusion may have been misinterpreted as signs of cell death. The similarities between muscle atrophy and apoptosis warrant a closer investigation into the presence and activation of the caspase cascade (Fig. 9.5) in muscle atrophy. Two pathways can activate caspases and both are relevant for muscle atrophy. The first mechanism of activation is through a large family of receptors that includes the tumour necrosis factor receptor. Mitochondria are involved in the second activation pathway.

9.4.5 Other proteases in the muscle

Other relevant proteolytic enzymes may be present in muscle cells. It is clear that the proteasome system explains most of the protein degradation that occurs in muscle. However, since *in vitro* experiments clearly showed that the proteasome cannot degrade intact myofibrils, this suggests that the myofibrillar proteins must be released from the myofibril by some strictly regulated mechanism or a continuous exchange prior to degradation. Calpain is one candidate enzyme that catalyses this process but many other mechanisms are possible (Fig. 9.4). One of these other mechanisms involves a myofibril-bound serine protease. Myofibril-associated serine proteases were purified from mouse muscle (Hori *et al.*, 1998; Sangorin *et al.*, 2000) and they can degrade myofibrils *in vitro* (Hori *et al.*, 2002; Sangorin *et al.*, 2002). However, the enzymes were not completely purified or identified and it remains uncertain where these proteases are located *in vivo*. Therefore, it is possible that these enzymes are localized in the sarcoplasm or even outside the muscle fibres in the extracellular matrix, connective tissue cells or macrophages.

With intracellular remodelling or breakdown the extracellular matrix is also remodelled or degraded to adjust to different fibre diameter and contractile forces. The proteolytic enzymes involved in the degradation and remodelling of the extra-

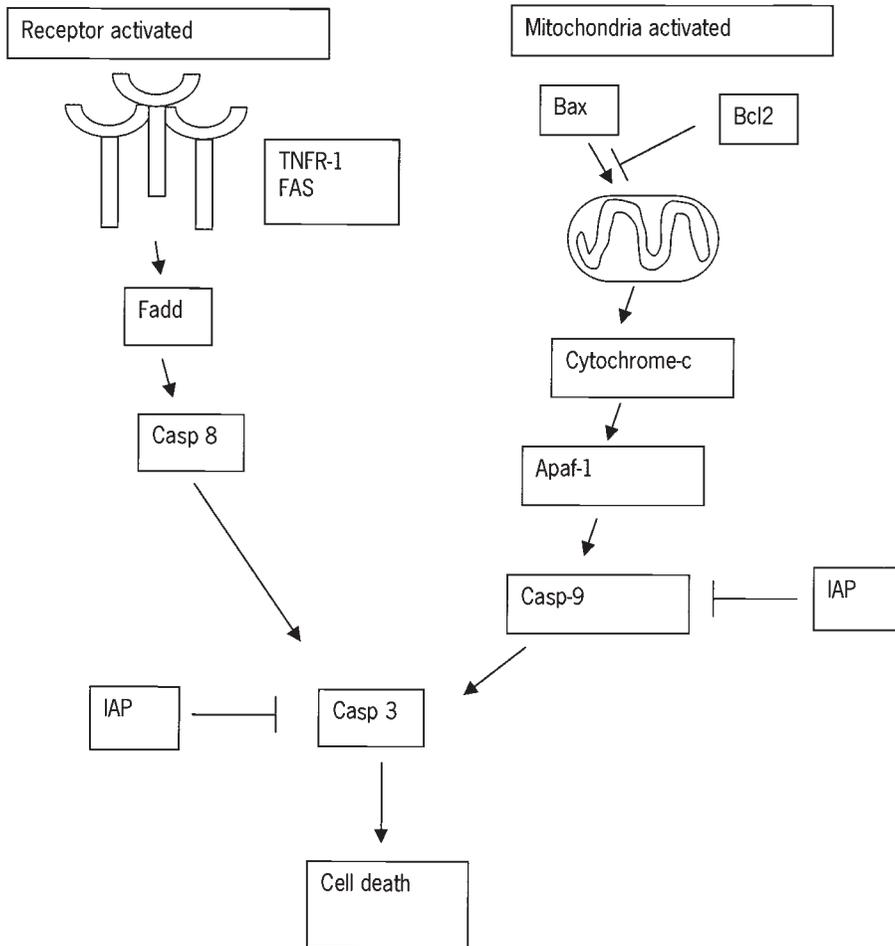


Fig. 9.5. Caspase regulation in skeletal muscle. Caspases can be activated by mitochondria-releasing activating factors (e.g. cytochrome-c) or by the specific receptors. The receptors are activated by ligand induced trimer formation. Release of caspase-activating factors from mitochondria can be induced by bax and inhibited by bcl-2 (Olive and Ferrer, 2000). Expression of the caspase-activating receptors Fas and tumour necrosis factor receptor I has been detected in skeletal muscle.

cellular matrix will not be discussed in this chapter, which is focused on intracellular remodelling.

9.5 Conclusion

The proteolytic capacity of the major proteolytic systems in muscle increases during remodelling and breakdown. This capacity increase occurs through augmented expression of the major proteolytic systems. Many studies have focused on this upsurge in capacity by measuring protease mRNA or expression, or activity meas-

urements with artificial substrates. However, these assay systems cannot be used to identify the proteolytic mechanism responsible for the degradation of specific substrates. If the substrates for the different proteolytic enzymes are unknown it remains possible that degradation increases for some non-myofibrillar substrates but remains unchanged for myofibrillar proteins. It is even conceivable that the increased capacity for proteolysis occurs without an increase in protein degradation.

Knowledge of the relative importance of the proteolytic enzymes was obtained with protein balance and inhibitor studies in incubated muscle. The results from these studies demonstrate that increased proteolysis in skeletal muscle is mainly caused by increased proteasomal protein degradation. It remains unclear why expression of lysosomal proteases (cathepsin L in particular) is often increased during muscle remodelling and breakdown. Possibly lysosomal proteolysis contributes mainly to extracellular matrix remodelling. A major problem is that protein balance studies cannot distinguish between substrates. Many minor myofibrillar components that may exert a regulatory function have been identified in recent years. It is likely that the degradation of important regulatory proteins is overlooked because only minor amounts of these substrates are present. Valuable insight into the mechanism of muscle proteolysis was recently obtained with transgenic mice overexpressing calpain in their skeletal muscle. In these mice both remodelling and breakdown were reduced during unloading. The fact that inhibition of calcium dependent proteolysis did not have a similar strong effect may be explained by the unspecific inhibitors used and the divergent effects of calcium-dependent proteolysis (both beneficial through p94 and adverse through m- and μ -calpain). Although the major proteolytic systems involved in muscle proteolysis are now known this does not directly lead to an insight into the mechanism for muscle proteolysis.

Possible mechanisms are summarized in Fig. 9.4. The only mechanism supported with experimental evidence is the mechanism in which myofilament release is induced by calpain. However, it is still possible that the first step in myofibril degradation involves another modifying enzyme or degradation by serine protease or caspase that requires calpains for activation or that even more than one mechanism exists. The major problem with all the proposed mechanisms is that, although the activation of degradation can be explained, no explanation is offered for the deactivation. For example, if calpain activates myofilament release what prevents it from releasing all the myofilaments from all myofibrils in the muscle cell? It is this spatial regulation of proteolysis in skeletal muscle that allows the muscle to remain active during breakdown and remodelling. A lot of these questions on the proteolysis in muscle could be answered if the activities of the enzymes involved in muscle proteolysis were combined with ultrastructural studies identifying their localization.

Proteolytic enzymes are still mentioned as potential targets for inhibition of muscle breakdown. It appears likely that muscle breakdown is performed by the same proteolytic enzymes (e.g. the proteasome, the lysosome and the calpain system) that degrade the majority of proteins in other tissues. The inhibition of these proteolytic pathways would have severe toxic effects on the tissues with a high protein turnover before any effect could be observed in skeletal muscle. The discovery of the muscle-specific ubiquitin ligases MAFbx and MuRF1 offers new possibilities for study of the regulation of muscle proteolysis. Inhibitors for these ubiquitin ligases are not yet available but have a great potential for reducing proteolysis caused by chronic disease, sepsis or disuse.

Increased knowledge about regulation of muscle proteolysis is not only of clinical importance. In farm animals, immobilization, emotional stress (both acute and chronic) and inflammation may also induce catabolism of muscle protein affecting both the quality and quantity of meat.

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10 The Muscle Regulatory Factors Gene Family in Relation to Meat Production

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10.1 Introduction

A defined number of skeletal muscle fibres are formed in two separate waves during prenatal development, while postnatal growth is restricted to hypertrophic muscle fibre growth (see Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume). The genes of the MRF (muscle regulatory factors) gene family, consisting of four structurally related transcription factors – *myogenin*, *MyoD1*, *myf-5* and *MRF4* – regulate both skeletal muscle fibre development and postnatal hypertrophic growth. In meat-producing animals, skeletal muscle tissue becomes meat after slaughtering. Skeletal muscle fibres are the major cell type of meat mass. Thus, differences in the activity of the MRF gene family may be very important for the amount of meat deposited in these animals. Therefore, the MRF genes can be considered as interesting potential candidate genes to investigate the relation between genomic variation in these genes and skeletal muscle mass, and thus meat mass.

The purpose of this chapter is to discuss the MRF gene family in relation to meat production, and show that information of genomic variation in these functional genes, and variation in their expression provide information that can be used in commercial breeding. Furthermore, we will describe a technique (chromatin immunoprecipitation or ChIP) that can be used to identify the genes regulated by the MRF transcription factors.

At the genomic level, genetic variation is a mutation leading either to a modified protein with a (slightly) changed activity, or to a (slightly) changed level of expression of an unmodified protein. Therefore the candidate gene approach (for a review see te Pas and Soumillion, 2001) investigates the effects of allelic variation of a gene on economically important traits. A whole genome approach alternative to the candidate gene approach is the genome scan method (see Rothschild *et al.*, Chapter 12, this volume).

In this chapter we will focus on the MRF gene family, which are candidate genes for meat mass production for which the genomic structure and physiologic data on regulation of skeletal muscle development and skeletal muscle growth rate are available, and briefly mention the genes NFAT and β -catenin of importance in this context. We will briefly review the genes of the MRF gene family in relation to skeletal muscle mass production, followed by a detailed review of the knowledge on the MRF genes in relation to meat production, at the level of both gene structure and expression. Some information about attempts to influence MRF gene expression *in vivo* and *in vitro* will be reviewed and its relevance for skeletal muscle mass determination indicated. Finally we will discuss future research needed to evaluate the role of these and other candidate genes in meat production in more detail.

10.2 Regulation of the Development of Skeletal Muscle Fibres: Importance for Livestock Production

By definition, meat is skeletal muscle tissue after slaughtering the animal. Skeletal muscle tissue consists of a number of different cell types, such as muscle fibres, adipocytes, connective tissue cells, endothelial cells and nerve tissue cells. The composition of all these cells together makes skeletal muscle tissue, and each cell type is

assumed to affect meat quality traits. However, for skeletal muscle mass – and thus meat mass – muscle fibres make up the major part. Two independent parameters, muscle fibre numbers and muscle fibre thickness, determine muscle mass. Three examples of extremely muscled livestock, the double-muscled cattle (see Kambadur *et al.*, Chapter 14, this volume), the callipyge sheep (see Freking *et al.*, Chapter 15, this volume) and the Piétrain pigs, show that both parameters have an important impact. Double-muscled cattle have greatly increased numbers of skeletal muscle fibres compared with normal cattle because of a mutation in the *myostatin* gene (Grobet *et al.*, 1997, 1998; Kambadur *et al.*, 1997), an inhibitor of skeletal muscle fibre development (McPherron *et al.*, 1997). Callipyge sheep show muscle fibre hypertrophy determined by a paternally inherited callipyge allele (Carpenter *et al.*, 1996; Freking *et al.*, 1998). Piétrain pigs show skeletal muscle fibre hypertrophy due to a presently unknown mechanism (Staun, 1963). Interestingly, the hyperplastic double-muscled cattle phenotype is more heavily muscled than the hypertrophied muscle fibre thickness in Piétrain pigs (double-muscled cattle have approximately 20–40% more meat on their carcass than normal muscle cattle while Piétrain pigs have 5–10% more meat than comparable pigs from other breeds), suggesting that skeletal muscle fibre hyperplasia is especially important for muscle – and thus meat – mass. Therefore, knowledge at the morphological, physiological and genomic levels about the development and growth of skeletal muscle fibres is of utmost importance for the improvement of meat production in livestock.

10.2.1 The origin of skeletal muscle fibres

Skeletal muscle fibre development starts in embryonic somites (for an excellent review see Perry and Rudnicki, 2000). Somites develop from the paraxial mesoderm. The ventromedial part of the somite develops into the scleroderm, giving rise to chondroblasts. The dorsolateral part of the somites develops into the dermomyotome, which further matures into the dermatome and the myotome (Sassoon, 1993; Smith *et al.*, 1994; Lyons *et al.*, 1996) (see also Maltin and Plastow, Chapter 13, this volume). Skeletal muscle development starts in the myotomal part of matured somites when pluripotent cells become determined to myoblasts – the muscle precursor cells. The existence of at least two separate groups of myoblasts has been demonstrated (Miller, 1992; Ordahl and Le Douarin, 1992; Stockdale, 1992; Braun and Arnold, 1996). Myoblasts proliferate thereby increasing their numbers, until differentiation, i.e. fusion of myoblasts to multinucleated myofibres, occurs (for reviews see the next paragraph). Part of the myoblasts relocate to the limb buds to form the limb musculature (for a review see Sassoon, 1993).

Skeletal muscle fibre development is restricted to fetal development. Postnatal muscle growth is restricted to hypertrophic growth of prenatally developed muscle fibres. Skeletal muscle tissue develops in two separate waves. In a primary wave of muscle fibre development muscle fibres are formed without a template. These primary muscle fibres serve as templates on which the secondary muscle fibres develop in a second wave of myoblast proliferation and differentiation (see also Section 10.4) (Handel and Stickland, 1987, 1988; Dwyer *et al.*, 1993; Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume).

Thus, the number of primary muscle fibres seems to be an important factor for

muscle mass determination. Furthermore, primary muscle fibre development is suggested to be regulated mainly genetically, while secondary muscle development can be influenced by environmental factors, such as nutritional level of the sow, and addition of hormones and growth factors to the sow (Dwyer and Stickland, 1991; Rehfeldt *et al.*, 1993; Dwyer *et al.*, 1994). Therefore, the genetic background of the mechanism regulating the skeletal muscle fibre development in livestock, especially in meat-producing animals, has been studied in more detail at the genomic level. A number of hormones, growth factors and regulatory genes were found to affect the diverse stages of skeletal muscle development and growth, but the MRF gene family takes a central position in the process (for reviews see Olson, 1990; Sassoon, 1993).

10.3 The Muscle Regulatory Factors Gene Family

The MRF gene family has been studied in detail in human, mouse, *Xenopus* and a number of avian systems and has been the subject of a number of excellent extensive reviews (Weintraub *et al.*, 1991; Lyons and Buckingham, 1992; Olson, 1993; Sassoon, 1993; Weintraub, 1993; Olson and Klein, 1994; te Pas and Visscher, 1994; Cossu *et al.*, 1996b; Rawls and Olson, 1997; Perry and Rudnicki, 2000; Sabourin and Rudnicki, 2000; te Pas and Soumillion, 2001). We will discuss the MRF gene family with a focus on the importance of the genes for meat-producing livestock.

The MRF proteins are skeletal muscle tissue-specific basic helix–loop–helix transcription factors that activate skeletal muscle differentiation stage-specific genes (reviewed in Edmondson and Olson, 1993). Furthermore, the MRF genes are also expressed in postnatal skeletal muscle tissues where expression has been related to repair of damaged skeletal muscle fibres, and probably also to hypertrophic skeletal muscle growth (Beilharz *et al.*, 1992; Fuchtbauer and Westphal, 1992; Grounds *et al.*, 1992; Robertson *et al.*, 1992; Koishi *et al.*, 1995).

The MRF gene family consists of four genes, *MyoD* (also called *MyoD1*, or *myf-3*), *myogenin* (also called *myf-4*), *myf-5* and *MRF4* (also called *myf-6*, or *herculin*) (reviewed in Olson, 1990; Montarras *et al.*, 1991; Weintraub *et al.*, 1991; Fig. 10.1). *MyoD* and *myf-5* are expressed in proliferating myoblasts. *Myf-5* expression precedes *MyoD* expression (Montarras *et al.*, 1991; Hannon *et al.*, 1992; Lyons and Buckingham, 1992; Pownall and Emerson, 1992; Sassoon, 1993; Weintraub, 1993; Olson and Klein, 1994; Lyons *et al.*, 1996; Rawls and Olson, 1997). However, the genes are activated in different myoblast subpopulations in the myotome (Yee and Rigby, 1993; Smith *et al.*, 1994; Braun and Arnold, 1996; Cossu *et al.*, 1996a,b), which probably gives rise to distinct myoblast populations (Miller, 1992; Ordahl and Le Douarin, 1992; Stockdale, 1992). *Myf-5* is activated in the dorsal subdomain of the myotome by factors derived from the neural tube, while *MyoD* is activated in the ventral subdomain induced by the notochord (Cossu *et al.*, 1996b). *Myogenin* is expressed during differentiation, i.e. fusion, of myoblasts to multinucleated myofibres (Olson, 1990; Montarras *et al.*, 1991; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). The relationship between *myf-5* and *MyoD* expression level and myoblast proliferation rate is not clear. Crush-injury-activated satellite cells show increased levels of *myf-5* and *MyoD* expression (Beilharz *et al.*, 1992; Fuchtbauer and Westphal, 1992; Grounds *et al.*, 1992). However, *in vitro* studies using glucocorticoid treatment of proliferating myoblast cultures show that while proliferation rate decreases, *myf-5*, *MyoD*

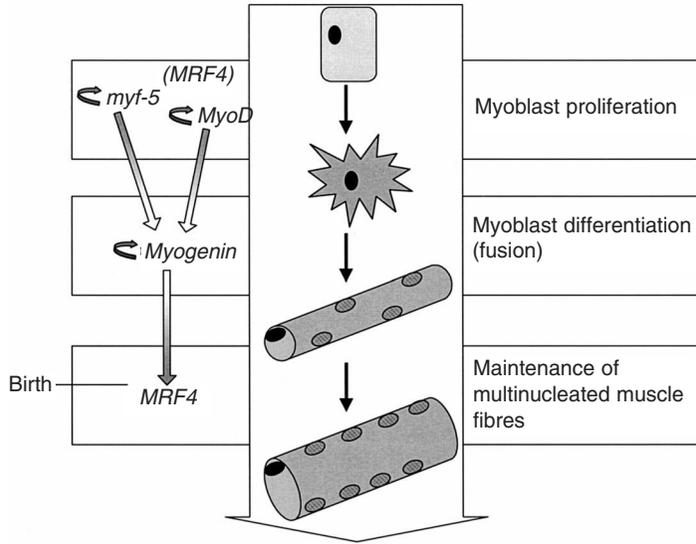


Fig. 10.1. Expression pattern of the genes of the MRF gene family during proliferation and differentiation of myoblasts, and maintenance of skeletal muscle fibres. *Myf-5* and *MyoD* are expressed in proliferating myoblasts. They autoregulate their own expression (curved arrows). *MRF4* is only transiently expressed during early myoblast phase. *Myogenin* is the only MRF gene expressed during myoblast differentiation. *Myogenin* also autoregulates its own expression (curved arrow). *MRF4* is the main MRF gene expressed in skeletal muscle fibres and is suggested to be involved in maintenance of the differentiation state of muscle fibres.

and *MRF4* mRNA expression level increases. This suggests that, at least under such artificial conditions, there is no direct relationship between *myf-5* or *MyoD* expression level and myoblast proliferation rate (te Pas *et al.*, 2000a). *MRF4* is expressed transiently during the early stages of myoblast proliferation, but is mainly expressed in postnatal skeletal muscle tissue where it is supposed to be important for maintenance of skeletal muscle fibre differentiation-specific phenotype. The importance of the transient *MRF4* expression during early skeletal muscle developmental stage is not exactly known, but a role in the development and maturation of the somites has been suggested. *MRF4* is also suggested to play a role in the transition from primary to secondary skeletal muscle fibre formation (for a review on *MRF4* specific effects see Konieczny, 1992).

How the MRF genes are switched on during embryonic development is not yet fully understood. Two mechanisms that are involved are briefly mentioned below. Recently, calcineurin has been implicated in regulating several aspects of skeletal muscle physiology, also in expression of MRF genes (Friday and Pavlath, 2001). The transcription factor nuclear factor of activated T cells (NFAT; for a review on NFAT see Rao *et al.*, 1997) has been mentioned as one downstream mediator of calcineurin signalling, and in skeletal muscle regulation of *myf-5*, a downstream target of the calcineurin substrate NFAT (Friday and Pavlath, 2001).

Also recently, the β -catenin protein has been shown to be potentially an important factor for skeletal myogenesis (Schmidt *et al.*, 2000; Petropoulos and Skerjanc,

2002). β -catenin was found in the myotome prior to *MyoD* expression. Wnt1 and Wnt3a are signalling factors that may transduce their signal by a conserved pathway that leads to transcriptional regulation by β -catenin/LEF1, where nuclear function was essential and sufficient for skeletal myogenesis in P19 cells (Petropoulos and Skerjanc, 2002). Furthermore it has been suggested that the Wnt/ β -catenin pathway is required for regulating myogenic gene expression in the presumptive mesoderm of *Xenopus* (Shi *et al.*, 2002). Possibly it directly activates the expression of the *myf-5* gene (Shi *et al.*, 2002).

10.3.1 Mode of action of the MRF proteins

MRF proteins dimerize with the ubiquitously expressed transcription factor proteins E12 and E2A to form heterodimers. Then, the heterodimers activate skeletal muscle differentiation stage-specific genes through binding to sequences called E-boxes (CANNTG) in the 5' upstream regulatory region of the genes (Olson, 1990; Weintraub *et al.*, 1991). This is exploited by ChIP (see Section 10.9) to find possible target genes of the MRFs. Thus, the muscle-specific gene expression pattern in muscle fibres is determined by the MRF gene expression pattern (Olson, 1990; Weintraub *et al.*, 1991). Furthermore, several of the MRF genes have been shown to auto-activate (enhance) their own expression too (Fig. 10.1) (Weintraub, 1993).

In the process of muscle development the Id protein (inhibitor of differentiation) is also important. Id is an MRF-like gene and as such Id is a strong regulator of MRF effects. The Id protein dimerizes with the E12 and E2A proteins preventing dimerization of the E12 and E2A proteins with the MRF proteins. Because Id heterodimers lack a transcription activation function necessary for activation of skeletal muscle-specific genes, the Id genes stimulate prolonged proliferation while delaying differentiation (Benezra *et al.*, 1990; Sun *et al.*, 1991; Jen *et al.*, 1992). Since generally the Id gene is not mentioned as belonging to the MRF gene family the role of this gene will not be reviewed here. Potentially, the gene has a strong capacity to influence skeletal muscle fibre numbers, since it may influence the length of proliferation periods during muscle fibre development.

10.3.2 Interactions with other proteins

The myocyte enhancer factor (MEF-2) gene family has been described as an important factor for the determination of the type of muscle-specific differentiation (including skeletal muscle, heart muscle and smooth muscle) (for a review see Olson *et al.*, 1995). Interactions between MEF-2 and the MRF have been shown in skeletal muscle fibres and differentiating myoblasts (Cserjesi and Olson, 1991; Edmondson *et al.*, 1992; Ornatsky *et al.*, 1997). MEF-2 cooperates with the MRF genes for the regulation of skeletal muscle-specific gene expression (Naidu *et al.*, 1995). Furthermore, MEF-2 directly influences MRF gene expression, and vice versa (Cserjesi and Olson, 1991).

A number of growth factors, such as insulin-like growth factor-I (IGF-I), hormones and proto-oncogenes such as *ski* are able to activate or repress members of the MRF gene family (Colmenares *et al.*, 1991; Florini *et al.*, 1991; Ichikawa *et al.*,

1997). These genes form a regulatory network influencing MRF gene expression pattern. We refer to the reviews for these aspects of MRF gene regulation (see above; see also Olson, 1990 for a review of factors affecting myoblast/satellite cell development in *in vitro* culture). The *ski* oncogene is the best-studied oncogene affecting muscle mass. *Ski* has been shown to increase muscle fibre thickness in *ski*-transgenic pigs probably through activation of MRF genes (Colmenares and Stavnezer, 1989; Hesketh and Whitelaw, 1992; Pursel *et al.*, 1992; Sutrave *et al.*, 1992).

10.4 MRF Genes and Regulation of Muscle Mass: Conclusions from the Knockout/Kick-in Mice

Although the physiology of the MRF genes is indicative of an important role in the determination of muscle mass, only limited direct evidence for this exists. Direct evidence for the involvement of the MRF genes in the determination of muscle mass was derived from the artificial knockout mice experiments; for reviews see Megeny and Rudnicki (1995), Rudnicki and Jaenisch (1995), Perry and Rudnicki (2000) and te Pas and Soumillion (2001).

What lessons can be learned from the MRF knockout mice for the function of the MRF genes for muscle mass, and thus meat mass, determination?

1. *Myogenin* seems to be a unique gene. Other MRF genes cannot, or only to a limited extent, replace myogenin functions. Therefore, if a mutation occurs in the *myogenin* gene in meat-producing livestock animals, affecting its amino acid sequence associated to its function, or its expression level or its expression pattern in the developing embryo, this will most probably directly affect muscle mass (meat) production.
2. Mutations in either the *myf-5* or *MyoD* genes will probably not directly affect meat mass, because they can at least partly substitute for each other's function. However, a combination of mutations will also give rise to differences in meat production. Furthermore, a recessive allele may affect muscle mass in the progeny of the animals.
3. The role of *MRF4* remains less clear. However, a mutation in *MRF4* affecting its transient early expression may affect *myf-5* expression, which may affect meat production indirectly. *MRF4* may be involved in the activation of secondary muscle fibre formation (Zhang *et al.*, 1995).

Therefore, we will focus first on the importance of genomic variation at the MRF gene loci with special attention to their role in genetic improvement of meat production. Thereafter, we will describe experiments aiming at elucidating the role of prenatal and postnatal MRF gene expression patterns and expression levels for skeletal muscle growth.

10.5 Relationships Between Genomic Variation in the MRF Gene Loci and Skeletal Muscle Mass/Meat Mass Determination in Livestock Meat Production

Although a porcine-specific *MRF4* polymorphism has been reported (Ernst *et al.*, 1994), presently information on association with production traits is not available. No information on other meat-producing livestock species is available either.

10.5.1 Myogenin

Myogenin is expected to be an important candidate gene for the determination of skeletal muscle mass based on the physiological knowledge of its expression pattern found in embryonic mice (Hannon *et al.*, 1992; Lyons and Buckingham, 1992; Pownall and Emerson, 1992; Yee and Rigby, 1993; Lyons *et al.*, 1996). *Myogenin* expression marks the end of myoblast proliferation, and induces differentiation of myoblasts, i.e. fusion to form multinucleated muscle fibres. Furthermore, *myogenin* knockout mice show that the number of *myogenin* alleles is directly related to the number of skeletal muscle fibres, thus muscle mass, and the amount of meat on the carcass. Finally, the importance of *myogenin* can be deduced from the fact that *myogenin* is expressed in all differentiating *in vitro* cell culture systems, while the other MRF genes are not (Edmondson and Olson, 1993).

The association between the influence of genetic variation in the *myogenin* gene locus and several phenotypic characteristics was investigated. Since a positive mutation in the *myogenin* gene is expected to yield more skeletal muscle tissue, the amount of meat on the carcass is an important trait to study. However, if an animal is born with an increased number of muscle fibres, an increased birth weight might also be expected comparable to the effects on birth weight seen in double-muscling cattle (Swatland and Kieffer, 1974). Additionally, data on delivery problems similar to the delivery problems described for double-muscling cattle (Swatland and Kieffer, 1974) should be recorded. Finally, higher skeletal muscle fibre numbers can be expected to be related to higher growth rate of the animal, especially in animals selected for growth rate and amount of lean meat on the carcass (Handel and Stickland, 1987, 1988; Dwyer and Stickland, 1991; Dwyer *et al.*, 1993).

Statistical analysis revealed that: (i) *myogenin* genotype was related to meat weight on the carcass, slaughter weight at a standard slaughter age, growth rate and piglet birth weight; while (ii) no association between *myogenin* alleles and back fat thickness was observed; and (iii) the same *myogenin* allele accounts for all positive effects. Moreover, the *myogenin* gene can be considered a gene with a major impact on meat production since the *myogenin* genotype explained 4–5.8% of the total genetic variation in these traits (te Pas *et al.*, 1999b). These data are in line with the expectation that *myogenin* alleles have important effects on skeletal muscle fibre numbers at birth. The consistency of the effects on birth weight, growth rate, carcass weight and meat weight increases the power of the analysis. Surprisingly, the allelic frequency of the *myogenin* allele with the favoured effects was relatively low in all populations tested. This is unexpected since the traits under investigation are favoured in selection. One possible explanation is that the allele that is favourable for meat mass in selection might also affect other, less favourable, traits. Such effects were, however, not noted in the several association studies. The reported delivery problem of double-muscling cattle (Swatland and Kieffer, 1974) was not observed with any pig *myogenin* genotype. It should be noted that the porcine birth weight was relatively much less increased compared with the birth weight in double-muscling cattle. However, since increased birth weight is related to decreased piglet mortality the reported difference in birth weight can still be associated with a 1% lower piglet mortality rate, which is a major economic factor in pig breeding (Bereskin *et al.*, 1973; English and Morrison, 1984; Kisner *et al.*, 1995).

Surprisingly, while the reported effects were found repeatedly in a number of

commercial pig populations, the effects could not be confirmed in an experimental cross between Meishan and white pig breeds (Professor Dr M.F. Rothschild and Dr C. Ernst, Iowa State University, USA, personal communication). One possible explanation for this might be that the commercial pigs were all selected for the meat production-related traits while the experimental cross pigs were not, at least the Meishan pigs were not. Furthermore, these experimental selection lines are founded by a limited number of founder pigs (Soumillion *et al.*, 1997; Professor Dr I.R.E.W. Brascamp, Wageningen University, The Netherlands, personal communication). If a polymorphism and associated trait are not segregating in these founder animals the experimental selection lines cannot be used for the association study. Thus, the genetic background and selection history of the animals used for an association study may be relevant to consider. Alternatively, traits could have been measured differently. Thus, biological traits like skeletal muscle development need to be properly related to meat production traits, and vice versa, before starting an association study, otherwise association will probably not be found and the different studies will give different results. This argues for standardization of phenotypical measurements in order to be able to compare different experiments.

10.5.2 *Myf-5* and *MyoD*

Using the same animals as described above for *myogenin*, no single association for *myf-5* was found for any of the traits recorded (te Pas *et al.*, 1999a; te Pas and Soumillion, 2001). Does the lack of association between genomic polymorphism and meat production traits imply that the *myf-5* gene is not important for meat production traits? We strongly argue against this statement. First, *myf-5* is involved in the normal skeletal muscle fibre development, and thus, biologically, the gene is important. However, no genomic variation has been found in the gene of the investigated breeding populations that can be used in animal breeding for improvement of meat production. Second, the reported data only included limited samplings of four inbred breeding populations. Therefore, additional breeding populations need to be studied before solid evidence for the lack of association can be presented. Third, the results also do not provide solid evidence that there is no relevant genomic variation in the gene at all. Considering the redundancy of *myf-5* and *MyoD* function as seen in gene knockout mice it is very well possible that the effect of a less favourable allele is compensated by *MyoD*. Therefore, these results argue for combined *myf-5-MyoD* genomic effects.

MyoD alleles affecting *MyoD* expression or function could affect myoblast proliferation rate and timing of differentiation (Yablonka-Reuveni *et al.*, 1999), which may affect muscle mass. A genome scan has mapped quantitative trait loci (QTL) to the region on chromosome 2 where the *MyoD* gene is located. QTLs for the carcass traits lean to fat ratio, back fat weight and weight of shoulder meat, and a QTL for meat quality – i.e. pH 24 h post mortem – were mapped to this chromosomal area with significance at the 5% chromosomal level. Lean cut percentage and bacon meat/half carcass weight QTLs were mapped to this chromosomal area at the approximately 5% genome wide significance threshold level (Lee *et al.*, 1998). Although the chromosomal area involved covers approximately half of porcine chromosome 2, the localization of *MyoD* to this area, combined with the known physiological function of

MyoD, suggests that *MyoD* is a good physiological and positional candidate gene for meat production and meat quality traits. Thus, genomic variation in the *MyoD* gene could be involved in the determination of carcass meat traits. These QTLs await further analysis refining the chromosomal area involved, and using *MyoD* as a candidate gene.

Alternatively, the insulin-like growth factor-II (*IGF-II*) gene maps to the same chromosomal region on pig chromosome 2 as the *MyoD* gene. An imprinted QTL has been reported on this locus that affects skeletal muscle mass (Jeon *et al.*, 1999; Nezer *et al.*, 1999). Therefore, it cannot be excluded that at least part of the reported effects of the *MyoD* QTL relate to the *IGF-II* gene locus QTL although no imprinting was reported for the *MyoD*-related QTL.

It remains to be analysed whether the porcine *myf-5* gene is also polymorphic in these populations, and whether these polymorphisms genetically affect these meat production traits similarly. This analysis will be important to elucidate the redundancy in function of the *MyoD* and *myf-5* genes for their relation to livestock meat production traits. Furthermore, since *myf-5* and *MyoD* play both unique roles and cooperative roles in the development of specific muscles (Kablar and Rudnicki, 2000), muscle specific effects of diverse genotypes may be expected.

The effects of genomic variation in the non-livestock model species mouse have been studied in more detail in relation to repair of skeletal muscle tissue after crush injuries of muscles. A *PvuII* polymorphism affecting a putative E-box (CANNTG) in the promoter region of the *MyoD* gene in mouse was associated with earlier expression of *MyoD* after crush injuries, suggesting a uniquely enhanced muscle tissue repair (Mitchell *et al.*, 1992; Kay *et al.*, 1993; Marlow *et al.*, 1997).

10.6 Use of Genetic Variation in the Prenatal and Postnatal Expression Level of MRF Genes to Enhance Muscle Mass/Meat Production

Genomic variation may influence either amino acid composition of proteins, or expression patterns and/or expression levels of the genes. An altered expression level may be indicative of genomic variation in the promoter area of the genes, or at other loci than the locus under investigation; for example, genomic loci for trans-regulatory genes affecting the expression of the gene under investigation. The recently developed microarrays provide a technique that will enable the investigation of the expression profiles of whole muscle transcriptomes, and comparison of the differences under influence of allelic variation (Pietu *et al.*, 1999).

10.6.1 Prenatal selection-related MRF gene expression patterns in relation to muscle mass determination

A quail line selected for high growth rate and high meat content showed myofibre hyperplasia associated with delayed somite formation and delayed MRF expression patterns suggesting a time frame with an increased proliferative phase and delayed differentiation phase (Marks, 1978a,b, 1989; Fowler *et al.*, 1980; Coutinho *et al.*, 1993).

Selection for increased carcass protein content in mice and index traits showed higher *in vitro* satellite cell proliferation rate than selection for body weight and the control line. Satellite cells showed increased responsiveness to growth factors. Cell fusion kinetics were increased in the body weight and carcass protein content selection lines (Walther *et al.*, 1998). In another study mice were selected for small and large body size (Penney *et al.*, 1983). Skeletal muscle fibre hyperplasia was observed (Penney *et al.*, 1983). No differences were observed in fibre size, timing of myoblast fusion or protein synthetic rate. However, skeletal muscle tissue of the mice of the large body size selection line contained more nuclei than comparable muscles in mice of the small body size selection lines. The authors concluded that muscle fibre hyperplasia in the mice of the large body size selection line was not due to a delay in fusion, but to increased myoblast proliferation rates before fusion (Penney *et al.*, 1983). These results suggest a mechanism involving *myf-5* and *MyoD* expression.

A mouse-specific *myogenin* allele showing severely reduced *myogenin* expression level (25% of wild type) was discovered and heterozygous and homozygous mice were bred (Vivian *et al.*, 1999). Heterozygous hypomorphic/wild type mice showed a normal phenotype. Homozygous hypomorphic *myogenin* mice showed severe muscle hypoplasia and a reduced muscle-specific gene expression level, and are not viable as neonates. Heterozygous hypomorphic/knockout *myogenin* mice show sternum defects, severe muscle hypoplasia, and are not viable. The effects resemble the homozygous *myogenin* knockout mice effect (see te Pas and Soumilion, 2001). These results provide further evidence for *myogenin* allele dosage effect, comparable to the *myogenin* knockout mice (see te Pas and Soumilion, 2001). Finally, *MRF4* knockout mice carrying a homozygous hypomorphic *myogenin* gene showed more severe abnormal musculature than mice carrying normal *myogenin* alleles (Vivian *et al.*, 2000).

10.6.2 Postnatal MRF expression in relation to skeletal muscle mass determination

MRF gene expression has been observed in postnatal muscle tissue in satellite cells (i.e. postnatal myoblasts expression of *myf-5*, *MyoD* and *myogenin*) and in muscle fibres (i.e. *MRF4*) (Fuchtbauer and Westphal, 1992; Grounds *et al.*, 1992; Hinterberger *et al.*, 1992; Konieczny, 1992; Koishi *et al.*, 1995). Muscle fibres also express *MyoD* and *myogenin*, although to a much lower level than in satellite cells (Hughes *et al.*, 1993, 1997).

Muscle tissue of slaughter weight pigs selected for high growth rate showed elevated *myogenin*, *myf-5* and *MyoD* mRNA levels (te Pas *et al.*, 2000b). Thus, it is concluded that selection for increased growth rate is associated with increased mRNA levels of the MRF genes expressed in satellite cells. These cells are responsible for postnatal hypertrophic muscle growth. These results show that increased overall growth rate is associated with skeletal muscle-specific growth by affecting muscle growth regulatory mechanisms.

Selection for increased growth rate in pigs has been reported to be associated with increased plasma growth hormone (GH) levels (Lund-Larsen and Bakke, 1975; Norton *et al.*, 1989; Clutter *et al.*, 1995). However, GH itself did not affect growth rate directly (Harbison *et al.*, 1976; Chung *et al.*, 1985; Etherton, 1988). Physiologically, GH is interacting with a number of other hormones and growth

factors such as insulin-like growth factor-I (IGF-I) (Evock *et al.*, 1990; Owens *et al.*, 1990). *In vitro* cell cultures of mouse myoblasts cells treated with IGF-I showed up to 60-fold induced *myogenin* mRNA expression (Florini *et al.*, 1991). Thus, selection for increased growth rate could act on GH levels, thereby inducing IGF-I expression, which induces *myogenin* (Mangiacapra *et al.*, 1992) (for a review of the role of IGFs in myogenic differentiation see Magri *et al.*, 1991). Higher IGF-I levels were also reported to delay skeletal muscle differentiation (Ewton *et al.*, 1994). Genomically, the variation affecting muscle tissue growth rate may be found on the GH locus, or even further upstream at the locus of the transcription factor regulating GH expression, the pituitary-specific transcription factor-1 (*Pit-1*) gene (Tuggle *et al.*, 1993; Yu *et al.*, 1995; Frohman and Kineman, 1999).

In a number of different muscles the *MRF4* mRNA expression level was higher in muscles from pigs selected against fat deposition – i.e. for increased lean meat content (lean (L)-line) compared with muscles from pigs selected for high growth rate (fast (F)-line). Increased *MRF4* expression levels may suggest higher muscle maintenance in L-line pigs. Also, increased *MRF4* expression may associate with more mature muscles with lower growth rate (te Pas *et al.*, 2000b). Alternatively, *MRF4* expression level was correlated with muscle fibre type in some mixed-fibre typed muscles in rat, with higher *MRF4* expression levels in white muscle fibres (Walters *et al.*, 2000). However, this explanation may be in contrast with the reported increase in red muscle fibre types of these pig selection lines (Brocks *et al.*, 1998) (for more information about muscle fibre types see Reggiani and Mascarello, Chapter 2, this volume).

These results show direct evidence for selection-affected mRNA expression levels. Selection also affects skeletal muscle fibre type composition (Brocks *et al.*, 1998). *Myogenin* and *myf-5* mRNA expression was also observed in muscle fibres, although muscle fibre expression was much lower than satellite cells expression level (Hughes *et al.*, 1997). More hypertrophied muscle fibres tend to become white fibres. Hughes *et al.* (1993, 1997) showed a direct relation between the *myogenin/MyoD* ratio in rats and muscle fibre type. White muscle fibres mainly express *MyoD*, while *myogenin* expression was predominantly found in red muscle tissue. In L-line pig muscles the *myogenin/MyoD* ratio correlated well with muscle colour, and the ratio was higher in red muscles. In F-line pigs no such relationship between muscle colour and *myogenin/MyoD* ratio was found (te Pas *et al.*, 2000b). Surprisingly, Brocks *et al.* (1998) found that selection increased the number of red muscle fibres relative to the number of white muscle fibres in F-line pigs. This effect was not observed in L-line pigs, which correlated well with the absence of a relationship with the *myogenin/MyoD* ratio.

In conclusion, selection can affect the expression of the MRF genes. Selection also affects skeletal muscle fibre type composition, and thus meat mass and meat quality traits. The effects may be related to each other, suggesting a genomic relationship between selection and meat and carcass characteristics.

10.7 Discussion: MRF Genomic Variation and Expression Affects Muscle Mass, and thus Livestock Meat Production

Myogenesis can be described as a three-step procedure (Fig. 10.1): (i) proliferation of predetermined precursor cells (myoblasts); (ii) differentiation of myoblasts into myofi-

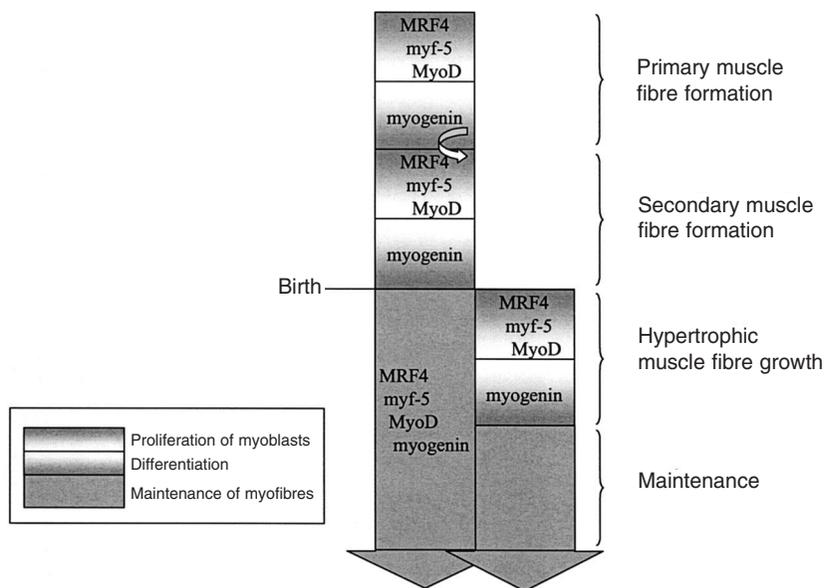


Fig. 10.2. View of the different phases of myogenesis in time in relation to skeletal muscle mass, and thus meat mass production. Myogenesis can be subdivided into four phases: (i) primary skeletal muscle fibre formation; (ii) secondary skeletal muscle fibre formation; (iii) postnatal hypertrophic skeletal muscle growth; and (iv) maintenance of skeletal muscle fibres. Phases i–iii can be further subdivided into a proliferative phase (prenatal: myoblasts; postnatal: satellite cells) and a differentiation phase (myoblasts: new skeletal muscle fibre formation; satellite cells: skeletal muscle fibre hypertrophic growth). The relevant MRF genes that are expressed during each phase are indicated (the lengths of the phases are not drawn to scale).

bres; and (iii) maintenance of existing skeletal muscle fibres. However, when applied to skeletal muscle mass determination, it is perhaps more correct to describe myogenesis as a four-step process (Fig. 10.2): (i) primary skeletal muscle fibre formation; (ii) secondary skeletal muscle fibre formation; (iii) hypertrophic skeletal muscle fibre growth; and (iv) maintenance of existing skeletal muscle fibres. Muscle fibre type composition is heritable (Suwa *et al.*, 1996). Therefore, phase 4 seems to be a static phase. However, remodelling of individual muscle fibres induces muscle fibre type transitions (Miller *et al.*, 1975; Yambayamba and Price, 1991; Weikard *et al.*, 1992; Cameron, 1993; Dwyer *et al.*, 1993; Karlsson *et al.*, 1993; Horak, 1995; Lefaucheur *et al.*, 1995; Muscat *et al.*, 1995; Hu *et al.*, 1997; Ruusunen and Puolanne, 1997), which shows that all four phases are highly flexible myogenic phases with potentials to affect muscle mass, and thus livestock meat production. In this section we will discuss the data of the MRF genes in relation to muscle mass determination as described above and try to fit them into a model describing the mechanism determining meat mass.

Muscle fibre hyperplasia was noted in a number of animal selection lines with different selection goals, such as body size, growth rate or muscle tissue mass maximization. Since skeletal muscle tissue is an important part of body mass (in selected pig breeds over 60% of carcass composition is muscle tissue), selection on all these diverse goals acts directly or indirectly on skeletal muscle tissue mass.

Figure 10.2 describes the steps in the myogenesis process that can be important for meat production. Prenatal skeletal muscle fibre formation occurs in two waves, the primary and secondary muscle fibre formation. Each wave of muscle fibre formation consists of a phase of proliferation of pre-determined myoblasts and differentiation to form multinucleated myofibres. After birth maintenance of muscle fibres takes place. Furthermore, a third wave of proliferation and differentiation of satellite cells (the postnatal equivalent of myoblasts) induces hypertrophic muscle growth. Each phase is under the control of the relevant MRF genes as shown in Fig. 10.2. Since secondary skeletal muscle fibre formation can be affected by environmental factors such as nutrition and hormonal composition of the sow (see te Pas and Soumilion, 2001), it is expected that the primary muscle fibre formation will be most affected by genetic effects. Because primary muscle fibres serve as templates for secondary muscle fibre formation, increased primary muscle fibre numbers will affect muscle mass during secondary skeletal muscle fibre formation too. Indeed, it was shown that primary muscle fibre formation is most affected by genetic factors.

With the exception of one mouse selection line where increased myoblast proliferation rate is suggested, all lines of evidence suggest that the length of the proliferative phase due to a delayed differentiation, rather than myoblast proliferation rate is important. Delayed development of somites has been shown in quails, and is suggested in a number of other lines. Genomic variation measured in markers close to the MRF genes or in the expression of the MRF genes accompanies these observations. Delayed differentiation of myoblasts by delayed development of somites can be achieved by delayed expression pattern of the *myogenin* gene. This suggests that the regulation of the expression pattern of the *myogenin* gene plays a central role in muscle mass determination. Indeed all lines of evidence point to the *myogenin* gene as a nodal point in the regulation of skeletal muscle mass (see also te Pas and Soumilion, 2001).

10.8 New Directions for the Research and Livestock Breeding for Meat Production

The studies described above will provide additional information for the evaluation of genomic MRF gene influence on muscle mass determination. It has been known for a very long time that muscle fibre numbers and muscle fibre hypertrophy affect meat quality traits such as tenderness (Staun, 1963). However, the genomic background affecting differences in tenderness remains largely unknown (for genomic information from the genome scan methodology see Rothschild *et al.*, Chapter 12, this volume). With the knowledge of the influence of genomic variation in the MRF genes on muscle mass and muscle fibre numbers reviewed above it is intriguing to speculate about the effects that the MRF genes may have on meat tenderness and other meat quality traits.

Presently, meat quality is receiving increasing attention from consumers. First of all because of food safety reasons, but also as a way to increase the quality of life. Meat quality traits are complex traits that may be influenced by all cell types in muscle tissue. Although the main part of skeletal muscle tissue are muscle fibres, which therefore determine muscle mass, other important cell types include adipocytes, connective tissue, neurones and endothelial cells. All these cell types can be important for meat quality: for example, adipocytes store fatty acids that are

reported to be important for meat colour, juiciness, tenderness, etc. (Staun, 1963). Thus, genomic variation in genes affecting the amount of intramuscular fat is important for meat quality parameters (Gerbens *et al.*, 1998, 1999). In the promoter area of at least one of these genes that is expressed in skeletal muscle fibres, the heart fatty acid binding protein, E-boxes are present suggesting regulation of the expression by the MRF genes (Gerbens *et al.*, 1997).

These studies can be broadened by evaluation of the effects of the numerous genes that affect, and are affected by, the expression levels and patterns of the MRF genes. Such studies are under way in the authors' laboratory. The newly developed DNA microarray technology enables the study of expression level and/or genomic variation in large numbers of genes simultaneously. In the near future also proteomics will be incorporated more routinely in research projects. Of the proteomics techniques, 2-D gel electrophoresis already gives the possibility of comparing the protein profiles of samples of interest. The interesting proteins can be excised from the gel and the amino acid sequence can be determined with mass spectrometry techniques. Such studies will increase our knowledge and insight into gene regulatory processes and their effects on animal physiology.

The MRF genes encode muscle-specific transcription factors that regulate the expression of muscle-specific genes. Genomic variation in the MRF genes may regulate groups of genes differently. Alternatively, genomic variation in the target genes may be affected differently by the MRF proteins. Thus, investigation of the MRF target genes may be very relevant. Recently, at the laboratory of the authors a study was started aimed at: (i) finding the genes that are regulated by the MRF proteins in muscle tissue; and (ii) finding the differences in the binding patterns of the MRF proteins to these genes related to meat mass and meat quality. Therefore, a technique called chromatin immunoprecipitation (ChIP) was used (see Section 10.9 for a description of how to find the relevant genes). In short, the DNA-bound MRF proteins are cross-linked to the DNA and the DNA of interest is subsequently isolated and analysed. For *myf-5* it was found that ChIP resulted in an enrichment of *myf-5* promoter sequences. Present research at our laboratory includes a microarray; also present on the array are the 96 DNA sequences enriched for the *myf-5* promoter sequences of unknown genes found after the ChIP with selection for *myf-5*. It is expected that novel genes will be found. Next, we will analyse the differences in the amount of MRF proteins cross-linked to the promoter region of specific genes to analyse the amount of MRF-related stimulation of the expression of the genes, which will be correlated with meat quality traits.

10.9 Towards Identification of Genes that are Regulated by the MRF Proteins in Muscle Tissue

A powerful feature of ChIP is that it does not destroy any specificity in target gene selectivity that is dictated by promoter context. To fully use this aspect we performed the ChIP assay on intact muscle samples that were immediately frozen upon sampling at slaughter and stored at -80°C .

10.9.1 Chromatin immunoprecipitation

Frozen 0.5 g pig extensor digitorum longus muscle was fixed and instantly homogenized using an ultra turrax homogenizing device in 4.5 ml 1% formaldehyde/PBS on ice. Fixation was allowed to proceed for 20 min at room temperature under rotation. To stop the fixation reaction 1 M glycine was added to a final concentration of 125 mM and allowed to rotate at room temperature for 5 min. After stopping the fixation the muscle material was collected by centrifugation (3000 rpm, 5 min, 4°C). The pellet was washed with phosphate buffered saline (PBS) two times, and finally resuspended in 4.5 ml cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40, 1 mM PMSF, 10 µg/ml aprotin, 10 µg/ml leupeptine) and allowed to rotate for 10 min at 4°C. After centrifugation (3000 rpm, 5 min) the pellet was resuspended in 4.5 ml nuclei lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS, 1 mM PMSF, 10 µg/ml aprotin, 10 µg/ml leupeptine) and allowed to rotate for 10 min at 4°C. The suspension was sonicated three times for 30 s with intermediate cooling on ice (resulting in an average fragment size of >0.5 kb) and clarified by centrifugation (15,000g, 20 min at 4°C). The collected supernatant (chromatin-DNA) was divided in aliquots, frozen in liquid N₂, and stored at -20°C for later use.

Immunoprecipitation of *myf-5* was performed. Five hundred µl of chromatin-DNA supernatant was diluted with 144 µl 5× RIPA buffer (5% Triton X-100, 0.5% Na-deoxycholate, 0.5% SDS, 700 mM NaCl, 1 mM PMSF), 0.2 µg of anti-Myf-5 pAb (Santa-Cruz) was added and allowed to incubate for 18 h at 4°C. Fifty microlitres 50% (v/v) protein A-sepharose CL4B beads (Pharmacia) were added and the incubation continued for 3 h at 4°C. The immunoprecipitates were washed at room temperature five times for 3 min each with 5× RIPA buffer, once for 3 min with LiCl/NP40 (250 mM LiCl, 0.5% NP40, 1 mM PMSF)/500 mM NaCl, once for 3 min with LiCl/NP40 buffer, and twice for 3 min with TE (10 mM Tris pH 8, 1 mM EDTA, 1 mM PMSF). Subsequently the beads were resuspended in 100 µl TE and treated with 5 µl RNase for 30 min at 37°C.

To retrieve the DNA the covalently linked proteins were removed by proteinase K treatment followed by heat treatment: 3 µl 10% SDS and 2.5 µl 1 mg/ml proteinase K were added to the resuspended beads and incubated for 16 h followed by heat treatment for 6 h at 65°C. After 20 s centrifugation at 5000 rpm supernatant was collected and after ethanol precipitation the pellet was resuspended in 100 µl TE and a second proteinase K treatment was performed as above for 4 h at 45°C. To remove protein from the solution a phenol extraction was performed followed by an ethanol precipitation step. The DNA was resuspended in 300 µl H₂O.

10.9.2 DNA amplification

To amplify the diversity of DNA molecules from the immunoprecipitation a two-step PCR reaction of the round A/B DNA amplification protocol (www.microarrays.org/pdfs/Round_A_B_C.pdf) was performed. In this PCR degenerative primers were used (primer A: GTT TCC CAG TCA CGA TCN NNN NNN NN; primer B: GTT TCC CAG TCA CGA TC). Input in round A was 7 µl in DNA purified as described in Section 10.9.1. After round B the product showed as a smear on an agarose gel ranging from 500 to 1500 bp (Fig. 10.3).

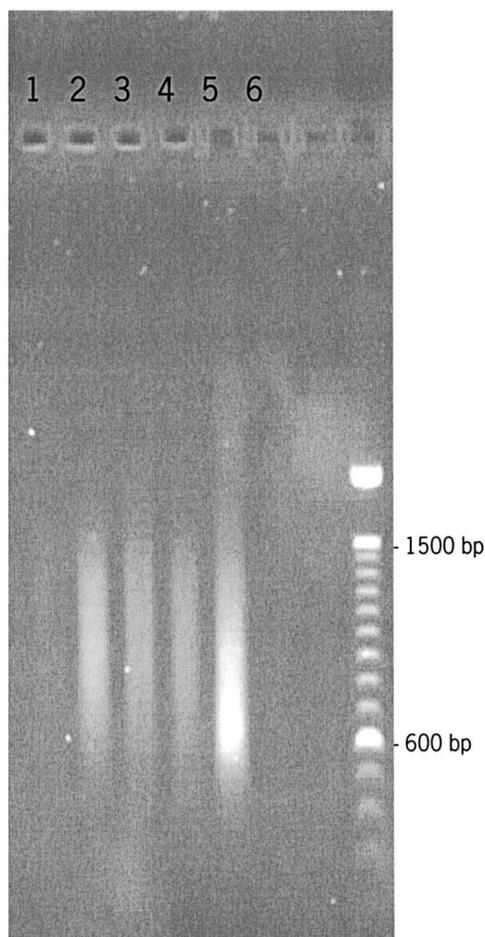


Fig. 10.3. Ethidium bromide-stained agarose gel of chromatin immunoprecipitated (ChIP) and round A/B PCR-amplified DNA. Lane 1: control antibody; Lanes 2–4: anti-myf-5 antibody, different DNA isolates; Lane 5: genomic DNA, positive PCR control; Lane 6: water, negative PCR control; Lane 7: empty; Lane 8: 100-bp DNA ladder.

10.9.3 Sequencing of DNA after ChIP

The amplified DNA was cloned in the pGEM-T easy vector (Promega) and 96 colonies were sequenced. Similarly 96 control clones isolated using a non-specific control pAb (anti-rabbit Ig) were sequenced.

All sequences from anti-myf-5 antibody and control antibody were analysed and compared with each other using two different methods. First, all the possible CANNTG E-box motifs were counted. At random one CANNTG E-box motif will be found for every 256 bp. For ChIP with control antibody 1.01 CANNTG E-box motif per 256 bp was found, as expected for the control antibody, that should not bind to any protein–DNA molecule. For ChIP with anti-myf-5 an average presence

of the CANNTG E-box motif of 1.25 per 256 bp was found. The value of 1.25 found for *myf-5* underlines an enrichment of *myf-5* promoters with the above described ChIP technique. Second, an algorithm was used (with 0.8 score cut-off; www.fruitfly.org/seq_tools/promoter.html) to predict possible eukaryote transcription promoters. For ChIP with control antibody ten predictions were made for every 50 sequences tested. For ChIP with anti-*myf-5* antibody 15.4 predictions were made for every 50 sequences tested. The higher prediction value for *myf-5* again underlines an enrichment of *myf-5* promoters with the ChIP technique.

Further analysis of the 96 possible target sequences of *myf-5* is currently ongoing in our laboratory. As part of this investigation all 96 sequences are spotted on microarrays and will be hybridized with prenatal pig muscle expression profiles. Knowledge of the target genes of the MRF transcription factors will help to elucidate the influence of different genetic variants of the MRF genes on meat deposition, muscle quality and meat quality.

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11 The Muscle Transcriptome

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11.1 Motives to Address the Muscle Transcriptome

Growth in general is a central biological process affected by many factors and characterized mainly by the deposition of adipose and connective tissue, bone and muscle. Muscle tissue, including cardiac, smooth and skeletal muscles accounts for most of the body mass and daily energy consumption. Skeletal muscle becomes meat after slaughter. It consists of three different types of muscle fibres each characterized by certain structural, biochemical and metabolic properties (see Reggiani and Mascarello, Chapter 2, this volume). The growth and weight of muscle is mainly determined by the number and size of the muscle fibres and prolificacy of satellite cells (see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume). The proportion of different muscle fibres, their structure and functional properties affect the animals' growth performance and are endogenous factors on post-mortem meat quality traits (Swatland, 1984; Lengerken and Pfeiffer, 1991; Lengerken *et al.*, 1997; see also Hopkins and Taylor, Chapter 17, this volume).

Growth rate and the kind of tissue deposited are major factors for economically important traits in meat-producing animals. This includes fattening traits (daily gain,

feed efficiency), body composition traits (meat content, fat content, loin eye area) and meat quality traits (colour, water-holding capacity, pH, tenderness, intramuscular fat). These are complex traits reflecting the action and interactions of many different physiological pathways.

There are currently three major production problems that breeders encountered when attempting to breed for improved performance, body composition and meat quality. First, these traits are to some extent negatively correlated (see also Gerbens, Chapter 16, this volume). Secondly, most of the traits, especially body composition and meat quality traits, can only be measured post mortem, which makes prior breeding selection difficult. Thirdly, the consumers' as well as the meat processing industry's conception of high meat quality is not uniform and their expectations are changing.

The identification of genes that regulate fattening, body composition and meat quality traits will assist efficient meat production and facilitate the resolution of existing production problems.

Researchers have used different strategies to detect such genes. One is the positional cloning approach that includes the observation of co-segregation of a large number of markers and a trait ('genome scan') and fine mapping of the suspect regions approaching the unknown gene more and more closely (see Rothschild *et al.*, Chapter 12, this volume). Candidate genes may be identified based on knowledge of physiology, biochemistry or pathology, which clearly indicates the mechanism of the trait ('direct candidate' approach). Indirect approaches to identify candidate genes are: (i) the 'positional candidate' approach (Ballabio, 1993), which combines linkage information for a particular trait and mapping information on genes exhibiting particular functional properties and/or patterns of expression ('functional candidates'); and (ii) the 'comparative mapping' approach, which combines the results of reverse genetics efforts and information on genes mapped in the corresponding syntenic group in other species.

Most of the successful approaches to detect quantitative trait loci (QTL) were based on genome scans performed in resource populations. The first QTL in pig detected by this approach was reported by Andersson *et al.* (1994). A QTL accounting for 10% of phenotypic variation for average backfat and abdominal fat on chromosome 4 was detected. A QTL for growth was also found on chromosome 13 accounting for about 10% of phenotypic variation. Subsequently these results were supplemented and confirmed (Rothschild *et al.*, 1995; L. Wang *et al.*, 1998; Milan *et al.*, 1998; Moser *et al.*, 1998; Marklund *et al.*, 1999). QTL for meat and carcass quality traits based on reverse genetics were also reported (Andersson-Eklund *et al.*, 1998; de Koning *et al.*, 1998, 2001a,b; Rohrer and Keele, 1998a,b; Walling *et al.*, 1998, 2000; Geldermann *et al.*, 1999; Rohrer, 2000; Bidanel *et al.*, 2001; Malek *et al.*, 2001a,b; for review see Rothschild *et al.*, Chapter 12, this volume).

Unfortunately, genome-wide scans cannot resolve the location of a QTL more precisely than 10–30 cM (Darvasi and Soller, 1997). As this is equivalent to a region containing 5–30 MB of DNA and several hundreds of genes it is a major task to fine map the QTL region in order to finally positional clone the gene. The identification of the polymorphic genes within the QTL regions detected by reverse genetics is the aim of many current studies. Furthermore it has to be proven that the QTL segregate and show any effect in other commercial and experimental populations than the resource population used to identify the QTL region.

The alternative ways, the direct candidate gene approach and the comparative mapping approach, have also been applied successfully. Polymorphism of porcine POU domain class 1 transcription factor 1 (POU1F1), alanyl (membrane) aminopeptidase N (ANPEPN), ryanodine receptor (RYR1), insulin-like growth factor (IGF-1), heart fatty acid binding protein (H-FABP), leptin, muscle regulatory factors, cholecystokinin (CCK), cholecystokinin type A receptor (CCKAR) and leptin receptor have been shown to be associated with variation in growth and performance traits (Yu *et al.*, 1995; Clutter *et al.*, 1996; Geldermann, 1996; Nielsen *et al.*, 1996; Casas-Carrillo *et al.*, 1997; Vincent *et al.*, 1997; Gerbens *et al.*, 1998; te Pas *et al.*, 1999; Hardge *et al.*, 2000; te Pas and Soumillion, 2000).

More recent approaches to detect candidate genes are based on the analyses of differences in the expression profile in particular subsets of cells/tissues and/or individuals with certain phenotypes.

This chapter reviews the current status of the analysis of the muscle transcriptome, the construction of transcript maps and studies aiming at the identification of genes controlling growth, body composition and meat quality traits by expression profiling of muscle tissue.

11.2 Complexity of the Genome, Transcriptome and Proteome: Strategic Implications

Now that the human and mouse genomes have been sequenced (Venter *et al.*, 2001; Mouse Genome Sequencing Consortium, 2002) there are reliable estimates that the mammalian genome contains about 35,000 coding sequences. The level of the transcriptome is even more complex since many genes give rise to different transcripts because of alternate splicing, alternative transcription starts and alternative reading frames, with the consequence that several different related messages are co-expressed and yield sets of different proteins. From many mRNA many protein isoforms, often extensively post-translationally modified are synthesized and furthermore in some instances altered by expression of other genes in the same metabolic pathway (Kettman *et al.*, 2002). A typical cell contains about 40,000 mRNA molecules at any time that represent about 5000 molecular species of mRNA. Most of the transcripts are present in only one or two copies per cell and they account in most instances for the proteins of low abundance. Each cell has about 1 billion protein molecules; the 100 most abundant represent about 90% of the mass of cellular proteins. Taking into account alternative splicing at the transcription level and co- and post-translational modifications of polypeptides the number of protein species is much higher than the number of active genes and mRNA species. It is estimated that each cell expresses up to 100,000 different proteins derived from 5000 mRNA species. Overall estimates are that a vertebrate organism comprises about 35,000 genes, 120,000 transcripts and 200,000–4,000,000 distinct proteins (Liang *et al.*, 2000; Fountoulakis *et al.*, 2001; Kettman *et al.*, 2002).

Analysing the transcriptome cannot fully reveal characteristics of the proteome that determine the phenotype. But elucidating the transcriptome and qualitative and quantitative differences of the mRNA species present in particular cells or tissues at specified times of development or under certain endogenous or exogenous conditions will allow the targeting of a subset of genes out of the 35,000 genes present in

the genome that are likely to be involved in the manifestation of distinct phenotypes. These genes are functional candidate genes because of their temporo-spatial distribution of expression or their expression in certain phenotypes. The functional candidate approach benefits from the fact that it only deals with cDNA, devoid of intronic and intergenic sequences, which represent only a few per cent of the total genome (about 3% in mammals). Differential expression screening approaches are therefore more function oriented.

Expression analyses have a great potential to support the identification of genes controlling traits that are important for animal production. The map-based and function-oriented approaches for detecting candidate genes support each other in the attempt to identify the underlying genetic mechanisms of phenotypic variation in economically important traits. As outlined in Fig. 11.1 different methods of expression profiling reveal expressed sequence tags (ESTs) that represent functional candidate genes with trait-associated and/or specific temporo-spatial distribution of expression. These genes can be screened for single nucleotide polymorphisms (SNPs) and mapped genetically and/or physically. Those of the differentially expressed ESTs mapping in QTL regions that were identified by the map-based approach of linkage analysis become positional functional candidates and are additional input to the final procedures of identifying and evaluating potential QTL.

The combination of population geneticists and molecular genetics has brought the livestock genomic field to a revolutionary time. The association between genotype and phenotype can be more completely understood but complex traits still have proved to be challenging, because it is impossible to follow all genomic regions that are responsible for the complex variation of the trait. Part of the existing genetic variation affects the activity of genes and how it changes; that is, there is polymorphism of the level of gene expression (expression level polymorphism, ELP; Doerge, 2002). Functional genomics approaches can be used to generate information about gene function, as well as data on genetic interactions, not only among and between gene complexes, but also in response to environmental stimuli. One of the major chal-

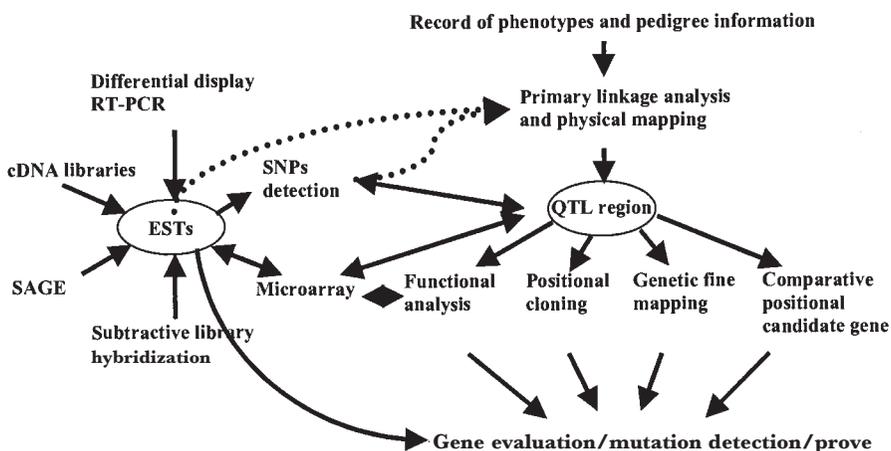


Fig. 11.1. Strategies to identify genes and further characterize loci controlling economic traits by using expressed sequence tags (ESTs) as functional candidate and positional candidate genes.

Challenges will be the combination of the data of DNA, RNA and protein levels to dissect complex traits or economic trait loci. New bioinformatics tools open the way to merge and make sense out of the combination of quantitative data of expression levels and information about the physiological function of particular genes and groups of genes representing certain metabolic pathways. These genomics data provide an understanding of metabolic, regulatory and developmental pathways but are limited to explanation of the complexities of segregating populations. A number of ideas to combine the genomics data with QTL methodologies have been proposed. Jansen and Nap (2001) propose to merge genomics and genetics into 'genetical genomics'. This involves expression profiling and marker-based fingerprinting of each individual of a segregating population, and exploits all the statistical tools used in the analysis of quantitative trait loci. Doerge (2002) proposes the use of current QTL methods and software to identify loci that affect the level of expression of a particular gene or a set of genes. That means that the quantitative data of gene expression analyses are handled like phenotypic data and their inheritance is compared with that of large numbers of marker genes applying methods of QTL analyses.

11.3 Identification of Skeletal Muscle Expressed Sequence Tags

Current sequencing efforts prove that it is logistically possible to derive the sequence of all 35,000 genes. However, in order to elucidate the transcriptome of each cell or tissue at particular times and under particular environmental conditions a suitable approach is to sequence only parts of the genes called ESTs. The concept of EST sequencing first came into public view in 1991 (Adams *et al.*, 1991). The basic idea is simple: create cDNA libraries from tissues of interest, pick clones randomly from these libraries, and sequence a large number of clones. Each sequencing reaction generates 300 base pairs or so of sequence that represents a unique sequence tag for a particular transcript.

In the last few years the identification of ESTs has largely contributed to the progress in defining the genetic control of particular traits in human and animals. There was a rapid increase of the number of ESTs having been sequenced in the past years. For example in mid-1999 there were 1,359,585 human ESTs and 472,938 mouse ESTs stored in the corresponding dbEST database, compared with 4136 porcine sequences. In winter 2002/2003 there were 4.9 and 3.6 million human and mouse ESTs, respectively, and about 115,000 porcine ESTs.

The strategy to randomly sequence cDNA library clones from tissues of interest to obtain ESTs is suitable for production of a collection of transcripts. EST sequencing can be accomplished using normalized or non-normalized cDNA libraries. A normalized cDNA library is one in which each transcript is represented in more or less equal numbers (Soares *et al.*, 1994). The principle is to submit the population of cDNA to a process of denaturation followed by re-association in such a way that the most abundant molecular species re-associate more rapidly and can be partly eliminated; in the remaining 'normalized' population, the number of copies of each of the more abundant species will have decreased by a factor of 1000 to 10,000. The advantage of using normalized cDNA libraries is that redundant sequencing of highly expressed genes is minimized, and the potential for identification of rare tran-

scripts is maximized (Bonaldo *et al.*, 1996). An advantage of non-normalized, non-amplified libraries is that the transcript abundance of the original cell or tissue is accurately reflected in the frequency of clones in the library. Non-normalized libraries can be used for an EST project to identify highly expressed, unknown genes and to compare the expression of highly expressed genes in different cell or tissue samples (Kawamoto *et al.*, 1996; Ji *et al.*, 1997). Given an extensive knowledge of gene sequences, the construction of serial analysis of gene expression (SAGE) libraries (Velculescu *et al.*, 1995) is an alternative efficient approach for expression profiling that provides qualitative and quantitative data of the transcriptome. In this method a short contiguous sequence of 10–11 base pairs (bp), derived from a defined location within each transcript and unique to each tag, is detected for identification of individual mRNAs. Concatenated series of these tags of 20–22 bp in length, which represent 20 or more genes, are cloned resulting in a SAGE library that gives an accurate picture of gene expression at both the qualitative and the quantitative level. The disadvantages of SAGE are related to the technical difficulty in generating good SAGE libraries and in analysing the data. In addition, identification of the tag requires previous sequence data.

First attempts to characterize the human muscle transcriptome revealed ESTs that represent close to 1000 genes expressed in muscle (Lanfranchi *et al.*, 1996; Pietu *et al.*, 1996). The attempts have been extended widely in human science and led to the release of several databases, which are publicly accessible. Current research work is aiming at the analysis of the abundance of different transcripts, specificity of the transcripts to the muscle transcriptome as well as the mapping of muscle expressed genes. The construction of a preliminary transcript map of human skeletal muscle was initiated by sequencing more than 11,000 ESTs from a cDNA library representing 1945 individual transcripts of which more than 500 were allocated on human chromosomes (Pallavicini *et al.*, 1997) and added to the existing human somatic cell and radiation hybrid genetic maps (Auffray *et al.*, 1995; Houlgatte *et al.*, 1995; Schuler *et al.*, 1996; Deloukas *et al.*, 1998). A follow-up more comprehensive map with 1078 entries was published in 1998 (Bortoluzzi *et al.*, 1998). The human muscle gene map is available on-line (see TRAIT, Table 11.1). The studies reveal a concentration of muscle expressed genes in five chromosomal regions of chromosomes 17, 19 and X (Pallavicini *et al.*, 1997; Bortoluzzi *et al.*, 1998).

Bortoluzzi *et al.* (1998) found that out of the 1078 muscle transcripts considered less than 4% represent genes known to be specifically expressed in muscle. Based on the assumption that the number of detected ESTs per gene is a function of the transcript frequency in the population of mRNAs, an *in silico* analysis covering more than 4000 transcripts found in human skeletal muscle annotated in the UniGene database showed that 9% of the entries represent genes highly expressed in muscle while 27% and 64% account for moderately and weakly expressed genes, respectively. Moreover only 1.2% of the entries were found to be muscle specific (Bortoluzzi *et al.*, 2000). The data support the hypothesis that the biochemical and functional properties of differentiated muscle cells may result from the transcription of a very limited number of muscle-specific genes along with the activity of a large number of genes, shared with other tissues, but showing different levels of expression in muscle. The low number of muscle-specific transcripts is possibly due to the facts that: (i) all cells have a cytoskeleton and share most metabolic pathways; (ii) most cells exhibit some contractile properties; and (iii) different tissues share certain types of cells. The group

of highly expressed genes includes a number of typically skeletal muscle genes. Together with the 47 putative skeletal muscle-specific genes a set of 417 transcripts is proposed to characterize the adult human skeletal muscle transcriptional profile (Bortoluzzi *et al.*, 2000). This information is useful as a reference for analysing the relationship between the muscle transcriptional profile and different endogenous and exogenous conditions as well as individual phenotypic differences, for example by microarray technology. Welle *et al.* (1999) used the SAGE method to generate a catalogue of more than 53,000 short (14 bp) expressed sequence tags from mRNA obtained from human adult muscle. Over 12,000 unique tags were detected and about 10% represented highly abundant transcripts. Transcripts of moderate or rare abundance appeared at 21% and 69%. The results of the *in silico* analysis (Bortoluzzi *et al.*, 2000) and the SAGE analysis (Welle *et al.*, 1999) are in good agreement. This was specifically tested on frequency data of transcripts that were covered by both studies (Bortoluzzi *et al.*, 2000).

Knowledge of the overall composition of the muscle transcriptome in farm animals is still limited since the biggest efforts to elucidate the transcriptome did not account for the specific tissue of origin of the transcripts. The largest proportion of ESTs known in cattle and pig were obtained from such cDNA libraries of pooled tissues (Smith *et al.*, 2001; Fahrenkrug *et al.*, 2002). About 68,000 bovine ESTs were derived from four cDNA libraries that contained cDNA reverse transcribed from mRNA of: (i) lymph nodes, post-pubertal ovary, fat tissue, hypothalamus and pituitary; or (ii) testis, thymus, skeletal muscle, pancreas, adrenal gland, endometrium; (iii) bone marrow, pre-pubertal ovary, fetal skeletal muscle; or (iv) whole day 20 and day 40 embryos (Smith *et al.*, 2001). In the pig, two pooled cDNA libraries that are made up of: (i) day 11, 13, 15, 20 and 30 fetuses; and (ii) pituitary, hypothalamus, ovary, endometrium and placenta collected at different oestrus and gestation stages, were used to obtain about 66,000 EST sequences. The results of both efforts together with EST sequences submitted to GenBank/EMBL have been assembled and annotated in a Cattle Gene-Index and a Porcine Gene-Index accessible via the web page of the Institute for Genome Research, TIGR (Table 11.1). The remaining sequences of the 237,293 bovine and 113,358 porcine ESTs that are currently available at dbEST (dbEST release 011003) are from smaller studies, some of which focus on muscle ESTs and are discussed below.

In chicken about 340,000 ESTs were sequenced from 64 cDNA libraries generated from 21 tissues including embryonic and adult muscle. This comprehensive collection allows deduction of the origin of the transcripts. These ESTs make up 85,000 contiguous sequences out of which 20% represent full-length cDNA sequences (Boardman *et al.*, 2002). Based on this data the authors estimate that the chicken genome has approximately 35,000 genes in total.

A considerable number of skeletal muscle-derived porcine ESTs were obtained in the framework of the GENETPIG project (Identification of GENes controlling Economic Traits in PIG, EU-Biotech, BIO4-98-0237), which aimed at the identification of porcine ESTs in order to improve the porcine gene map and to contribute to the listing of candidate genes for economically important traits (Hatey *et al.*, 2000). In total 701 porcine muscle ESTs representing 306 non-redundant cDNA species were obtained (Davoli *et al.*, 1999, 2002). The ESTs with homology to known genes cover those involved in cell division/DNA synthesis, cell signalling and communication, cell structure and motility, defence and homoeo-

stasis, gene and protein expression as well as metabolism. The proportions of genes belonging to these classes as proposed by Adams *et al.* (1995) fit with those obtained in human. Out of 306 porcine transcript species, 161 matched a human sequence analysed by Bortoluzzi *et al.* (2000). Similar to earlier findings in human (Welle *et al.*, 1999; Bortoluzzi *et al.*, 2000), 62% of these transcripts represent highly expressed genes and 22% and 15% account for moderately or weakly expressed genes, respectively (Davoli *et al.*, 2002). Yao *et al.* (2002) used a non-normalized and a normalized cDNA library from porcine skeletal muscle to isolate ESTs. In total 782 ESTs (687 from the normalized and 95 from the non-normalized library) were obtained, which represent 742 unique transcripts with 139 that revealed no significant matches to porcine ESTs in public databases, suggesting possible muscle-specific expression (Yao *et al.*, 2002).

The attempts to detect ESTs specifically from muscle tissue in cattle are even scarcer. Screening a bovine fetal skeletal muscle library revealed 77 ESTs (Grosse *et al.*, 2000).

A number of databases publicly accessible via the Internet have been established, containing information on ESTs from a number of tissues including skeletal muscle. A list is given in Baxevanis (2003); Table 11.1 gives an overview of the current content and features of some of these databases.

11.4 Methods to Illuminate the Transcriptome and to Relate Muscle Differential Expression and Phenotype

The functional genomics era approaches the completion of the genomic sequencing phases. There are millions of ESTs from different organisms in databases. However, the function, expression and regulation of more than 80% of them have yet to be illustrated. So the next phase of genome projects will place strong emphasis on assigning function to these genes. One of the routes for exploring the function of a gene is by determining its pattern of expression. Because the relative abundance of gene expression often reflects specific cellular needs, a sufficiently large and diverse set of gene expression profiles from different stages of development, mutants, treatments and conditions would provide insight into gene functions.

Novel and powerful techniques are now available to analyse the global gene expression patterns. Relating expression profiles to phenotypic characteristics will elucidate the effect of particular genes and the network of coordinated gene expression necessary to express a particular phenotype. A single expression analysis in a given stage and/or under given conditions has the potential to identify the essential physiological functions, but, more interestingly, when groups of genes are taken together, their coordinated gene expression has the potential to uncover associated regulatory segments of the genome. In recent years, a variety of techniques has been developed to analyse differential gene expression, such as differential display reverse transcriptase-polymerase chain reaction (RT-PCR), subtractive library hybridization, SAGE and cDNA microarrays. In the livestock field, the use of gene expression profiling techniques to elucidate tissue-specific differential gene expression in relation to particulate developmental stages and/or exhibition of certain phenotypes is still at the beginning. Several projects are under way and their results will significantly improve and enlarge the list of candidate genes for economically important

Table 11.1. Databases accessible via the Internet with information about the muscle transcriptome.

Name	Short description	Content	Features	Access
TRAIT database, TRAnscript Integrated Table	Muscle TRAIT is a collection of information about transcripts expressed in human skeletal and cardiac muscle, blood and leukaemia cells	28,893 ESTs derived from a 3' end cDNA library of human skeletal muscle; 970 (705) differentially expressed in adult skeletal muscle, 21 years old (40 years old) compared with eight other tissues	Query for nucleotide sequence, gene, map position, protein domain, tissues expression levels, gene ontology definitions	http://muscle.cribi.unipd.it/
Rochester Muscle Database	Inventory of adult human skeletal muscle mRNA by the SAGE method	A total of 53,875 tags were catalogued, representing 12,207 unique tags	Listing of all tags or identified tags in order of abundance or in alphabetical order	http://www.urmc.rochester.edu/smd/crc/old%20site/welle/swindex.html
Human and mouse gene expression database bodymap	Bodymap contains expression information of human and mouse genes, novel or known, in various tissues or cell types and various timing created by random sequencing of clones in 3'-directed cDNA libraries	Human: 18,998 independent clusters; 1,862 clones expressed in skeletal muscle; 702 muscle-specific; mouse: 16,772 independent clusters; no skeletal muscle library	Query for cells within tissues; gene name or definition, primer ID, sequence, accession numbers in GenBank, and UniGene	http://bodymap.ims.u-tokyo.ac.jp/
Mouse genome informatics – gene expression database, GXD	GXD contains gene expression information from the laboratory mouse. GXD stores and integrates different types of expression data with particular emphasis on endogenous gene expression during mouse development	5,910 genes, 877 entries of muscle expressed genes	Query for genes; gene classifications; chromosomal location; expression in/at developmental stage or anatomical structure; assay type(s); reference	http://www.informatics.jax.org/menus/expression_menu.shtml
TIGR Gene Indices	The TIGR Gene Indices integrate research results from international gene research projects in order to ultimately represent a non-redundant view of all genes of 20 species and data on their expression patterns, cellular roles, functions and evolutionary relationships	Human: 806,582 unique sequences, 187,287 arranged in assemblies, 288 assemblies specific to muscle; mouse: 734,653 unique sequences; 146,273 arranged in assemblies, 200 assemblies specific to muscle; pig: 49,201 unique sequences; 17,354 arranged in assemblies, 25 assemblies specific to muscle; cattle: 71,652 unique sequences; 26,931 arranged in assemblies, 18 assemblies specific to muscle; chicken: 107,869 unique sequences; 35,790 arranged in assemblies, 142 assemblies specific to muscle	Query for nucleotide or protein sequence; tissue; cDNA library; gene product name; search by radiation hybrid map location; functional classification based on the gene ontology assignments; linking to metabolic pathways; putative identifications through the nature genome directory	http://www.tigr.org/tbl/tgi/
UniGene	UniGene is a system for automatically partitioning GenBank sequences of several species into a non-redundant set of gene-oriented clusters each representing a unique gene. UniGene contains also related information such as the tissue types in which the gene has been expressed and map location	Human: 3,966,221 total sequences in clusters; mouse: 3,348,641 total sequences in clusters; pig: 60,575 total sequences in clusters; cattle: 131,576 total sequences in clusters; chicken: 123,838 total sequences in clusters	Query for gene; nucleotide and protein sequence accession numbers; tissue, organism; number of ESTs in a cluster; UniGene cluster identifier; mapping information; any word appearing in the title/description	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene

traits including growth, carcass composition and meat quality for which muscle is the major target tissue.

11.4.1 Differential display

Differential display RT-PCR or RNA fingerprinting is a PCR-based method for detection of differentially expressed genes in various cells or under altered conditions (Liang and Pardee, 1992; Welsh *et al.*, 1992). The differential display RT-PCR is based on the use of arbitrary primers to amplify partial cDNA sequences from subsets of mRNAs (see also Maltin and Plastow, Chapter 13, this volume). The approach involves amplification of 15,000 cDNA fragments that represent an essentially complete set of expressed genes. The first step is the reverse transcription of a subpopulation of RNA molecules, which is achieved through the use of an oligo-dT. Subsequently, an arbitrary primer is used to prime second strand synthesis by DNA polymerase and to amplify cDNA fragments. Finally the cDNA fragments are separated on a polyacrylamide gel. This method was further improved by Bauer *et al.* (1993) who established a set of 26 decamer random primers that theoretically will result in representation of almost all mRNA species by at least one band. Differential display is relatively simple to execute, and is efficient for analysing small amounts of RNA. As little as 5 or 10 ng of total RNA can be used to perform the experiments, and many samples can be analysed on a single gel. The differential display RT-PCR method also facilitates the cloning of rare mRNAs that may be difficult to obtain with techniques such as the differential screening of cDNA libraries. The approach is also suitable for minimizing the redundant isolation of ESTs representing abundantly transcribed housekeeping genes. A limitation of the differential display approach is that false positive results are often generated during PCR with lower annealing temperatures.

In order to identify ESTs that represent candidate genes for carcass traits in pigs, the differential display approach was used (Ponsuksili *et al.*, 2000; Zhao *et al.*, 2001). Ponsuksili *et al.* (2000) used the carcass trait 'eye-muscle area', which has high heritability ($h^2 = 0.4-0.6$; Sellier, 1994), as a valuable model case to apply the approach of analysing discordant sib pairs of the breed German landrace and an experimental F2 cross of Duroc and Berlin miniature pig in order to obtain candidate ESTs. The comparison of about 4000 differential display bands obtained with the anchored primer (d)T₁₁VA (V: A,C,G) and 26 arbitrary primers (Bauer *et al.*, 1993) revealed 27 bands with differential expression between discordant sib pairs. Out of these, seven bands were analysed further and trait-associated differential expression was confirmed by semiquantitative RT-PCR for six fragments. Two clones showed high homology to known genes, two were homologous to an EST and a SINE sequence. Two clones did not show any homology (Ponsuksili *et al.*, 2000). Zhao *et al.* (2001) used the mRNA differential display technique to identify differentially expressed genes in longissimus muscle between Duroc and Erhualian breeds. RNA pools were made from three animals in each breed. Five 3' anchored primers in combination with ten different 5' arbitrary primers were used and nearly 2000 bands were examined, among which 12 differentially displayed cDNAs were found. Two bands uniquely expressed in Erhualian pigs were cloned and sequenced. One band showed significant homology to the porcine myosin heavy chain gene.

Semiquantitative RT-PCR confirmed its differential expression. Another band showed homology to porcine NADH₄ and no difference was found in its expression level between the two breeds.

By differential display Maak *et al.* (2001) identified genes that are differentially expressed between healthy and splay-leg newborn piglets in order to identify candidate genes for this inherited disorder.

Using RNA arbitrary-primed polymerase chain reaction (RAP-PCR) Ashwell *et al.* (1999) identified liver and muscle transcripts that are differentially expressed between two White Plymouth Rock chicken lines that have been divergently selected for body weight at 8 weeks of age (41 generations). Among the more than 50 differentially expressed genes are key enzymes involved in glycolysis, gluconeogenesis, pro-hormone processing and muscle fibre formation.

11.4.2 Subtractive hybridization

Subtractive cloning methods have been in use for many years and new methods based on PCR are rapid and easy to execute (Hubank and Schatz, 1994; Diatchenko *et al.*, 1996). Subtraction methods aim to enrich the cDNA populations specific to a particular tissue or cell type by eliminating sequences common to several tissues or cell types and thus are non-specific. Subtractive cloning offers an inexpensive and flexible alternative to EST sequencing and differential display, and can be performed in any laboratory equipped with basic molecular biology tools.

One of the PCR-based subtractive cloning methods has been adapted from representational difference analysis (reverse transcription RDA, RT-RDA) (Lisitsyn *et al.*, 1993; Hubank and Schatz, 1994). In this approach, double-stranded cDNA is created from the two cell or tissue populations of interest, linkers are ligated to the ends of the cDNA fragments, and the cDNA pool is then amplified by PCR. The cDNA pool from which unique clones are desired is designated the 'tester', and the cDNA pool that is used to subtract away shared sequences is designated as the 'driver'. Following initial PCR amplification, the linkers are removed from both cDNA pools, and unique linkers are ligated to the tester sample. The tester is then hybridized to a vast excess of driver DNA, and sequences that are unique to the tester cDNA pool are amplified by PCR. This method requires several rounds of hybridization and does not resolve the problem of the wide differences in abundance of individual mRNA species (Hubank and Schatz, 1994).

The other new PCR-based subtractive cloning method is suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996). SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non-target DNA amplification. This technique is based on the suppression PCR effect in combination with subtraction. In general, cDNA is synthesized from total cellular RNA from the two types of tissues or cell being compared. To optimize the subtraction the cDNA is digested with a restriction enzyme to generate small DNA fragments (approximately 500 nucleotides in length). Specially designed DNA adapters are ligated on to one of the tester cDNAs, which is followed by two rounds of hybridization with excess of driver DNA and the cDNA pools are then amplified by suppression PCR. Since this method is suppression PCR-based it is sensitive as well as efficient for identifying genes of interest even from minute amounts of starting

material. Furthermore it will to some degree preferably amplify rare transcripts that will lead to normalization of amplified differentially expressed cDNA.

The primary limitation of subtractive cloning lies in its insufficient suitability to detect transcripts with quantitative differences in the level of expression while transcripts with qualitative differences are detected with high reliability. In addition, each experiment is a pairwise comparison, and since the subtractions are based on a series of sensitive biochemical reactions it is difficult to directly compare a series of RNA samples.

In an attempt to elucidate the molecular mechanisms whereby inactivation of myostatin results in increased skeletal muscle mass and to identify QTL that interact with myostatin, suppressive subtractive hybridization has been used. Numerous genes involved in cell proliferation, transcription and protein synthesis were shown to be differentially expressed between double-muscling and wild-type bovine embryos. Some of the genes reside within the previously identified QTL that interact with myostatin (Reecy *et al.*, 2002).

In a project aiming at the detection of prenatal differentially expressed muscle transcripts for the Piétrain and Duroc breeds at seven key developmental stages (day 14, day 21, day 35, day 49, day 63, day 77 and day 91) in order to obtain genes affecting meat quality, several techniques for expression profiling are applied, including subtractive hybridization, differential display RT-PCR and cDNA microarrays. Lists of several hundred genes with breed-specific, stage-specific and phenotype-associated expression have been established. These will be screened for polymorphism and performance tested commercial slaughter pigs will be genotyped as the functional candidates for validation of association between the candidate genes and technological meat quality traits (Wimmers *et al.*, 2002).

11.4.3 Microarrays

Microarray technology represents a major advance in regard to exploiting the coordinated expression of genes under various endogenous and exogenous conditions. It provides a strategy to monitor the expression of thousands of genes in one experiment allowing investigators to consider addressing some important biological questions that have not been easily addressed with traditional expression-based technologies, such as Northern blots, *in situ* hybridization or RNase protection assays, which examine gene expression changes of only a few genes at a time. Microarray analyses offer static information about gene expression (that is, in which tissue the gene is expressed) and dynamic information (that is, how the expression pattern of one gene relates to those of others) (Duggan *et al.*, 1999).

Two array-based technologies have been developed: oligonucleotide and cDNA arrays. Oligonucleotide-based microarrays can be manufactured by directly synthesizing oligonucleotides on the glass surfaces or by deposition of pre-synthesized oligonucleotides on to the chip (Hacia, 1999; Lipshutz *et al.*, 1999). Oligonucleotide-based microarray technology can not only detect expression profiles but is also used for analysing sequence variations in (genomic) DNA (D.G. Wang *et al.*, 1998). The two main limitations of this technology are its high cost and the necessity of comprehensive gene sequence information. Currently sufficient sequence information is available from humans and some laboratory rodent species but within the next

several years plenty of sequence information will be available for farm animal species as well. cDNA microarrays are produced by spotting the PCR products representing specific genes on a glass slide or nylon membrane (Eisen and Brown, 1999). The cDNA is then individually arrayed on a single matrix. The construction of these kinds of arrays is more laborious due to the need for physical intermediates compared with the oligonucleotide arrays but they do not depend on the complete knowledge of gene sequences and are likely to be more suitable for application in heterologous conditions due to the higher length of the gene representing cDNAs that are spotted. The possibility of cross-species hybridization of porcine target on human microarrays has been demonstrated (Mathialagan *et al.*, 2002; Medhora *et al.*, 2002; Moody *et al.*, 2002). This will widen the applicability of the microarray technology in animal science as at the current status of research there is a lack of commercially available DNA arrays/chips of farm animals.

Microarrays are differentially hybridized using fluorescent labelled (Cy3 and Cy5) cDNA populations representing the transcriptome of the samples of interest. The two labelled cDNA pools, representing all of the RNAs expressed in each cell or tissue sample, are then simultaneously hybridized to the microarray under stringent conditions. The intensity of the hybridization signals are read by CCD cameras, and the relative expression level of each gene is represented by the intensity of the hybridization signal.

There are currently several attempts to apply microarrays to obtain genes that are differentially expressed in muscle tissue either between different developmental stages/ages or breeds or housing/feeding conditions in pig and cattle. These projects will reveal candidate genes for muscle growth and properties affecting meat quality. Due to the lack of knowledge of gene sequences in farm animals the construction of cDNA microarrays is preferred. The gene-specific cDNAs can be derived directly from databases (GenBank or dbEST) including sequenced cDNAs or ESTs, or cDNAs may be randomly chosen from any library of interest. While the targeted approach of spotting cDNAs derived from databases provides knowledge about the expression of selected candidate loci, the random approach of spotting unsequenced clones from cDNA libraries will allow identification of novel differentially expressed genes.

To study differential gene expression in porcine skeletal muscle, a cDNA macroarray containing 327 cDNAs derived from whole embryo and skeletal muscle was produced. Forty-eight genes were identified as having different expression in two or more of the four muscle tissue targets derived from 75- and 105-day fetal hind limb muscles, and 1- and 7-week postnatal semitendinosus muscles. These results were confirmed by hybridizing a second microarray and RNA blots (Zhao *et al.*, 2003). Other projects are on their way aimed at the identification of candidate genes for muscle growth and meat quality by analysing the expression profile of prenatal muscle tissue. Ernst *et al.* (2002) constructed a normalized porcine skeletal muscle cDNA library from hind limb muscle of pigs at 45 and 90 days gestation, birth, 7 weeks and 1 year of age and have sequenced 782 clones. A cDNA microarray containing 28 clones previously derived from differential display RT-PCR experiments and 740 clones of the normalized library was constructed. Hybridizing this array with targets derived from total RNA from skeletal muscle of pigs at 60 days gestation and 7 weeks of age revealed 55 clones that were overexpressed by at least twofold (41 by at least 2.5-fold) in 60-day fetal skeletal muscle compared with 7-week postnatal

muscle in all four experiments. No clones were identified as being overexpressed in 7-week postnatal muscle. To monitor gene expression throughout skeletal muscle development, muscle tissue from several different gestational and postnatal stages was collected. Focus was on tissue samples of early (29, 35, 43, 49 days), middle (56, 64, 70, 78 days) and late (84, 93, 99, 106 days) gestation, and 160 days of age. Microarrays from these cDNA libraries will allow the study of expression patterns throughout skeletal muscle differentiation and development in swine (Seo and Beaver, 2001).

To monitor the gene expression changes in seven tissues contributing to the regulation of metabolism and growth collected on 28, 56, 90 and 165 days of age in pigs with low and high lean growth rates, pooled RNA from each tissue from the high lean and low lean group was reverse transcribed and hybridized to human microarrays (UniGEM V2; Incyte Genomics, California). Expression profiles of skeletal muscle genes γ -actin, troponin and tropomyosin were found to be upregulated in loin and ham muscles of high lean group at all four time points examined. In total 394 regulated genes were identified in multiple tissues at all four time points examined. The genes identified are candidate genes that are contributing to lean growth and fat deposition (Mathialagan *et al.*, 2002).

A comprehensive study of muscle expression profiles in pigs by microarray technology was published by Bai *et al.* (2003). A cDNA microarray made up of 3500 and 2000 randomly chosen moderately to weakly expressed clones from a 50-day-old fetal longissimus dorsi and a 3-day-old piglet gastrocnemius cDNA library, respectively, was hybridized with transcripts of red (psoas) and white muscle (longissimus dorsi). Seventy genes more highly expressed in psoas and 45 more highly expressed in longissimus were sequenced. These included mitochondrial genes and genes involved in gluconeogenesis, transcription, translation and signal transduction on the one hand and genes coding for sarcomeric structural proteins and enzymes of glycolysis on the other hand as well as novel genes. The loci represent candidate genes that could influence muscle phenotype.

The usefulness of the microarray technology for the generation of new knowledge on the genetic, biochemical and environmental interactions that determine beef quality has been evaluated using a microarray of 9300 cattle cDNAs comprising about 1900 probes for which EST information is known, as well as *c.* 7400 anonymous cDNAs from skeletal muscle and subcutaneous fat. The microarray has been evaluated in hybridizations of tissue-derived RNA from breed comparisons, feeding trials, developmental time courses and on RNA samples from *in vitro* studies in order to survey gene expression changes associated with skeletal muscle and fat development in the bovine (Wang *et al.*, 2003).

A cattle cDNA microarray containing 7653 elements has been used to analyse expression profiles in 17 different cattle tissues sampled from a healthy, normal 1-week-old Jersey calf. Extreme expression ratios for 29 genes with 50-fold or greater and 13 genes with 50-fold less than the reference standard were identified as well as a large number of genes expressed primarily in a single tissue. A comprehensive matrix of all possible pair-wise comparisons for individual genes among tissues was constructed to further identify genes with tissue-specific behaviour and possibly unique function. Cluster analysis revealed groups of genes common to nerve, muscle, immune or digestive tissues (Band *et al.*, 2003).

11.5 Prospective of Functional Genomics in Livestock Species

In addition to map-based reverse genetic approaches (QTL scans) and direct candidate gene association and linkage studies, expression analyses have been shown to be especially useful: (i) to improve the gene maps and therefore the comparative maps; and (ii) to increase the number of (functional, positional) candidate genes and therefore facilitate the identification of genes controlling economically important traits. While there are many advantages of the techniques described here to analyse differential gene expression with a focus on the transcriptome, there are some potential limitations of these technologies. Expression analyses are sensitive to heterogeneity of tissues that generally consist of different proportions of various cell types. So expression profiling requires knowledge of the cell types responsible for changes in profiles produced by *in situ* hybridization (Hampson and Hughes, 2001). Level of mRNA does not always reflect protein concentration and a polymorphism in the coding region might not affect the expression level of a gene, but instead, it might cause a change of amino acid sequences of the protein coded by the gene. Expression data as well as polymorphism data on the level of genomic and copy DNA are not the endpoint of studies towards the elucidation of the control mechanisms that are active on the level of the genome and transcriptome that finally lead to the phenotype of any organism. The final definition of the phenotype depends on the proteome, therefore proteomics is needed as an alternative technology to complement and enhance the effectiveness of cDNA expression profiling technologies (Aardema and MacGregor, 2002).

Proteomics is a complementary technology to expression profiling on the level of the transcriptome for monitoring gene expression at the protein level. The proteome has been defined by Wilkins and colleagues as the complete set of proteins encoded by the genome (Wilkins *et al.*, 1996). Proteins are frequently the functional molecules and, therefore, the most likely to reflect differences in gene expression. Genes may be present, they may be mutated, but they are not necessarily transcribed. Some messengers are transcribed but not translated, and the number of mRNA copies does not necessarily reflect the number of functional protein molecules (Anderson and Seilhamer, 1997). In addition, the analysis of protein product, post-translational modification, subcellular localization, turnover and interaction with other proteins as well as functional aspects can only be approached by proteomics tools (for further discussion on proteomics see Maltin and Plastow, Chapter 13, this volume).

Novel and powerful techniques are now available to analyse the global gene expression patterns. Each of these technologies has its own advantages and limitations, but in combination they should provide a detailed gene expression phenotype at both the transcription and translation levels. Such technology will be changing livestock genomics dramatically and these will have significant impact on animal breeding in the future.

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12 Genome Analysis of QTL for Muscle Tissue Development and Meat Quality

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12.1 Introduction

Modern molecular biology and the science of genomics have opened up new and exciting possibilities for the dissection of complex phenotypic traits. These advances in molecular biology first made it possible to develop comprehensive genetic linkage maps in the pig (e.g. Ellegren *et al.*, 1994; Rohrer *et al.*, 1994, 1996; Archibald *et al.*, 1995). To date, over 4000 genes and anonymous markers have been added to the gene map of the pig. In addition to identifying and mapping genes and markers, animal geneticists have begun to search for the individual genes that affect traits of interest in the pig.

Muscle tissue, meat quality and carcass traits are complex characteristics of considerable importance to the producer, meat scientist and consumer. Measurement of

these traits usually includes objective measurements assessing backfat, intramuscular fat (marbling), loin-eye area, water-holding capacity, light reflectance and pH and subjective measurements for colour, tenderness, juiciness and flavour (see also Hopkins and Taylor, Chapter 17, this volume). For many of these traits the heritabilities are moderate to high (Sellier, 1998). While it is clear that most of these traits are likely to be controlled by many genes, some individual genes may have large effects. Furthermore, selection for lean growth by most breeders may have large effects on meat quality (Loneragan *et al.*, 2001). To find these genes three approaches have been employed. The first has been to find or observe whether 'major' genes are segregating in a population. The second approach is the 'genomic scan' method, which uses specialized crossbred resource families and random genetic markers to scan regions of the genome that are associated with meat quality and carcass traits. The final approach is called the candidate gene approach, which uses genes that are presumed to be associated with physiological or biochemical functions affecting the traits of interest (see Houba and te Pas, Chapter 10, this volume). The purpose of this chapter is to review the progress made in identifying genes and genomic regions affecting muscle tissue, meat quality and carcass traits in the pig.

12.2 Major Genes Identified in Populations

The best-known and well-described gene affecting meat quality in the pig is the *HAL* gene (Christian, 1972). Pigs that are homozygous for the recessive *HAL*ⁿ allele have porcine stress syndrome (PSS) and they are subject to sudden death from stress. In addition, those surviving and those heterozygous for the condition have many meat quality problems including pale, soft and exudative (PSE) meat caused by the rate and/or the extent of post-mortem pH. Unfortunately, the recessive deleterious meat quality allele is also associated with more lean meat. It is this association that has allowed the gene frequency to be increased initially through selection for increased muscularity. What has not been confirmed is whether this association is a direct effect of the *HAL* locus or a closely linked gene. Recent attempts to dissect the varied effects that *HAL* has on meat quality to determine whether they are direct effects and not due to linkage have failed to produce alternative genes responsible for the effects seen. The mutation causing PSS is now known and is caused by the porcine ryanodine receptor (*RYR1*) that maps to chromosome 6. A DNA test for the defective allele (*HAL* 1843TM) is patented and used widely throughout the world (Fujii *et al.*, 1991). Worldwide the frequency of this defective allele has decreased to nearly zero, though some lines maintain the gene in order to capture the increased lean produced from heterozygous pigs.

The second meat quality major gene found segregating in populations is the so-called 'acid meat gene'. It was first noticed in France that meat from Hampshire pigs often had extremely low pH and had a much lower level of yield of the cured-cooked ham called the 'Paris Ham'. Once also termed the 'Hampshire Effect', the effect was found to be dominant and hypothesized to be a single mutation (Naveau, 1986). It has now been named the Rendement Napole (*RN*) gene. Further analysis has shown that the *RN*⁺ allele increases the amount of glycogen by about 70% in white muscle. The existence of the *RN* locus was confirmed by breeding experiment and was later mapped to chromosome 15 in the pig (Mariani *et al.*, 1996; Milan *et al.*, 1996; Looft

et al., 1996). Anonymous DNA markers genetically linked to the mutation were first used to begin to remove the negative allele. After several years and considerable effort, a consortium led by L. Andersson, D. Milan and C. Loof reported the identification of the causative mutation (Milan *et al.*, 2000). The gene involved is a new member of a gene family coding for one of the regulatory subunits of the AMP-activated protein kinase complex (named *PRKAG3*). Interestingly, the same gene might explain certain forms of diabetes in humans and the consortium is looking at opportunities for their research to benefit human health. This result represented a tremendous effort by this group of laboratories to move from the chromosomal position to the gene itself. The test for the RN^- mutation is being used to remove the defect from primarily Hampshire-based lines and this genetic test represents another important additional tool to be used by pig breeders to improve meat quality. The RN^- allele has also been found to be associated with leaner carcasses (about 1 standard deviation effect without dominance for backfat thickness, 0.5 standard deviation effect with dominance for carcass lean cuts; Le Roy *et al.*, 2000), which might explain its relatively high frequency in Hampshire-based populations. Highly significant interactive effects of *HAL* and *RN* loci on some meat technological quality traits have recently been evidenced by Le Roy *et al.* (2001). Cure-cooked hams from pigs carrying *RN* and *HAL* n alleles had much higher slicing losses when transformed using the 'superior ham' sliced-packed-up process than expected based on the individual effect of each gene. Conversely, the *HAL* n allele significantly reduced the unfavourable effect of RN^- on slice cohesiveness. Interestingly, additional mutations (Ciobanu *et al.*, 2001) within the gene have been discovered and are of importance to the industry (see later section of this chapter).

12.3 Genomic Scanning and Candidate Gene Approaches

12.3.1 QTL mapping programmes in the pig

A significant number of QTL mapping programmes have been developed in the pig over the last 5–8 years; most are associated with growth and performance traits. Those studies dealing with the traits of interest with published results are indexed in Table 12.1. Some other experiments are currently under development in Australia, France (within Large White and Landrace populations), Spain (Iberian × Meishan cross) and presumably several other countries. In most cases, pig QTL resource families were produced by crossing phenotypically divergent founder populations. Statistical analyses were generally performed assuming that different QTL alleles were fixed in founder populations. The QTL identified are consequently those explaining the genetic differences between populations.

Experimental size varies from 200 (Swedish programme) to approximately 1000 individuals (France, Germany, The Netherlands). The traits investigated also widely differ according to the programme. Carcass, muscle tissue and meat composition traits are more difficult to measure than growth traits, and their representation in QTL experiments has been more limited. Furthermore, only some of these experiments represent published whole genome scans. The other results only concern a limited number of chromosomes where QTL had been identified in previous studies. As a consequence, results concerning the most widely studied chromosomes such as

SSCr 1, 2, 4, 6, 7 or X are over-represented. Each experiment uses its own panel of markers and marker distances computed from the experimental data. In order to be able to compare QTL localizations between experiments, the most probable position of each QTL was recomputed on a reference map, published by Rohrer *et al.* (1996), by interpolation from the relative positions of flanking markers on the reference map. It should be kept in mind that these positions are rather imprecise, as they cumulate the low accuracy of QTL localization and an additional inaccuracy due to the estimation of their position on the reference map. Two QTL positions differing by 20 or 30 cM may consequently correspond to the same locus.

12.3.2 Candidate genes in pigs

Initially, the ‘candidate gene approach’ (Rothschild and Soller, 1997) used genes that by their very nature are expected to be associated with certain physiological or biochemical functions. Other candidate genes may be chosen based on the fact that mutations seen in one species, in a particular area of a gene, might exist in the same gene in other species and could have similar effect. The candidate gene approach can also be used as a ‘positional comparative candidate gene approach’ in which QTL regions are first identified and then candidate genes are used to find the underlying genes responsible for the variation of the traits of interest. To date several genes have been investigated by both procedures. Results from these two approaches are combined by trait to facilitate a more coherent discussion.

12.4 QTL and Candidate Gene Results

12.4.1 Backfat thickness

The localization of the QTL detected for backfat thickness is presented in Fig. 12.1. Backfat thickness QTL were detected on all porcine chromosomes except SSCr 16 and 17, with genome-wide significant effects on ten different chromosomes. Very clear results were also obtained for the three same regions of chromosomes 1, 4 and 7 as for growth traits (Andersson *et al.*, 1994; Knott *et al.*, 1998; Rohrer and Keele, 1998a; Walling *et al.*, 1998a,b; Wang *et al.*, 1998; de Koning *et al.*, 1999, 2001a; Pérez-Enciso *et al.*, 2000; Rattink *et al.*, 2000; Rohrer, 2000; Wada *et al.*, 2000; Bidanel *et al.*, 2001; Grindflek *et al.*, 2001; Malek *et al.*, 2001a). Due to the *MC4R* effect on feed intake, the candidate gene approach was used and it was shown that variation in this gene is significantly associated with 5–8% differences in backfat and relates to one QTL for backfat thickness on chromosome 1 (Kim *et al.*, 2000). Other regions with backfat QTL include the region carrying the *IGF2* locus at the end of the short arm of chromosome 2 (Knott *et al.*, 1998; Jeon *et al.*, 1999; Nezer *et al.*, 1999; Rattink *et al.*, 2000; Bidanel *et al.*, 2001; de Koning *et al.*, 2001a) and the central region of chromosome X (Rohrer and Keele, 1998a; Rohrer, 2000; Bidanel *et al.*, 2001). QTL with significant effects on backfat thickness were also obtained in other regions of chromosomes 1 (Malek *et al.*, 2001a) and 2 (Rohrer, 2000).

A number of other significant QTL have been detected. Two of them were detected in different regions of chromosome 6 by Bidanel *et al.* (2001) in a Meishan

Table 12.1. Main QTL mapping programmes in pigs for muscle, meat quality and carcass composition.

Country	Institution	Population ^a	Animals	Markers	Traits ^b	References
Belgium	Liège University	LW × PI		137	GR, CC	Nezer <i>et al.</i> (1999); Nezer <i>et al.</i> (2002)
France	INRA	MS × LW	1103	137	GR, CC, MQ	Bidanel <i>et al.</i> (2000, 2001, 2002); Milan <i>et al.</i> (2002); Quintanilla <i>et al.</i> (2002, 2003)
France	INRA	PI × LW	378	8 (SSCr2)	GR, CC, MQ	Sanchez <i>et al.</i> (2003)
Germany	Hohenheim University	PI × (MS or WB)	979	121	GR, CC	Moser <i>et al.</i> (1998)
Germany	University of Bonn	Du × Miniature pig	438	8 (SSCr 4)	CC	Murani <i>et al.</i> (2002)
Japan		MS × MP	215	318	GR, CC	Wada <i>et al.</i> (2000)
The Netherlands	Wageningen University	MS × LW	800	127–132	GR, CC, MQ	Rattink <i>et al.</i> (2000); de Koning <i>et al.</i> (1999, 2000, 2001); Harlizius <i>et al.</i> (2000); Hirooka <i>et al.</i> (2001)
Norway	Agricultural University of Norway	(DU × LR) × LW	305	11 (SSCr 4); 9 (SSCr 6); 9 (SSCr 7)	MQ	Grindflek <i>et al.</i> (2001)
Scotland	Roslin Institute (RI)	MS × LW	390	9 (SSCr 4)	GR, CC	Walling <i>et al.</i> (1998a,b)
South Korea	National Livestock Research Institute	Korean native × LR	240	80 (SSCr 1 to SSCr 10)	GR, CC, MQ	Kim <i>et al.</i> (2002); Park <i>et al.</i> (2002)
Spain	IRTA, INIA, University of Barcelona	LR × IB	500 369	92 (all autosomes) + 5 (X chromosome)	CC, MQ	Ovilo <i>et al.</i> (2000, 2002b); Pérez-Enciso <i>et al.</i> (2000, 2002); Ovilo <i>et al.</i> (2002a)
Sweden	University of Agricultural Sciences, Uppsala (UAS)	WB × LW	191	117–236	GR, CC	Knott <i>et al.</i> (1998); Andersson-Eklund <i>et al.</i> (1998, 2000); Jeon <i>et al.</i> (1999)
UK, Spain, Sweden	IRTA, UAS, RI, Sygen, COPAGA, Quality Genetics	LW, LR, H, PI, MS, CoL,	4400	25 (SSCr 1, 2, 3, 4, 6, 7, 8, 9, 10, 13)	GR, CC, MQ	Evans <i>et al.</i> (2003)
USA	Iowa State University	CH × YO	294	5 (SSCr 4); 10 (SSCr 7)	GR, CC	Rothschild <i>et al.</i> (1995); Wang <i>et al.</i> (1998)
USA	Iowa State University	BE × YO	500	125	CC, MQ	Malek <i>et al.</i> (2001a,b)
USA	University of Minnesota	MS × LW	298	119	GR, CC	Paszek <i>et al.</i> (1999); Wilkie <i>et al.</i> (1999)
USA	USDA	MS × SL	540	157	GR, CC	Rohrer and Keele (1998a,b); Rohrer <i>et al.</i> (1999, 2001); Rohrer (2000)

^aBE, Berkshire; CH, Chinese breeds; Du, Duroc; H, Hampshire; IB, Iberian breed; CoL, commercial lines; LR, Landrace; LW, Large White; MS, Meishan; MP, miniature pig; PI, Piétrain; SL, synthetic line; WB, wild boar; YO, Yorkshire. ^bGR, growth traits; CC, carcass composition; MQ, meat quality. The authors apologize for any studies missing from this review.

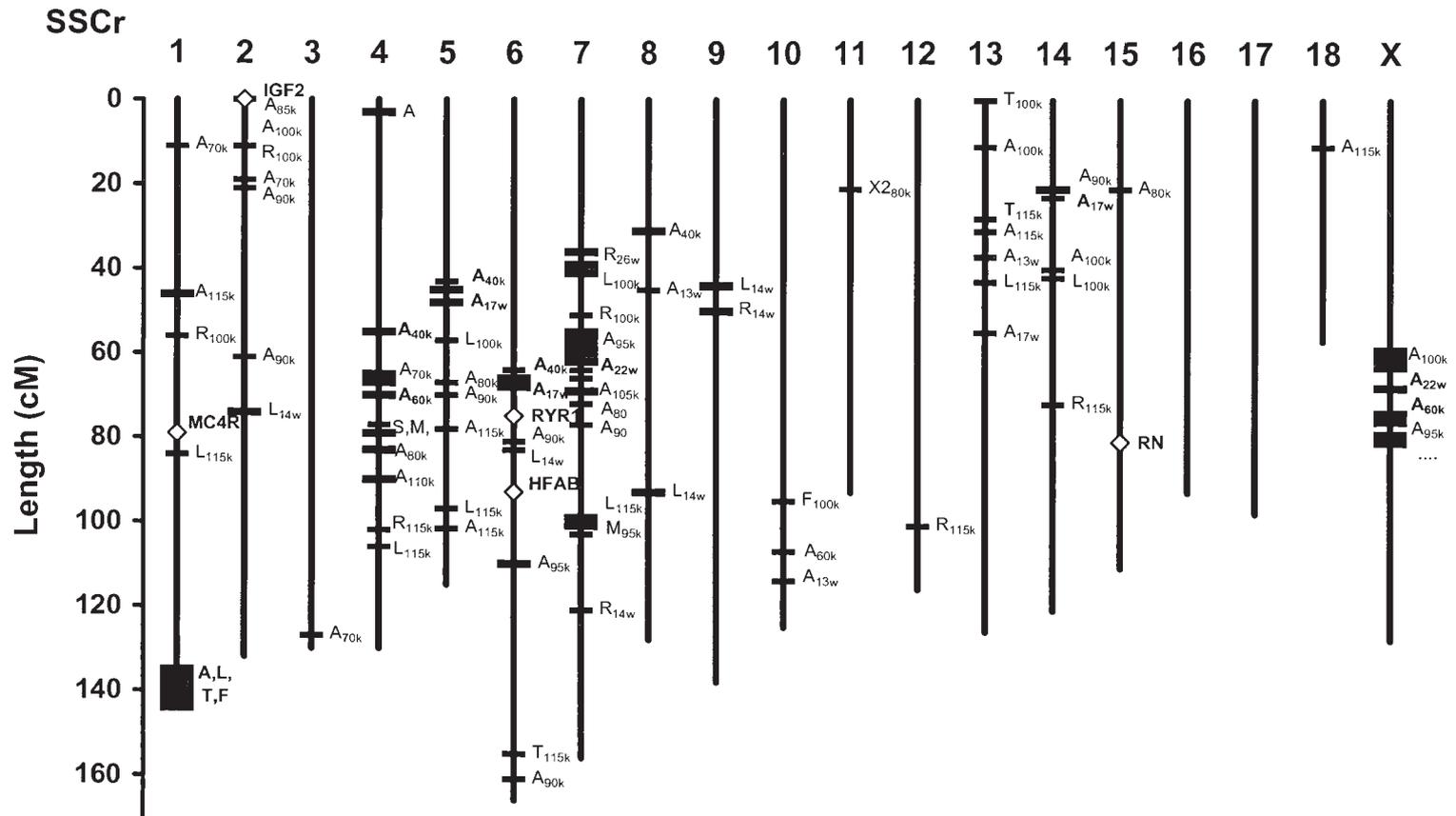


Fig. 12.1. Candidate genes and quantitative trait loci detected for backfat thickness. A (average), L (lumbar), R (last rib), T (tenth rib), S (shoulder), M (mid-back), F (first rib), backfat thickness at xx kg (k) or xx weeks (w) of age; locus names (in bold characters): MC4R, melanocortin-4 receptor locus; IGF2, insulin growth factor 2 locus; RYR1, ryanodine receptor locus; HFAB, heart fatty acid binding protein locus; RN, 'acid meat' locus.

× Large White cross and Ovilo *et al.* (2000) in a Landrace × Iberian F2 population. Several candidate genes were considered including *LEPR*, *HFABP* and *MC5R* (Gerbens *et al.*, 1997; Grindflek *et al.*, 2001; Ovilo *et al.*, 2002b). Similarly, two QTL have been obtained on chromosome 8 by Rohrer (2000) and Bidanel *et al.* (2001). The three other significant QTL are localized on chromosomes 5 (Bidanel *et al.*, 2001), 8 (Bidanel *et al.*, 2001), 9 (Rohrer, 2000) and 14 (Bidanel *et al.*, 2001). One QTL for backfat was also found on chromosome 13, near *PIT1* (Yu *et al.*, 1995).

The chromosome X QTL has the largest effects, explaining up to 50% of the phenotypic variance of backfat thickness measurements based on one experiment. The QTL located on chromosomes 1, 2, 4 and 7 explain 5–23%, 2–20%, 2–17% and 6–33%, respectively, of the phenotypic variance of backfat thickness. The other identified QTL explain less than 5% of backfat thickness variability. The important variations in QTL effects between experiments are in some cases related to the likely differences in the founder populations used. In a joint analysis of chromosome 4 effects in several QTL experiments, Walling *et al.* (2000) clearly showed that the effects of wild boar alleles on backfat thickness were significantly larger than those of Meishan alleles. Similarly, the QTL located in the *IGF-2* region on chromosome 2 explain 1–3%, 10% and 20% of the phenotypic variance backfat thickness in Meishan × Large White, wild boar × Large White and Piétrain × Large White crosses, respectively (Jeon *et al.*, 1999; Nezer *et al.*, 1999; Rattink *et al.*, 2000; Bidanel *et al.*, 2001).

On the whole, QTL allele effects are consistent with breed differences. However, negative, i.e. favourable, effects of Meishan compared with Large White alleles were obtained for the QTL located on chromosome 7 (Rohrer and Keele, 1998a; Wang *et al.*, 1998; de Koning *et al.*, 1999, 2001a; Rohrer, 2000; Bidanel *et al.*, 2001), on chromosome 10 (Rohrer, 2000) and on chromosome 6 (de Koning *et al.*, 1999, 2001a), though a favourable effect of Large White alleles is reported by Rohrer (2000). This QTL is near the location of *H-FABP* (heart fatty acid binding protein), which has been suggested as a candidate gene for fatness (Gerbens *et al.*, 1997). Only one of the six significant QTL detected in Large White × wild boar crossbred pigs showed favourable wild boar alleles (Knott *et al.*, 1998). Conversely, six of the nine QTL suggested in a Berkshire × Yorkshire cross presented unfavourable alleles of the leaner Yorkshire breed (Malek *et al.*, 2001a). Though the genetic determinism of backfat thickness is essentially additive (e.g. Sellier, 1998), significantly favourable dominance effects were evidenced on chromosome 1 (de Koning *et al.*, 1999; Bidanel *et al.*, 2001; Malek *et al.*, 2001a), chromosome 6 (de Koning *et al.*, 1999; Bidanel *et al.*, 2001), chromosome 13 (Bidanel *et al.*, 2001; Malek *et al.*, 2001a) and chromosome 7 in some Meishan × Large White crosses (Walling *et al.*, 1998b; Bidanel *et al.*, 2001). Significant imprinting effects were obtained for the QTL in the *IGF2* region on chromosome 2 (Jeon *et al.*, 1999; Nezer *et al.*, 1999; de Koning *et al.*, 2000).

Of particular importance is whether these results will translate to useful findings in commercial lines. The results of Malek *et al.* (2001a,b) offer some information since QTL in that population have yielded results useful for the industry. A new study using ten commercial pig populations from research herds and breeding companies (Evans *et al.*, 2003) has examined 11 chromosomal regions (1p, 1q, 2, 3, 4, 6, 7, 8, 9, 10 and 13). Of these 11 chromosomal regions, three were considered control and eight as regions in which previous QTL had been verified (Evans *et al.*, 2003). Results confirmed a number of the fat QTL on chromosomes 1, 3, 4, 6 and 13 in some but not all of the commercial populations.

12.4.2 Carcass composition

The localization of QTL affecting carcass composition is shown in Fig. 12.2. Marker-trait associations were detected on all chromosomes except SSCr 10, 17 and 18. The halothane sensitivity allele at the *RYR1* locus and the *RN* allele at the *RN* locus both have favourable effects on carcass length, on carcass leanness and, for *RYR1*, on dressing percentage (Sellier, 1998; Le Roy *et al.*, 2000). Two other QTL with significant effects on dressing percentage were detected on chromosome 4 in a Berkshire \times Yorkshire cross (Malek *et al.*, 2001a) and chromosome 7 in a Meishan \times Large White (Rohrer and Keele, 1998b). Other QTL have also been suggested by Rohrer and Keele (1998b) on chromosome 3, by Malek *et al.* (2001a) on chromosomes 7, 8, 13 and 14 and by Milan *et al.* (2002) in another region of chromosome 4. The QTL explain in all cases a relatively limited fraction (less than 6%) of the phenotypic variance. The effects of Large White alleles were always favourable compared with Meishan alleles but, except for the chromosome 8 QTL, were unfavourable with regard to Berkshire alleles. Significant dominance (chromosomes 7 and 8) and even overdominance (chromosomes 13 and 14) effects were evidenced in the Berkshire \times Yorkshire cross (Malek *et al.*, 2001a), but not in Meishan \times Large White crosses.

Five QTL located on chromosomes 4, 7, 8 and X had significant effects on carcass length. The chromosome 8 QTL was detected in a Large White \times wild boar F2 population (Knott *et al.*, 1998). Two QTL were detected on chromosome 7: the first one in the swine leucocyte antigen (SLA) region by Rohrer and Keele (1998b) in a Meishan \times Large White cross and the second one in the middle on the long arm of the chromosome by Nezer *et al.* (2002) in a Piétrain \times Large White cross. The chromosome 4 QTL was detected in both Meishan \times Large White and Duroc \times Miniature pig crosses (Rohrer and Keele, 1998b; Murani *et al.*, 2002). Their most likely position is in all cases close or similar to those of growth, backfat thickness or carcass composition QTL detected on the same chromosomes. Allele effects are essentially additive and, with the exception of the two chromosome 7 QTL, which explain more than 15% of carcass length variability and present positive effects of Meishan and Piétrain alleles, respectively, the others have moderate effects (3–6% of the phenotypic variance) in agreement with breed differences. In commercial populations Evans *et al.* (2003) also saw QTL on chromosomes 2, 3, 4, 6, 8, 9, 10 and 13.

Two QTL with significant effects on head and/or feet weight were detected on chromosomes 1 and 7 in Meishan \times Large White F2 pigs (Milan *et al.*, 2002). Two other significant QTL affecting another indicator of skeletal development, the bone/lean meat in ham ratio, were obtained in a Large White \times wild boar cross on chromosomes 4 and 8 (Andersson-Eklund *et al.*, 1998). A suggestive QTL affecting feet weight was reported in the same area of chromosome 8 by Milan *et al.* (2002). The chromosome 7 QTL explains between 40 and 50% of the phenotypic variance of head and feet weight. The effects of the other QTL are moderate (5–8% of the phenotypic variance). With the exception of the suggestive QTL on chromosome 8, the effects of Large White alleles are negative compared with Meishan alleles and positive compared with wild boar alleles. Significant dominance effects were obtained for chromosomes 1 and 7, as well as for chromosome 8 in the Large White \times wild boar cross.

Seven different chromosomes carried QTL with significant effects on the rela-

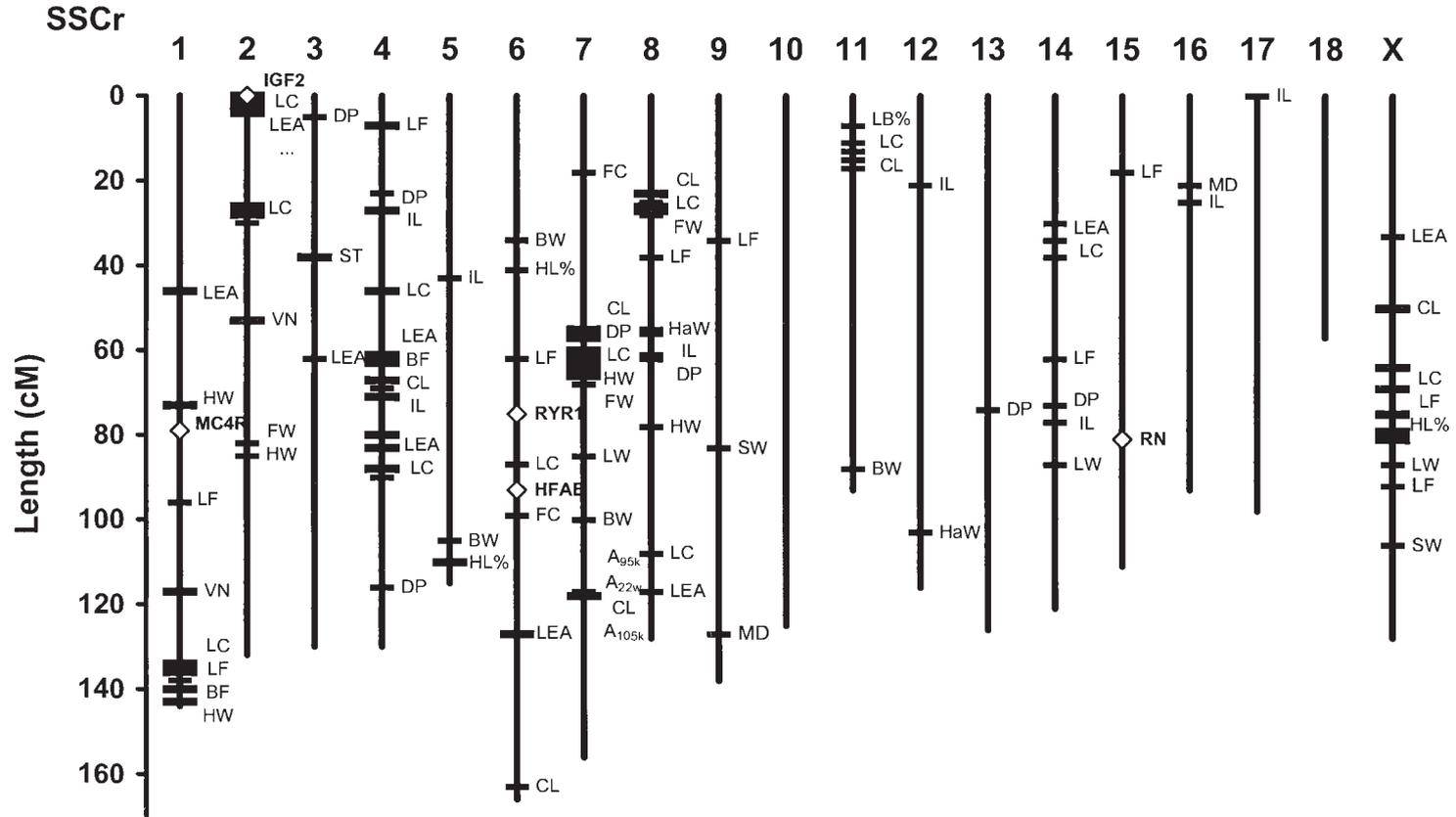


Fig. 12.2. Candidate genes and quantitative trait loci detected for carcass traits. BF, backfat weight; BW, belly weight; CL, carcass length; DP, dressing %; FC, carcass fat content; FW, feet weight; HaW, ham weight; HL%, (ham + loin) %; HW, head weight; IL, length of small intestine; LC, carcass lean content; LEA, loin-eye area; LF, leaf fat weight or %; LW, loin weight; ST, skin thickness; SW, shoulder weight; VN, vertebra number; locus names (in bold characters): **MC4R**, melanocortin-4 receptor locus; **IGF2**, insulin growth factor 2 locus; **RYR1**, ryanodine receptor locus; **HFAB**, heart fatty acid binding protein locus; **RN**, 'acid meat' locus.

tive proportions of carcass lean and fat tissues. The chromosomal regions detected are in most cases the same as those containing growth and backfat QTL: two regions of chromosome 1 (Rohrer and Keele, 1998b; Malek *et al.*, 2001a; Milan *et al.*, 2002), the end of the short arm of chromosome 2 near *IGF-2* (Jeon *et al.*, 1999; Nezer *et al.*, 1999; Milan *et al.*, 2002; Sanchez *et al.*, 2003), chromosome 4 (Knott *et al.*, 1998; Pérez-Enciso *et al.*, 2000; Milan *et al.*, 2002; Murani *et al.*, 2002), chromosome 6 (Ovilo *et al.*, 2000), chromosome 7 (Milan *et al.*, 2002) and chromosome X (Rohrer and Keele, 1998b; Milan *et al.*, 2002). Only one QTL affecting ham weight on chromosome 5 (Milan *et al.*, 2002) did not correspond to any backfat QTL. The largest effects were evidenced on chromosome X. The QTL explained more than 40% of the phenotypic variance of carcass lean content and of (loin + ham) percentage in Meishan × Large White crosses, but had a limited effect on fat tissue weight or proportions (Milan *et al.*, 2002). Conversely, the chromosome 7 QTL had larger effects on carcass fat content (22% of the phenotypic variance of (back + leaf) fat percentage) than on lean content (5% of the phenotypic variance of (loin + ham) percentage) (Milan *et al.*, 2002). Large variations in QTL effects were also observed between experiments. The QTL located in the *IGF-2* region on chromosome 2 explained 5–6%, 15–30% and 30% of the phenotypic variance, respectively, of carcass lean proportions in Meishan × Large White, wild boar × Large White and Piétrain × Large White crosses (Jeon *et al.*, 1999; Nezer *et al.*, 1999; Bidanel *et al.*, 2001). The chromosome 4 QTL explained 3–4% of the phenotypic variance of body composition traits in Berkshire × Yorkshire and Meishan × Large White crosses (Malek *et al.*, 2001a; Milan *et al.*, 2002) vs. 12–13% in the wild boar × Large White and Iberian × Landrace crosses (Knott *et al.*, 1998; Pérez-Enciso *et al.*, 2000). The chromosome 6 QTL also had large effects (21% of lean-eye area variance) in the Iberian × Landrace cross (Ovilo *et al.*, 2000). The QTL located on chromosomes 1 and 5 have in all cases moderate effects (less than 10% of the phenotypic variance).

The effects of QTL alleles are generally consistent with breed differences. The only exceptions concern the SLA region on chromosome 7, where Meishan alleles have favourable dominant effects on Large White alleles (Milan *et al.*, 2002), and one of the chromosome 1 QTL, which exhibits favourable and dominant Berkshire alleles compared with Large White alleles (Malek *et al.*, 2001a). Significant dominance effects were also reported for the QTL located at the end of the long arm of chromosome 1 (Milan *et al.*, 2002). The other QTL have essentially additive effects. Significant imprinting effects were reported for the QTL located in the *IGF-2* region on chromosome 2 (Jeon *et al.*, 1999; Nezer *et al.*, 1999).

12.4.3 Muscle tissue and meat quality

Chromosomal regions with significant effects on meat quality traits were detected on chromosomes 1, 5, 6, 7, 8, 12, 15, 17 and X (Fig. 12.3). Significant effects on intramuscular fat content or marbling were detected on chromosome 1 (near *MC4R*) in a Berkshire × Yorkshire cross (Malek *et al.*, 2001b), on chromosome 6 (near *H-FABP*) in a Landrace × Iberian cross (Ovilo *et al.*, 2000, 2002a,b), in a Landrace × Korean native cross (Kim *et al.*, 2002) and in a commercial population (Grindflek *et al.*, 2001), on chromosome 7 in Meishan × Large White pigs (Bidanel *et al.*, 2002) and on chromosome X in Meishan × White (Harlizius *et al.*, 2000) and Landrace × Iberian

(Pérez-Enciso *et al.*, 2002) crosses. Two QTL affecting this same trait were also obtained on chromosome 6 by de Koning *et al.* (2001b), but at more than 40 cM of the previously reported QTL. The chromosome 6 and 7 QTL explain 14–18% of the phenotypic variance. Large White or Landrace alleles have unfavourable additive effects compared with Iberian, Korean native or Meishan alleles. Conversely, Yorkshire alleles have favourable effects compared with Berkshire alleles for the chromosome 1 QTL, but this QTL only explains 3–4% of the phenotypic variance of intramuscular fat content or marbling.

Two QTL located on chromosomes 5 and 15 have significant effects on meat ultimate pH in Berkshire × Yorkshire F2 pigs (Malek *et al.*, 2001b). The QTL located at the end of the long arm of chromosome 5 explains approximately 5% of the phenotypic variance and presents favourable dominant Large White alleles. The same chromosomal region also has suggestive effects on meat colour. The chromosome 15 QTL explains 4–6% of ultimate pH variance and presents favourable, but partly recessive, Berkshire alleles. This QTL, which also affects muscle glycolytic potential, is localized in the same region as the *RN* locus. The *RN* mutation evidenced by Milan *et al.* (2000) was not present in the population studied. The observed effect is due to additional mutations inside the *RN* locus (Ciobanu *et al.*, 2001). Further study has demonstrated that the three mutations when combined into haplotypes produce differences in pH that may be as high as 0.1 pH units in all breeds except Berkshires in which the differences may exceed 0.2 units. Unlike the *RN* mutation, which is essentially only in Hampshires, these three new mutations are in all breeds and this makes them extremely important economically. Only one QTL for 24 h post-mortem pH was observed on chromosome 3 in the Iberian × Landrace (Ovilo *et al.*, 2002a). Suggestive QTL on ultimate pH were also reported in the same region of chromosome 6 by Malek *et al.* (2001b) in Berkshire × Yorkshire and Park *et al.* (2002) in Korean native × Landrace crosses. This region also has a suggestive effect on drip loss in Meishan × White pigs (de Koning *et al.*, 2001b). Confirmation of some of these QTL was demonstrated in commercial populations (Evans *et al.*, 2003). Conductivity and pH at either 45 min or 24 h was seen on chromosomes 2, 3, 4, 6, 7, 9, 10 and 13 and chromosomes 1, 2, 3, 4, 6, 7, 9, 10 and 13 for the two sets of traits, respectively.

Significant marker–trait associations were detected for meat colour on chromosome 12, 15 and 17 in Berkshire × Yorkshire pigs (Malek *et al.*, 2001b). The chromosome 15 QTL effects are due to the additional mutations found in the *RN* locus (Ciobanu *et al.*, 2001). The chromosome 12 QTL explained about 10% of the variance of a subjective colour score, with favourable Large White alleles, but does not seem to affect reflectance measurements. The chromosome 17 QTL affects both colour score and reflectance measurements. Berkshire alleles are favourable compared with Large White alleles and explain approximately 4% of the phenotypic variance of both traits. Genome-wide significant Minolta reflectance (colour) QTL were observed in Iberian × Landrace pigs on chromosomes 4 and 7 (Ovilo *et al.*, 2002a). The same two chromosomal regions also significantly affected muscle pigment (haematin) content. The QTL explained 11% and 4%, respectively, of the variance of Minolta reflectance, and 3% of the variance of haematin content, with positive effects of Iberian alleles on meat colour and pigment content.

Conversely, no genome-wide significant QTL have so far been detected for water-holding capacity or drip or cooking loss. Some suggestive QTL were reported

for drip loss on chromosomes 1, 2 and 11 by Malek *et al.* (2001b) and on chromosomes 4, 6, 14 and 18 by de Koning *et al.* (2001b), for water-holding capacity on chromosomes 2 and 13 (Malek *et al.*, 2001b) and for cooking loss on chromosomes 6 (Park *et al.*, 2002), 7 and 18 (de Koning *et al.*, 2001b), but results are not consistent between traits and across experiments.

Two QTL, with significant effects on the number of muscle fibres of the semi-membranosus muscle, were detected on chromosomes 7 and 8 by Bidanel *et al.* (unpublished results). They explain, respectively, 9 and 6% of the trait variance. Meishan alleles had a positive, but recessive, effect for the chromosome 7 QTL, and a negative and largely additive effect for the chromosome 8 QTL. This latter QTL also affects the relative surface of type I fibres, with favourable dominant effects of Large White over Meishan alleles. A single experiment (Malek *et al.*, 2001b) has carried out a genome scan for meat sensory quality traits, including sensory panel scores. Only suggestive QTL were obtained but they correlate well with more objective measures like pH or instron measures of tenderness. Some of them are close to significance, such as the QTL located at the end of the long arm of chromosome 2, which affects also tenderness and chewiness scores. In addition, a small but distinct QTL for tenderness was detected in the middle of chromosome 2 by Malek *et al.* (2001b). Further investigation revealed that calpastatin (*CAST*), which is a specific inhibitor of calpains, a Ca^{2+} -activated protease family and considered to be the major cause of initiation of myofibrillar protein degradation in living muscle, mapped under the QTL (see Hopkins and Taylor, Chapter 17, this volume). Extensive analysis of the *CAST* gene revealed several polymorphisms that altered the protein, and these had a large effect on tenderness (see Table 12.2). Further analysis in commercial lines has revealed significant differences in firmness (correlated to tenderness) measured in commercial facilities.

12.4.4 Fat quality

Similarly, the only published whole-genome scan for fat quality traits concerns fat androstenone level (Bidanel *et al.*, 2000; Quintanilla *et al.*, 2003). Six genome-wide significant QTL were detected in Meishan \times Large White F2 males on chromosomes

Table 12.2. Association results between genotypes of *CAST Hpy1881* and meat quality traits in BxY F2 animals.*[†]

Traits	Genotype			P
	11	12	22	
Firmness	3.21 ^{e,c}	3.44 ^f	3.43 ^d	0.001
Juiciness	6.23 ^a	6.05	5.76 ^b	0.05
Tenderness	8.01 ^a	7.74 ^b	7.75	0.11
Chew score	2.32	2.51	2.54	0.11
Instron force (kg)	4.39 ^a	4.45 ^a	4.63 ^b	0.05

* $n = 136$ (11), 228–233 (12) and 129–130 (22).

[†] Significant differences: a–b, $P < 0.05$; c–d, $P < 0.005$; e–f, $P < 0.0005$.

3, 4, 7, 14 and at the end of the long arm of chromosomes 6 and 9. The largest effects have been obtained on chromosomes 7, 4, 14 and 3 (respectively, 11–15, 7–13, 7–12 and 7–11% of the phenotypic variance). Meishan alleles are associated with higher fat androstenone levels, except on chromosome 7, where Meishan alleles are associated with low androstenone levels, and on chromosome 6, where effects differ according to half-sib families, suggesting segregation within the Meishan breed in addition to segregation of effects between breeds. Meishan alleles are recessive with respect to Large White alleles for chromosomes 4, 5 and 14 QTL, partly dominant for the chromosome 3 QTL and dominant over Large White alleles on chromosome 7.

Several fat quality traits have also been considered in the Landrace \times Iberian experiment carried out in Spain. A QTL affecting fatty acid composition has been reported by Pérez-Enciso *et al.* (2000) for chromosome 4, and both *FAT1* and *H-FABP* genes are located on this chromosome. The QTL has a highly significant effect and explains 25% of the phenotypic variance of linoleic acid percentage in subcutaneous adipose tissue. Iberian alleles are additive and associated with lower proportions of linoleic acid, in agreement with breed differences. A close to significance effect was also reported for the proportion of fatty acids with double bonds (Pérez-Enciso *et al.*, 2000). A QTL affecting the percentage of heptadecanoic acid has been reported in the same chromosomal region in a commercial population (Grindflek *et al.*, 2001).

12.5 Future Research Approaches

New technical developments continue to provide novel tools that may yield exciting results. In particular, sequencing efforts in all species have now allowed the identification of tens to thousands of individual genes that may be responsible for the traits of interest. These gene projects involve the development of genomic libraries from specialized tissues. Using these specialized tissues including muscle tissue from the ham and loin in which specific genes are expected to be expressed, researchers can select expressed sequence tags or ESTs and sequence them. To date (late 2002) several such projects are under way in the pig and over 100,000 ESTs from a variety of tissues have been deposited; it is likely that a million more could be deposited soon because of the large Chinese–Danish EST project. These include identification of ESTs from muscle tissue, reproductive tissue and embryos and from immune response tissue. Public deposit of such partial gene sequences can be found in GenBank (www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html) or in more specialized databases (pigest.genome.iastate.edu/data.html). Many of these ESTs will be mapped so that the comparative map of the pig will advance rapidly allowing faster utilization of information from the human and mouse genomes (see also Wimmers *et al.*, Chapter 11, and Maltin and Plastow, Chapter 13, this volume).

The ESTs can also be used to examine gene expression. The genes or ESTs are spotted on microarrays or gene chips in order to study the expression of many genes in parallel. RNA from excellent or poor meat quality animals or other sets of treatments affecting meat quality is then hybridized to the arrays or chips and expression is compared. The genes that show significant differences between treatments or states become guides to candidate genes and pathways that are important for the

trait of interest. Some companies have funded projects with specialized tissues from animals challenged by specific diseases. Such an example is the EC funded project called Quality Pork Genes (www.qualityporkgenes.com). The use of DNA chips or arrays to examine gene expression will offer new glimpses into the complex traits of economic importance in the pig.

The connection between microarray work and that of QTL scans is now being considered (Jansen and Nap, 2001). This approach has been termed 'genetical genomics' and combines arrays, segregation analysis and QTL scan information to identify candidates and crucial steps in biochemical and physiological pathways. Such methods are likely to offer better explanations for understanding genetic control of muscle growth and post-mortem differences in muscle quality. The initial success of QTL scans will also be followed by the development of densely covered single nucleotide polymorphism (SNP) maps. This approach is well advanced in human research to find genes associated with disease and novel methods to aid in discovery and validation of SNPs are being suggested. SNP maps are beginning to be developed in the pig (Fahrenkrug *et al.*, 2002). Efforts to obtain these SNP maps have been proposed by companies like Celera and competitors and an international effort between Denmark and China to sequence the pig genome is well under way. Other similar efforts are being discussed in part in the USA. These and other yet to be developed methods offer great promise for the future of swine improvement as long as cost is in line with the value that can be generated.

12.6 Practical Applications in the Pig Industry

Information at DNA level can help producers and pig breeders to select against specific major mutations such as the negative *HAL* allele or *RN* allele. DNA information can also be used to assist in the selection of quantitative traits including those that can be selected by traditional means (e.g. using *MC4R* to increase marbling). Molecular information can increase the accuracy of selection, allow for selection for sex-limited traits or imprinted genes and allow for selection for traits like meat quality. Such opportunities using DNA technology can therefore increase the selection response in the population. The size of the extra response obtained through marker-assisted selection (MAS) schemes has been considered by many workers. Researchers have consistently shown that there is a short-term benefit in using MAS, but some researchers also show that in some cases this can lead to a long-term penalty. However, this is over a relatively long time frame. Importantly, these responses can be sustained if new markers are continually identified. For example, new markers can be added to the selection index as old markers begin to reach fixation.

In the meantime we anticipate that significant progress will be made by utilizing candidate genes and searching for population-wide linkage disequilibrium, using tools such as random markers and building up haplotype blocks covering interesting genomic areas. Overall these approaches have led to a number of genes and markers being used in the swine industry as shown in Table 12.3. These and others will be utilized at all levels of the pork chain to improve carcass composition, meat quality and cooking properties. Such developments will help producers and packers but ultimately benefit the consumer most of all.

Table 12.3. Molecular genetic tests used by the swine industry.

HAL	Meat quality – non-exclusive use
KIT	White colour – exclusive use (PIC)
MC1R	Red/black colour – exclusive use (PIC)
MC4R	Growth and fatness – exclusive use (PIC)
RN ⁻	Meat quality – non-exclusive/exclusive tests (several companies)
PRKAG3	Meat quality – exclusive use (PIC)
AFABP, HFABP	Intramuscular fat – non-exclusive use (IPG)
CAST	Tenderness – exclusive use (PIC)
IGF2	Carcass composition – exclusive use (Seghers)
Trade secret tests	Several traits – many companies

A list of genetics companies providing routine genotyping in livestock can be seen at: www.genome.iastate.edu/community/genetest.html

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13 Functional Genomics and Proteomics in Relation to Muscle Tissue

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13.1 Introduction

Many of the major livestock producing organizations are becoming increasingly interested in the application of new tools for the investigation and identification of the molecular basis of economically important traits. For the red meat industry, a major objective is to focus on breeding and/or selecting animals that will produce meat of high and consistent eating quality. Genomic work in livestock began more than 10 years ago and has been concentrating on the identification of quantitative trait loci (QTL) and candidate genes and their potential application for selective breeding through QTL and gene mapping, cloning and sequencing and association studies (Rothschild and Plastow, 1999; Haley and Visscher, 2000; Harlizius and van der Lende, 2001; Andersson, 2001; Bidanel and Rothschild, 2002; Garnier *et al.*, 2003). Although these approaches have been successful in identifying a number of muscle genes which have relevance to the breeding of livestock for meat production (Table 13.1), they are time consuming, expensive and a number of studies have failed to identify genes with utility. This chapter reviews some of the recent developments

Table 13.1. DNA markers for meat quality. Candidate genes and their potential application for selective breeding through QTL and gene mapping, cloning and sequencing and association studies. These approaches have been successful in identifying a number of muscle genes with relevance to the breeding of livestock for meat production.

Animal	Gene	Trait	Reference
Beef cattle	<i>CAST</i>	Tenderness	Barendse (2002b)
	<i>Myostatin</i>	Yield and eating quality	Grobet <i>et al.</i> (1997)
	<i>Thyroglobulin</i>	Marbling	Barendse (1997, 2002a)
Pigs	<i>CAST</i>	Tenderness	Ciobanu <i>et al.</i> (2002)
	<i>CRC1</i>	PSE (halothane sensitivity)	Fujii <i>et al.</i> (1991)
	<i>hFABP</i>	Intramuscular fat content	Gerbens <i>et al.</i> (1999)
	<i>cKIT</i>	Appearance (reduced process cost)	Marklund <i>et al.</i> (1998)
	<i>MC4R</i>	Lean content	Kim <i>et al.</i> (2000)
	<i>Myogenin</i>	Meat yield	te Pas <i>et al.</i> (1999)
	<i>PRKAG3</i>	pH, colour, drip loss, yield of cooked ham	Milan <i>et al.</i> (2000) Ciobanu <i>et al.</i> (2001)
Sheep	<i>Callipyge</i>	Yield and tenderness	Freking <i>et al.</i> (2002)

in functional genomics and proteomics technologies and focuses on their potential use to understand the genes (genomics) or gene products/proteins (proteomics) that regulate the development, growth and performance of muscle tissue as well as the potential implications for breeding for meat quality.

In order to explore the potential application of these technologies to muscle tissue and livestock breeding it is important to consider the key processes in the development and growth of muscle. The development and growth of muscle is a huge topic in its own right, but in the current context of muscle production in livestock four key areas will be described very briefly: the specification of the myogenic lineage; the control of proliferation; fibre type setting and maintenance; and the regulation of hypertrophy.

13.2 Muscle Development and Growth

13.2.1 The specification of the myogenic lineage

In vertebrates, it is generally agreed that skeletal muscles (except those in the head) are derived from mesodermal precursor cells in the somites, although some studies indicate that myogenic specification could occur before somitogenesis (Devoto *et al.*, 1996; George-Weinstein *et al.*, 1996; Maxfield *et al.*, 1998a,b). The dorsal cells in the somite form the dermomyotome, a sheet of cells which gives rise to the progenitor lineages for all of the body musculature. Cells at the dorsal medial lip of the dermomyotome give rise to epaxial progenitors that form the deep back muscles (Denetclaw *et al.*, 2001), while cells in the lateral aspect of the dermomyotome are the progenitors of both hypaxial muscles and migratory cells, which form limb muscles. Gene expression studies using *in situ* hybridization techniques have revealed that a number of genes are expressed in the somite and act to regulate both myogenic specification and migration. Particular attention has focused on the myogenic regulatory

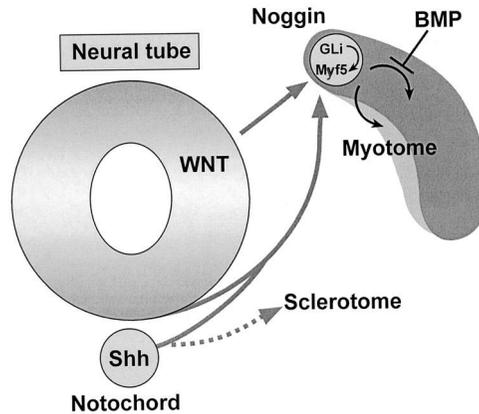


Fig. 13.1. A schematic illustration of muscle development adopted from Gustafsson *et al.*, 2002. Evidence to date suggests that Wnt, expressed in the dorsal neural tube, regulates the initiation of Shh signalling from the ventral notochord and the floor plate and thereby coordinates *Myf 5* activation in the epaxial progenitors with rostrocaudal somitogenesis. During somitogenesis *Gli2* and *Gli3* (the transcription factor effectors of Shh signalling) are activated by Wnt signals (Borycki *et al.*, 2000). Shh activates *Gli1* as well as controlling the localization of *Gli2* and *Gli3* to the epaxial progenitors (Borycki *et al.*, 2000) where *Myf5* is activated. Long-distance Shh signalling (Zeng *et al.*, 2001) then directly controls *Myf5* activation in the epaxial progenitors through positive *Gli* regulation of the epaxial specific *Myf5* enhancer (Gustafsson *et al.*, 2002). The localization of *Myf5* and *MyoD* in the dermomyotome is controlled by *Noggin*. The expression of *Wnt1* in the dorsal neural tube induces *Noggin* in the dorsal lip of the dermomyotome (McMahon *et al.*, 1998; Reshef *et al.*, 1998). *Noggin* blocks the action of *BMP4* to antagonize Shh-mediated *Myf5* and *MyoD* activation (Pourquie *et al.*, 1996) possibly via an interaction with Smad proteins (von Bubnoff and Cho, 2001).

factor (MRF) of transcription factors, *MyoD*, *Myf 5*, *myogenin* and *MRF4* (Pownall *et al.*, 2002) (see also Houba and te Pas, Chapter 10, this volume). It appears that *Myf 5* and *MyoD* are activated in all the somite-derived muscle progenitor lineages, and that a complex set of signals (Fig. 13.1) involving at least *Shh*, *Wnts*, *BMP4*, *Noggin* and *Glis* allows precise temporal and spatial regulation of myogenic cell determination and the formation of myogenic precursor cells (MPCs) (Reshef *et al.*, 1998; Tajbakhsh *et al.*, 1998; Borycki *et al.*, 1999, 2000; Wilson-Rawls *et al.*, 1999; Maltin *et al.*, 2001; Pownall *et al.*, 2002; Teboul *et al.*, 2002).

Studies of the expression, localization and function of the proposed components in the developmental cascade have identified a central role for the MRF genes in myogenesis. *Myf 5* and *MyoD* are essential for specification: *Myf 5* is involved in early progenitor specification and cell migration, while *MyoD* is active in the initiation of differentiation and contractile protein gene activation. However, expression of these genes alone is not sufficient to confer myogenic specification, and it is clear that other factors are necessary for the regulation of myogenesis (Pownall *et al.*, 2002). Identification of these and other possibly non-muscle factors that contribute to progenitor specification as well as further elucidation of the roles of known positive and negative signalling networks is an essential step towards an understanding of the regulation of muscle mass and is the first step in determining what will ultimately be the yield of meat from an animal. Parallel gene expression analysis using

microarrays together with proteomic analysis will provide some of this missing information.

13.2.2 The control of proliferation

The extent of proliferation and the timing of the switch to differentiation controls cell number and probably affects fibre number and hence muscle mass in the adult. A key element in the switch may be myogenin as it is a marker of entry into the differentiation programme. An association was found between variation in the *myogenin* locus and meat yield as well as birth weight and growth rate (te Pas *et al.*, 1999). When levels of growth factors/mitogens are high the expression of *Id* (inhibitor of differentiation) is induced. *Id* prevents heterodimerization between the MRF and the E type factors (e.g. E12) and inhibits DNA binding. Dimerization between MRF and E12 leading to transcriptional activation of specific gene programmes occurs only when growth factor levels fall. Cells that co-express myogenin with the cell cycle inhibitor p21 irreversibly withdraw from the cell cycle (Andres and Walsh, 1996). Myostatin (McPherron *et al.*, 1997) may also be an important regulator of proliferation because targeted deletion of *Mstn* affects both cell number and cell size, possibly through actions on p21 and the pro-apoptotic gene *bax* (Zimmers *et al.*, 2002), and results in a significant increase in muscle mass because of changes in both hyperplasia and hypertrophy. Double-muscling in cattle is the result of naturally occurring inactivation of this gene (e.g. Grobet *et al.*, 1997; see also Kambadur *et al.*, Chapter 14, this volume). Recent work, however, suggests that in proliferating cells a myostatin-mediated upregulation of p21 causes cell cycle arrest through hypophosphorylation of Rb (Thomas *et al.*, 2000). Under differentiation conditions p21 is downregulated through a loss of promoter activity (Langley *et al.*, 2002) and myostatin inhibits MyoD activity via the induction of Smad3 phosphorylation and increased Smad3–MyoD association (Langley *et al.*, 2002). These data suggest that the role of myostatin may be complex and myogenic stage dependent.

Smad signal transduction is also important in the BMP (bone morphogenetic protein) inhibition of myogenesis. Recently identified BMP-response elements in *Id* have provided an explanation for the observations that BMPs influence *Id* expression to inhibit myogenesis (Katagiri *et al.*, 2002; Korchynski and ten Dijke, 2002; Lopez-Rovira *et al.*, 2002). The data suggest that BMP2 or BMP6 signalling is transduced via receptor binding, phosphorylation of Smad1 or Smad5, binding with the co-Smad, Smad4, and the formation of a complex which binds to DNA and induces *Id* (Katagiri *et al.*, 2002; Korchynski and ten Dijke, 2002; Lopez-Rovira *et al.*, 2002). Thus the regulation of cell proliferation is complex and may be influenced by factors from tissues other than muscle.

Although it is clearly possible to increase muscle fibre number (and hence muscle mass) (Rehfeldt *et al.*, 1993, 2001) most strategies lead to an increase in secondary fibre numbers (Dwyer *et al.*, 1994; Maxfield *et al.*, 1998a,b), while primary fibres numbers are not increased. Similarly, retrospective examination of changes in fibre type through selective breeding clearly shows a reduction in the proportions of slow-twitch oxidative fibres (derived from primaries) and an increase in fast-twitch fibre types (derived from secondaries) (Oksbjerg *et al.*, 2000; Maltin *et al.*, 2001). This may have resulted in related changes in meat quality. These data might suggest that

the factors that regulate primary and secondary fibre numbers may be different. Recent evidence for the mechanisms controlling fibre type setting and maintenance suggest that this may be the case. The new technologies described in this chapter offer the potential for further dissection of these complex interactions and processes.

13.2.3 Fibre type setting and maintenance

It is widely suggested that the fibre type diversity (see also Reggiani and Mascarello, Chapter 2, this volume) seen in the adult is dependent on the various myogenic populations formed during the muscle fibre formation (Stockdale, 1992; Edom-Vovard *et al.*, 1999). The final stage of myogenesis occurs in two temporally discrete phases. The first is a wave of synchronous myoblast fusion forming the primary fibres (or primaries). This is followed by a second, asynchronous, phase during which secondary fibres (or secondaries) are formed on the surface of each primary fibre at the site of primary myotube innervation (Duxson and Sheard, 1995; Wigmore *et al.*, 1996). Data show that embryonic myoblasts, most numerous during primary myogenesis, only contribute to primary myogenesis and are thus the main progenitors of slow-twitch oxidative fibres in the adult. Fetal and adult myoblasts, occurring later in myogenesis, show greater pluripotency fusing with both primaries and secondaries to form, in the adult, either slow- or fast-twitch fibres (Dunlison *et al.*, 1999).

The signals from innervation appear to play a major role in the setting and maintenance of fibre type as well as hypertrophy (see below). Calcineurin (CaN), a Ca^{2+} /calmodulin-regulated serine/threonine phosphatase, has been implicated in the regulation of myogenic differentiation, fibre type and muscle hypertrophy (Chin *et al.*, 1998; Musaro *et al.*, 1999; Semsarian *et al.*, 1999; Friday *et al.*, 2000; Bodine *et al.*, 2001; Dunn *et al.*, 2001; Kegley *et al.*, 2001; Torgan and Daniels, 2001; Horsley and Pavlath, 2002). Recent evidence suggests that the contraction-dependent expression of the slow myosin heavy chain is mediated by the CaN/NFAT pathway (see below and Houba and te Pas, Chapter 10, this volume) (Torgan and Daniels, 2001), and that this pathway is one of three parallel intracellular signalling pathways that acts non-redundantly to control myogenin expression (Xu *et al.*, 2002a).

13.2.4 The regulation of hypertrophy

In differentiated tissue, nerve-activity-dependent differences in intracellular calcium concentrations appear to differentially activate CaN (Chin *et al.*, 1998; Dunn *et al.*, 2001; Liu *et al.*, 2001). Sustained elevations of intracellular calcium slow motorneuron firing, or IGF-I stimulation of the L-type calcium channel, activates CaN, which dephosphorylates NFATs (nuclear factors of activated T cells) and results in rapid nuclear translocation (Horsley and Pavlath, 2002). In the nucleus, NFATs interact with transcription factors to activate transcription leading to slow fibre gene expression (Chin *et al.*, 1998) and hypertrophy (Musaro *et al.*, 1999). Since both fibre type and muscle mass are implicated in the determination of meat quality and quantity, then understanding the factors that control CaN activity may be central in livestock selection and breeding.

Postnatal hypertrophic increase in muscle mass is mediated through an increase

in fibre size and the incorporation of satellite cell nuclei into the growing fibres (see Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume) to maintain the DNA:protein ratio. The precise factors that control the activation and subsequent incorporation of daughter satellite cell nuclei into the parent fibre are unknown. However, if myostatin regulates proliferation and hypertrophy then it may be speculated that myostatin could be involved in the maintenance of DNA:protein ratios postnatally through effects on p27Kip protein (a protein similar to p21), which appears to regulate satellite cell proliferation (Spangenburg *et al.*, 2002).

Changes in gene transcription entraining increases in muscle gain are associated with changes in protein synthesis and/or protein degradation. In developing muscle, protein synthesis rates appear to be high (consistent with observations *in vitro*), but rates fall with increasing gestational age. Myogenic cell fusion and differentiation is associated with high proteolytic activity, particularly of the calpains, which appear central to the fusion process (Dedieu *et al.*, 2002). Postnatally, rates of both synthesis and degradation appear to fall with increasing age (Fiorotto *et al.*, 2000) and decreasing growth rate. In livestock species it has been suggested that high growth rates attributed to high rates of both synthesis and degradation are desirable because at slaughter upregulated rates of protein degradation may contribute to increased tenderness. In fact for ruminants, where growth rates are within the range of those found in normal agricultural practice, this does not appear to be the case (Lobley *et al.*, 2000), and rates of turnover do not relate to eating quality. Selection of animals on a growth rate basis is therefore perhaps less useful than selection for efficiency and hence best economic production of muscle.

Consequently, there are a number of stages of muscle development where gaining a greater understanding of both the genes and gene products involved, together with greater understanding of their regulation, would be of considerable value in the prediction and selection of genotypes of livestock for muscle growth, development and meat deposition. Recent technological advance in both functional genomics and proteomics may provide the necessary tools to answer some of these questions. This will require analysis at the different stages of development as well as at the time of slaughter. As a caution, however, it should be pointed out that the production of increased muscle mass is not necessarily synonymous with quality, and this should be considered in designing selection strategies for breeding.

13.3 Functional Genomics

The preceding section has introduced the complex nature of muscle development and the processes that need to be considered in relation to manipulating muscle growth for meat production. As the term implies, functional genomic approaches relate to investigations of gene function, and in its broadest definition can be interpreted to encompass the majority of molecular genetic and biological disciplines. Indeed there appear to be almost as many interpretations of the phrase 'functional genomics' as there are scientists working in the field. However, the concept of the 'function' of a gene is difficult because, as is evident from the outline of muscle development above, genes rarely 'function' alone or even have a single function; the influence of a gene usually depends on complex interactions with other genes and

proteins. Since a DNA sequence does not necessarily translate directly to the function of the gene, for the purposes of this chapter, the 'function of the genome' is considered to be that mediated through the transcriptome. The transcriptome reflects the qualitative and quantitative aspects of mRNA levels and hence permits the study of gene expression in different physiological conditions. Since transcriptomic changes are likely to be the major influence on meat quality, for the purposes of this chapter, functional genomics is defined as the study of the transcriptome.

The phenotypic characteristics of muscle depend on differential polygenic expression, the precise nature of which may vary both within individuals and between individuals. Consequentially, while the application of a functional genomics approach is appropriate to elucidate candidate genes as determinants of muscle development and growth, care must be taken in experimental design to take account of the nature and sources of animal- and sampling-based variation. For developmental studies in livestock species sampling can present some challenges since it is quite difficult to obtain samples rapidly, and there can be a number of problems in obtaining samples of only myogenic tissue. In some studies this may not be a specific concern since there are techniques that can be used to produce tissue-specific transcriptomic tools (see below), but where strict muscle–muscle comparisons are necessary for a developmental study then careful dissection techniques or laser capture microdissection techniques (Mikulowska-Mennis *et al.*, 2002; Xu *et al.*, 2002b) may be necessary. In particular, it is important to note that, in addition to the rostrocaudal gradient of developmental progression, within the limb and even within a muscle, developmental progression is evident (Fig. 13.1). Thus consideration must be given to the site of sampling as an important source of variation.

There are several techniques that allow identification of differentially expressed genes between and within animals. These all analyse differences in the transcriptome through methods such as subtractive hybridization, differential display, serial analysis of gene expression (SAGE) and microarray analysis (for review see Moody, 2001). Each of these techniques has problems and merits.

Subtractive hybridization and in particular suppression subtractive hybridization (SSH; Diatchenko *et al.*, 1996), provides for the identification of differentially expressed genes on a wide scale and allows the isolation, sequencing and identification of unknown genes as well as normalizing for mRNA abundance. The drawback with SSH is that it only allows for pair-wise comparison of samples, but this limitation does not prevent pooling of mRNA prior to the reverse-transcription to cDNA. This technique has considerable potential for the investigation of developmentally regulated genes, subtractions being made on a developmental basis for example. SSH libraries also provide a ready source of interesting clones for the production of cDNA microarrays (see below).

In contrast, the techniques broadly known as differential display (Liang and Pardee, 1992; Welsh *et al.*, 1992; Stein and Liang, 2002) allow direct and simultaneous comparison of multiple samples so that relative changes in gene expression between and within individuals can be readily identified (Fig. 13.2). This permits, for example, comparison of gene expression between muscles of different developmental stages or from highly muscled or less muscled genotypes and the potential identification of genes, the differential expression of which correlates with development or muscle mass respectively. However, both SSH and differential display have some major disadvantages for a high throughput functional genomics approach. Neither

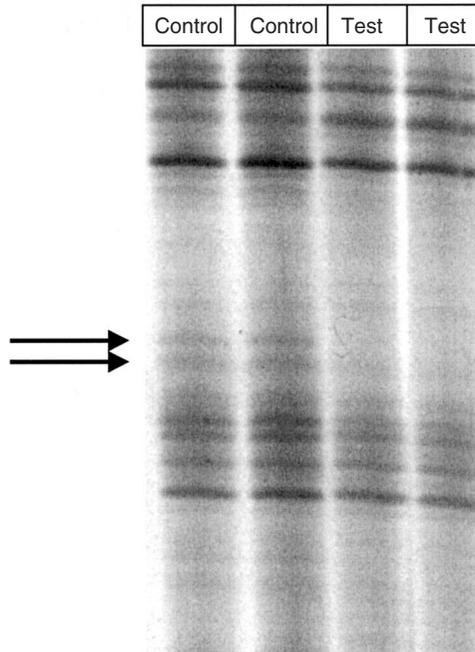


Fig. 13.2. Differential display techniques allow direct and simultaneous comparison of multiple samples so that relative changes in gene expression between and within individuals can be readily identified. The image shows differential expression of genes (arrowed) between two groups of samples.

SSH nor differential display are fully quantitative, additionally differential display has a tendency for a high rate of false positives and PCR-associated errors and finally neither method is particularly rapid; both require additional strategies to confirm differential expression.

As high throughput sequencing has developed, several projects have been set up to develop expressed sequence tag (EST) libraries (see also Wimmers *et al.*, Chapter 11, this volume). These are produced by picking clones from cDNA libraries at random and generating a single sequence of up to 500 bp per clone. Differences in gene expression are based on estimating the number of times a sequence occurs in EST libraries of genes from different sources. Accurate quantitative gene expression data can be obtained directly from ESTs derived from un-normalized libraries (Moody, 2001), since the process of normalization will equalize the abundance of clones from different transcripts (Soares *et al.*, 1994).

EST sequencing programmes in livestock species (Grosse *et al.*, 2000; Shi *et al.*, 2001; Smith *et al.*, 2001; Band *et al.*, 2002) have provided extremely valuable resources for other methods of gene expression analysis. For example, EST sequences provide information on both sequence and annotation for a similar, but more rapid and quantitative, technique known as SAGE (Velculescu *et al.*, 1995; Patino *et al.*, 2002). The concept underpinning SAGE is that short sequence tags can be used to identify a gene transcript, if a tag represents a known location in a gene (Velculescu *et al.*, 1995). Thus a unique SAGE tag will correspond to a unique tran-

script. SAGE tags are derived from mRNA by preparing a short sequence from a known location, which is ligated together with other SAGE tags in a cloning vector, thus creating a composite of all the genes expressed in the original sample. Differential gene expression is then quantified by comparison of the abundance of SAGE tags in various libraries; the identity of genes or EST sequences represented by the SAGE tags is determined by searching databases for ESTs of genes with SAGE tags in the appropriate location (Lash *et al.*, 2000).

The number and extent of livestock EST databases is increasing rapidly, and several interactive websites have been developed to allow viewing and analysis of livestock genes. For example, The Institute for Genomic Research (TIGR) hosts the Cattle Gene Index (BtGI) and the Pig Gene Index (SsGI), which contains information on both ESTs and assemblies. Much of the information for the BtGI has been provided from four normalized cDNA libraries based on pooled RNA from several dissimilar tissues of animals of various developmental stages and physiological states (Smith *et al.*, 2001). The approach of combining different tissues before normalization is believed to be particularly useful in reducing library redundancy, but there may be some loss of rare, low abundance messages. The application of these approaches to muscle development depends on the specific question to be answered, but the possibility of losing rare, low abundance messages that may be central to the complexities of developmental regulation is not trivial.

Microarray-based technologies for evaluation of gene expression (Schena *et al.*, 1995) are increasing in popularity and have several advantages over other techniques. In particular, microarray techniques allow a global assessment of gene expression. It is therefore possible to assess not only the expression status of a particular gene, but also the expression pattern of that gene relative to that of other genes; clearly a useful approach for understanding the polygenic regulation of a complex process such as muscle development (see Section 13.2). A number of reviews discuss the basis of microarray experiments (Duggan *et al.*, 1999; Quackenbush, 2001; Cook and Rosenzweig, 2002; Tefferi *et al.*, 2002; Yang and Speed, 2002), so this chapter will largely consider specific issues related to the study of muscle tissue.

Briefly, the technology is based on three types of microarray: two types of oligonucleotide arrays made either by direct synthesis of oligonucleotides on glass support, or by the printing of pre-made oligonucleotides on to nylon membranes or glass slides and cDNAs made by printing PCR products from library clones on to nylon membranes or glass slides. For muscle studies, as with most studies, the key issue is the selection of the probes for arraying. Since no livestock species has a completely sequenced genome, it is often most useful to prepare a cDNA library from the tissue of interest.

In studies of muscle development the selection of the tissue from which to prepare the cDNA library is a crucial component of the experiment and will again depend on the questions to be answered. In some recent studies of differential expression in cells (Yang *et al.*, 1999; Rho *et al.*, 2002) the cDNA microarray has been generated from cDNA clones produced by SSH and this has provided a reliable and more rapid means to identify differentially expressed genes. One potential advantage of this approach for muscle studies is that the procedure to some extent normalizes mRNA abundance so reducing the dominance of some of the highly abundant transcripts in the array. SAGE has also been used to create an inventory of high-abun-

dance mRNAs in normal adult human vastus lateralis muscle (Welle *et al.*, 1999). The data reveal that mitochondrial transcripts represented ~20% of the polyA RNA, transcripts encoding the myofibrillar proteins were the most abundant nuclear-encoded RNAs and that transcripts for ribosomal proteins and proteins involved in energy metabolism were also very abundant (Welle *et al.*, 1999). Unfortunately, there does not appear to be such a resource covering the changes in transcript abundance throughout myogenesis for livestock species.

One concern for the application of microarray technologies is the potential failure to detect low copy number genes. This may be particularly relevant in developmental studies of muscle where variation in the transcription factors for example, which may occur with low abundance, could have a major impact on developmental programming and outcome. However, the requirements of medical diagnosis to assess gene expression from fine needle aspirate samples or small amounts of tissue have now led to the development of amplification protocols. In addition to requiring only 1 µg of RNA, these protocols have also been shown to enhance the signals of low copy number genes (Hu *et al.*, 2002). This may have potential for the livestock industries, since it is possible that, with appropriate amplification, muscle gene expression information might be obtained from very small samples of embryonic/fetal tissue or from adult muscle via needle biopsy samples *in vivo*. This type of information could be important in evaluating the impact of production and welfare strategies, transport and peri-mortem handling on gene expression and may help provide information on the genetic determinants of meat quality.

Livestock functional genomic approaches are currently compromised by the lack of commercially available microarrays particularly for developmental studies. However, recent evidence suggests that for differentiated tissue at least cross-species microarray hybridization may have potential. The hybridization of pig longissimus dorsi muscle RNA to the GF211 Human GeneFilters (ResGen) nylon microarrays containing 4324 human genes of known function has been evaluated recently (Moody *et al.*, 2002). The data indicate that, for most genes, the results were highly reproducible; only around 6% of the genes gave highly variable results. It was concluded that, provided at least three replicate hybridizations are performed for each experimental treatment, the use of cross-species hybridization may have utility, at least for the adult pig (Moody *et al.*, 2002). The large knowledge base of the human muscle transcriptome (Pietu *et al.*, 1999) combined with potential access to commercial human muscle arrays (Bortoluzzi *et al.*, 1998, 2000) could significantly accelerate gene expression studies for differentiated muscle tissue. However, for developmental studies, at present, no evidence has been found to suggest that commercial alternatives exist or have been evaluated.

Given that the genetic regulation of myogenesis is not fully understood, that there are clear differences between and within individuals, and that there is more variation between microarray slides than within them, the design of the microarray experiment is crucial. Moreover, since it is not usually possible to address all the various comparisons and achieve sufficient replication within one microarray, several microarrays are generally required; thus it is important to design the experiments with this in mind. A recent review (Yang and Speed, 2002) highlights the issues fully. The key factor for a microarray experiment is clearly the scientific question to be answered, but given that, then the main design issues are the mRNA samples which are to be hybridized together on the same slide and how much repli-

cation there is and the use of dye swap experiments (Yang and Speed, 2002). There are a number of obvious designs which could be employed to study the changes in myogenic gene expression during development. Both single factor and multifactorial designs are possible but each requires a different statistical approach for analysis. For the majority of developmental studies, time course studies are useful. In this case a number of options exist (Yang and Speed, 2002), the simplest of which are when hybridizations can be carried out between consecutive time points, or with one time point as a common reference. As microarray technology evolves and more complex experiments are carried out, it is certain that issues of slide design and replication will become increasingly important in enabling robust statistical analysis to be applied to ever more challenging data.

The raw data produced from an array experiment is the hybridization image that must be analysed to obtain information on gene expression levels. Microarray analysis is based on the assumption that the intensity of the hybridization signal for any sequence on the array is proportional to the amount of mRNA in the target. Analysis involves the identification of each spot and measurement of spot intensity compared with background and in relation to a control spot replicated across arrays to allow scaling for comparison of different arrays (Quackenbush, 2001). Gene expression data of course are contextual, only relating to the particular nature of biological sample and the conditions of the specific experiment. Comparison between microarray data sets is therefore difficult and made more so by the lack of established standards for microarray experiments and raw data processing. To address these and other issues a number of resources have been set up. A system termed Minimum Information About a Microarray Experiment (MIAME) (Brazma *et al.*, 2001) has been set up by the Microarray Gene Expression Database (MGED) consortium (Ball *et al.*, 2002). In addition, RESOURCERER is a database that has been developed using EST analysis and gene sequences from TIGR gene index, to allow annotation for commonly used microarray platforms and to permit the genes represented to be compared both within and across species (Tsai *et al.*, 2001). RESOURCERER is freely available on the TIGR website (pga.tigr.org/tigr-scripts/nhgi_scripts/resourcerer.pl); the importance of such a database in standardizing and accelerating muscle gene expression studies for livestock species cannot be overemphasized.

Despite careful design, microarray experiments are often described as having considerable noise or measurement variability, so that the biological variation of interest is often confounded by technical variation. Consequently identification of statistically significant differences in gene expression is dependent on a combination of biological and technical variables including: (i) the type and physiological state of the sample; (ii) the extraction of mRNA and the reverse transcription reaction; (iii) the choice and fidelity of the microarray (hence the genes to be assessed); (iv) the reproducibility, sensitivity and resolution of the microarray; (v) scanning and image processing; and (vi) the statistical treatment of the data (Jenssen *et al.*, 2002; Kothapalli *et al.*, 2002; Wittwer *et al.*, 2002). Many of these sources of variability can be resolved through careful quality control. For example, in muscle studies it is essential that samples are collected quickly post mortem and as RNase free as possible. Contamination and cross-contamination must be avoided during collection and the same anatomical site must be sampled in each animal. In a study of variability in expression profiling in human muscle (Bakay *et al.*, 2002), the greatest source of variability appears to arise from both intra-patient variation (due to heterogeneity of

fibre type) and inter-patient variation (due to polymorphic noise). It would be expected that similar problems would be encountered in the study of both undifferentiated and differentiated muscle samples from livestock. Although these types of variation can be controlled by analysing large numbers of profiles on multiple individuals and multiple samples, it is also possible to normalize some of these variables through pre-profile mixing of samples.

Alternative splicing occurs in a number of genes – between 35 and 59% of human genes (Xu *et al.*, 2002c) – and this suggests an important role in the functionality of the genome. Alternative splicing can show strong tissue and developmental stage specificity (Chen *et al.*, 1999; Xu *et al.*, 2002a). For example, in humans, muscle accounts for at least 4% of the tissue-specific alternative spliced forms, and has 2.4 times more tissue-specific splice forms than average (Xu *et al.*, 2002c). Consequently, probes should not be selected solely from the 3' end of a given gene because this neglects the possible identification of splice variants if the alternative splicing occurs at the 5' region of the gene.

A considerable number of problems associated with inconsistent sequence fidelity of the cDNA printed on the arrays have been reported (Halgren *et al.*, 2001; Taylor *et al.*, 2001). These problems can be overcome by sequence verification for each clone prior to printing and the use of secondary methods such as Northern blotting, PCR and RNase protection assays to validate changes in gene expression. The importance of these checks is clear when it is considered that false positive rates as high as 47% have been reported (Kothapalli *et al.*, 2002).

As with the traditional measures of gene expression, normalization of gene expression signals to 'housekeeping' genes is a difficult area. Traditionally glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been used for many tissues, but for muscle, use of the glycolytic enzyme is not appropriate. Indeed, GAPDH expression levels are clearly affected by the physiological state of the muscle (Cros *et al.*, 1999; Wittwer *et al.*, 2002) and are therefore completely useless as a means to achieve physiological normalization and alternatives must be used (Wittwer *et al.*, 2002). The identification of housekeeping genes is particularly challenging in developing tissue, particularly in early embryos where genomic silencing and the gradual resumption of embryonic transcription lead to a rapid increase in RNA levels at the blastocyst stage. In pre-implantation bovine embryos the quantification of transcript levels for eight putative housekeeping genes using real-time PCR showed that histone H2a levels remained stable (Robert *et al.*, 2002), providing for the first time in bovine embryos a potential housekeeping gene for developmental studies in the early embryo. Progress has also been made through results from studies of the human genome suggesting that housekeeping genes show strong clustering within regions of the genome (Lercher *et al.*, 2002). This finding may be useful in identifying appropriate housekeeping genes for muscle studies. However, it should also be recognized that comparison between genes within microarray experiments will also need internal standards and controls to supplement those chosen for this purpose.

The science underpinning the statistical and analytical treatment of arrays is rapidly evolving in an attempt to extract functional information. For example, work to reduce variability in cDNA microarray experiments has identified systematic non-linear, intensity-dependent sources of variation that are present in both oligonucleotide and cDNA microarray data (Workman *et al.*, 2002). To resolve these issues new non-linear normalization methods have been developed, which are better than

present linear normalization approaches and reduce the variability between replicate arrays (Workman *et al.*, 2002). Having achieved a basis for analysis, the most common current method for the identification of genes and their function is cluster analysis, which is used to assess changes in the relative expression pattern. The assumption behind this approach is that genes with a similar expression profile across a set of conditions may share commonality of function. However, there are some difficulties with this approach since genes, the products of which may have the same function, do not necessarily share a similar transcription pattern. For example, in muscle the protein kinase C family of gene products are enzymes which act to phosphorylate other proteins, but it is clear that the transcription pattern of the individual isoforms may be different in any given situation (Sneddon *et al.*, 2000). A major challenge for microarray analysis will be the identification of gene interactions in situations where genes have dissimilar transcriptional profiles (Zhang, 2002; Zhou *et al.*, 2002).

Nevertheless current cluster analyses are widely used and have produced some interesting results for muscle gene expression. Of particular relevance to questions of muscle growth are those genes that change in response to alterations in growth stimuli (i.e. hypertrophic or atrophic drive) (Table 13.2; Carson *et al.*, 2001; Jagoe *et al.*, 2002; Wittwer *et al.*, 2002). The data in Table 13.2 illustrate two of the problems in the field. First, it is difficult to make a comparison across studies because only in a few cases are expression data available for the same gene, and secondly, different paradigms may give different changes in gene expression while the phenotypic changes appear similar. Given these restrictions, however, the available data do confirm existing beliefs that changes in muscle mass are accompanied by changes in the expression of genes involved in protein turnover, metabolism, neuromuscular transmission and transcription. The key for the livestock industry is, however, the identification of genes and pathways that influence both the mass of muscle and its subsequent eating quality as meat.

In all living systems the genotype is static, defined and heritable, whilst the phenotype is a dynamic, polygenic product of the interaction between the genome and 'environmental' factors. Thus analysis of the transcriptome will only inform about gene expression at the time of sampling; a functional genomics approach cannot inform on the post-translational modifications that occur following transcription. So a functional genomics or transcriptome approach will only provide limited information. This is particularly true for the study of meat quality. The quality of meat is heavily influenced by the way in which peri-mortem events affect both the transcriptome and the phenotype. Specifically, events that alter the post-mortem metabolism and activation of proteolytic enzymes have a significant impact on the subsequent eating quality of the meat. While it is possible that some of these effects may be seen in the transcriptome, the majority of the changes will not affect gene activity, but rather influence the products of the genes – the proteins.

13.4 Proteomics

Proteomics is the study of the proteome, or 'total protein complement of a genome' (Wasinger *et al.*, 1995). In practice this involves investigations of a number of characteristics of proteins including levels of abundance, post-translational modifications

Table 13.2. An outline of some of the significant changes in gene expression associated with changes in muscle mass through hypertrophy or atrophy.

Cell function/gene name	Abbreviation	Hypertrophy	Atrophy	Reference ^a
<i>Metabolism</i>				
Glyceraldehyde 3 phosphate dehydrogenase	GAPDH		↑↑	2
Muscle type (heart) fatty acid binding protein	H-FABP		↓↓	2
Long-chain specific acyl-CoA dehydrogenase	LCAD	↓	–	1,2
P450 IVB2	P4504B2	↑↑		1
Muscle specific beta enolase–Eno3	ENO3	↓↓	↓	1,3
Na ⁺ ,K ⁺ -ATPase alpha 2 subunit	Na,K-ATPase α 2		↑↑	2
Na ⁺ ,K ⁺ -ATPase beta 1 subunit	Na,K-ATPase β 1	↓↓	–	1,2
Calcium ATPase	SERCA2		↑↑	2
<i>Protein turnover</i>				
60S ribosomal protein L21	RPL21			
Cathepsin S	Cat S	↑		1
Cathepsin L	Cat L			
Ubiquitin-conjugating enzyme E2	UBE2B		↑	2
Tissue inhibitor of metalloproteinase 1	TIMP-1	↑		1
Tissue inhibitor of metalloproteinase 2	TIMP-2		↑	2
Kallikrein-like serine protease	RSKG-50	↑↑		1
<i>Transcription</i>				
<i>Myogenin</i>				
MyoG	MyoG	↑↑		1
<i>Jun-B</i>	JUNB	↑↑	↓	1,3
<i>c-Fos</i>		↑		1
<i>Cell Cycle</i>				
p21	P21	↑↑	–	1,2
<i>Growth factors</i>				
Insulin-like growth factor I	IGF-I	↑	–	1,2
Insulin-like growth factor-binding protein 5	IGFBP5		↑,↓	2,3
<i>Miscellaneous</i>				
Myosin-binding protein C	MYBPC2	↓	↓	1,3
Metallothionein		↑		1
<i>Neural</i>				
Nicotinic acetylcholine receptor α -subunit		↑	↓	1,2
Nicotinic acetylcholine receptor δ -subunit		↑	↓	1,2

^a1, Carson *et al.* (2001); 2, Wittwer *et al.* (2002); 3, Jagoe *et al.* (2002).

and protein–protein interactions, which are applied at the level of cells or tissues. This approach is particularly useful for investigations of myogenesis, muscle growth and meat quality where the examination of changes in the proteome may not only provide information about phenotypic differences, but also provide clues about proteolytic cleavage post mortem (Hopkins and Taylor, Chapter 17, this volume).

The technology on which many current proteomic approaches are based is not new, but rather an extension and improvement of techniques that were developed some 25 years ago (O'Farrell, 1975) and have been widely applied to the study of muscle in health and disease (Isfort, 2002). The developments in the field now permit a strict quantitative analytical approach not possible in earlier studies (Gorg *et al.*, 2000). Modern proteomic analysis comprises the separation of the proteins according to their isoelectric point (pI) and molecular weight through the combined use of isoelectric focusing (IEF) and SDS-PAGE, the mass-spectrometric identification of the proteins and data interpretation. Differential protein abundance is determined by comparative analysis of digital two-dimensional electrophoresis (2DE) images using various automated or operator-assisted software programs (Gorg *et al.*, 2000; Godovac-Zimmermann and Brown, 2001; Hamdan *et al.*, 2001; Asirvatham *et al.*, 2002; Brookes *et al.*, 2002).

There are two main challenges with current 2DE techniques. First, there is no standard 2DE system that can deal equally effectively with high and low molecular weights and basic proteins. This problem can result in spots containing overlapping proteins due to poor resolution because of attempts to cover the full pI range for all cell proteins (Godovac-Zimmermann and Brown, 2001). This is a particular problem if low abundance proteins are masked. Second, the total proteome of a cell or tissue is generally not monitored in 2DE. To examine the total proteome, all proteins in the tissue must be quantitatively extracted from the sample, and each protein must be resolved, displayed, quantitated and identified. Thus sample preparation is crucial. General recommendations are that samples should be as fresh as possible, free from contamination and where possible free from high levels of lipid, salt and polysaccharides. Lipids can bind proteins and alter both pI and molecular weight, high concentration of salt can compromise the integrity of the IEF gel and polysaccharides can block pores, or if charged can bind proteins. The problems of variable protease activity should also be considered, as should nucleic acids, which can block gel pores and bind proteins, which will therefore not be fully represented in the gel. In general the proteins are subjected to some degree of subcellular fractionation prior to IEF; usually a soluble fraction is prepared. Sample preparation is particularly important for the analysis of membrane proteins where specific preparative techniques may be necessary to facilitate analysis of highly basic, hydrophobic and low solubility proteins (Chich, 2001; Godovac-Zimmermann and Brown, 2001).

For studies of muscle these requirements present obvious problems for sample preparation. Ideally samples should be taken (and analysed) as quickly as possible and should be free from contamination with other tissue. If post-mortem samples are to be studied then it is essential that they should be derived from animals/muscles that have been subjected to identical peri- and post-mortem handling and conditions. Muscle contains relatively high levels of nucleic acids, glycogen and, in some muscles, lipid; thus preparative protocols should consider strategies such as ultracentrifugation to improve sample preparation. However, for all tissues including muscle, the inability to reliably monitor proteins that occur at low abundance, are hydrophobic, very acidic or basic in nature is a limitation of 2DE. Consequently there are problems in reliably assessing changes in low abundance proteins such as regulatory or signalling molecules. For myogenic proteomics, the diversity and various solubilities of the different proteins in the tissue present a challenge. For differentiated muscle, extraction procedures applied very often report the preparation

of a solubilized fraction (Isfort, 2002; Lametsch *et al.*, 2002) from which unextracted cellular components, high molecular weight protein complexes and insoluble proteins have been removed by centrifugation. This approach may produce some problems for reproducibility and necessitates assessments of both within and between sample coefficients of variation (CVs). For muscle, changes in the 'insoluble' protein pool may be of significance since it is clear that the extracellular matrix proteins play an important role in growth and mechanotransduction, as well as contributing to the toughness of meat. Methodologies to address the 'insoluble' proteins are an active area of investigation (Santoni *et al.*, 1999, 2000; Watarai *et al.*, 2000) as are methods for enhancing the relative abundance of low abundance proteins (Cordwell *et al.*, 2000; Corthals *et al.*, 2000).

For quantitative 2DE, both the staining and the scanning methods must provide a linear measure of protein abundance. Radiolabelling and phosphorimaging give a wide dynamic range. Coomassie G-250 staining shows linearity over a 20-fold range in 1D gels (Neuhoff *et al.*, 1990) and new matrix assisted laser desorption ionization-time of light mass spectrometry (MALDI-TOF) compatible silver staining methods have been developed (Mortz *et al.*, 2001). The use of a high quality laser scanning densitometer can give a linear measure of optical density over a 10,000-fold range (Mahon and Dupree, 2001). The challenges of image analysis for proteomics are well described elsewhere (Voss and Haberl, 2000; Mahon and Dupree, 2001; Asirvatham *et al.*, 2002; Brookes *et al.*, 2002), and it appears that the choice of software may critically affect the reproducibility of the data. Despite the considerable range of available software, there is little independent critical evaluation of the capabilities and weaknesses of the offerings from each manufacturer; there does not appear to be any direct comparison of packages applied to identical images. This is a major problem for the interpretation of published data, which needs to be addressed. In particular, it is obligatory for quantitative protein analysis that the relationship between protein abundance and integrated optical density (IOD) output from the analysis program is linear over a wide dynamic range. This essential feature is clearly present in some software, but possibly not in all. Indeed, it is suggested that software using Gaussian fitting of spots may be non-linear in response, thus raising questions about quantitative conclusions from work using this type of software (Mahon and Dupree, 2001).

For the image analysis of the muscle proteome, there appear to be two related challenges. First, it is likely that samples will be grouped on a phenotypic basis leading to the need to analyse large sets of spot patterns. Secondly, if between-group assessments are to be made, then it is important to be able to make comparative analyses between groups. The grouping of samples and the analysis of large sets is not necessarily a problem provided the software permits the desired size of group to be formed. However, the task of aligning and matching spots is not trivial and requires operator intervention, but if the spot patterns and intensities in the gel set are highly reproducible then matching efficiency can be improved (Voss and Haberl, 2000). This can be facilitated by running batches of gels to minimize gel-to-gel variation. If comparison between groups of gels that have been run at different times is to be made, then control of gel-to-gel variation is essential. Estimates of gel-to-gel relative errors range from 20 to 90%; 'typical' average values are around 20–30% (Mahon and Dupree, 2001). This target is achievable in muscle tissue analyses, provided care is taken at all stages, from the site of sample collection through high quality sample preparation to staining, to maintain strict protocol control

(Anandavijayan and Mathieson, personal communication). In addition, since it is suggested that protein expression levels should differ by at least two standard deviations for significance (Asirvatham *et al.*, 2002), adequate replication is necessary, and analysis in triplicate is suggested (Isfort, 2002).

The addition of mass spectrometry (MS) and database searching to 2DE provides the opportunity of relating protein sequences to the genes that encode them and hence the discovery of new protein/gene functions in a result-driven, hypothesis-independent manner. This strategy is more difficult for livestock species in which the genome is not fully sequenced. However, in a recent study of the proteome of *Bos taurus* serum MS has been used to generate *de novo* sequences that permitted, through the use of similarity searching and EST data, cross-species identification of proteins (Wait *et al.*, 2002). In this type of approach the FASTS and FASTF algorithms that use multiple short peptide sequences to identify homologous sequences in protein or DNA databases (Mackey *et al.*, 2002) proved particularly helpful. Although rapid progress is being made towards sequencing the genome of livestock species, these findings have clear applicability to the study of muscle such as myogenesis, growth and meat quality.

New emergent technologies taking advantage of expertise available in the semiconductor industries are leading to the development of two categories of new tools for proteomic analysis. Array-based tools and microfluidic-based devices for improved sample preparation and sensitivity (Lee, 2001) are, in combination with MS technology, especially the surface-enhanced laser desorption and ionization (SELDI) techniques, beginning to allow the investigation of protein–protein interactions including antibody–antigen binding reactions. For the study of myogenesis these techniques may be of particular value in the assessment of protein–protein interactions such as those taking place during the assembly of sarcomeric structures during development, or for the study of the substrate/protease interactions which must occur as part of the process of protein turnover during growth.

Recent interest in the study of the genetic, structural and functional identification and characterization of proteases has led to the introduction of the term ‘degradomics’ (Lopez-Otin and Overall, 2002). This is an exciting new field for muscle biology and meat science. Traditionally the action of proteases in muscle tends to be considered in a narrow context, despite the fact that proteases act as part of a multiprotease system comprising related and unrelated proteases, their substrates and cleavage products, inhibitors, cell receptors and binding proteins. The considerable potential for developing protease-specific protein chips based on current and emerging proteomics technology will allow the investigation of a wide range of protease–protein interactions. This will aid the understanding of both myogenic fusion and protein degradation as well as contributing to knowledge of the post-mortem proteolytic process.

The final challenge for the application of proteomics in relation to muscle tissue is that abundance profiling of enzymes does not inform about the level of enzyme activity. Thus to advance understanding of the regulation of meat quality, activity profiling will be necessary. Activity profiling is distinguished from current assay-based approaches by its global nature in surveying the activity of the different enzymes in a complex sample (Liu *et al.*, 1999; Greenbaum *et al.*, 2002). Activity profiling is also designed to distinguish between active enzymes and inactive-precursor or inhibitor bound forms (Lopez-Otin and Overall, 2002) so offers considerable

advantage over some of the current assay methods and has clear applicability to rapidly remodelling tissues such as developing muscle.

The application of 2DE and MALDI-TOF MS to pig longissimus dorsi has already led to the identification of 18 peptides that are formed post mortem from proteins involved in the structure or metabolism of muscle. As might be expected, these include the structural proteins actin, myosin heavy chain and troponin T, together with the metabolic proteins glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase and dihydrolipoamide succinyltransferase (Lametsch *et al.*, 2002). An interesting feature of the study was that by taking samples at intervals from slaughter to 48 h post mortem, it was possible to construct post-mortem degradation profiles for each of the proteins (Lametsch *et al.*, 2002). However, the results of the study did not confirm previous reports of degradation of structural proteins such as titin and nebulin (see also Hopkins and Taylor, Chapter 17, this volume). The reasons for this are not clear, but it is possible that cleavage of these proteins produces a large number of low abundance products that are not detected by 2DE. Alternatively, it may be that sample preparation (see above), and in particular the apparent lack of protease inhibitors in the homogenization buffer, has contributed to the observed results.

It is clear therefore that the current proteomic techniques have applicability to meat quality investigations and indeed the newer chip-based technologies offer exciting areas for development. However, since meat is derived from living muscle that is a highly plastic tissue *in vivo*, investigations of the factors that affect muscle growth and metabolism *in vivo* are pertinent. Consequently the development of techniques which allow dynamic measures of changes in the proteome are of interest and importance. Current strategies employ the incorporation of stable isotope-labelled amino acids into proteins (Ong *et al.*, 2002) that are then used to define changes in protein breakdown, for example by examination of mass shifts in tryptic fragments (Pratt *et al.*, 2002). These approaches have so far been applied in cultured cells but their potential for application to muscle development and growth in livestock species *in vivo* is clearly exciting and is now under investigation in a number of different centres.

13.5 The Application of Functional Genomics and Proteomics to Livestock Breeding

One purpose of any livestock breeding programme is to optimize the production of the tissue of major economic importance. For meat production, the focus is then on muscle, with aims being to increase the quantity, efficiency of production and the quality of the tissue as meat. Breeders have already made good progress on the selection for meat quality (see Knap *et al.*, 2002). This is achieved by collecting phenotypic information using sib slaughter schemes as full assessment can only take place after slaughter. For this reason meat quality is one of the best opportunities for marker-assisted selection; once associations between DNA markers and the trait have been identified this information can be routinely incorporated into selection indices. Another advantage is the potential to incorporate this information at a younger age (theoretically at birth or even in embryo selection). Although progress has been made using QTL studies and candidate gene approaches (see Table 13.1), functional genomics and proteomics are clearly useful technologies with which to

investigate the genetic basis of muscle development, growth and meat quality. It is anticipated that such studies will more rapidly increase the understanding of muscle biology and its influence on meat quality providing important information that will be exploited either directly or indirectly in association studies.

As already discussed, the techniques must be applied in a manner that ensures reproducibility and validity of the data. However, there are several additional aspects generic to both these approaches that must be considered. For developmental studies, the importance of comparing like samples with like is obvious but does pose some challenges. For example, it is important to be confident of the age of embryos/fetuses used for tissue recovery, but in pig production many units serve sows over three consecutive days. Thus gestational age may be difficult to ascertain with sufficient confidence for experimental purposes. It is clear from the brief outline of development given above that myogenesis is regulated by a complex mixture of signals. The origin of these signals in adjacent or distant tissues means that accurate and reproducible tissue sampling is important to avoid confusion between signal production and receipt. This will be particularly important for studies of diffusible signals such as Shh or myostatin.

The functional genomics/transcriptomic and proteomic approaches provide the opportunity to investigate global changes in known or unknown gene/gene product expression in muscle and to associate them with known phenotypic characteristics (Fig. 13.3). The major advantages of these techniques over more traditional QTL approaches are the facts that the very many genes/gene products can be studied in one experiment and that global and tissue-specific investigations can be carried out without the need for the generation of a segregating population. As a result, the information flow is more rapid and the timescale for studies is greatly reduced. In addition, because the techniques identify tissue-specific gene expression and relate them to tissue phenotype, the probability of identifying a gene with tissue-specific

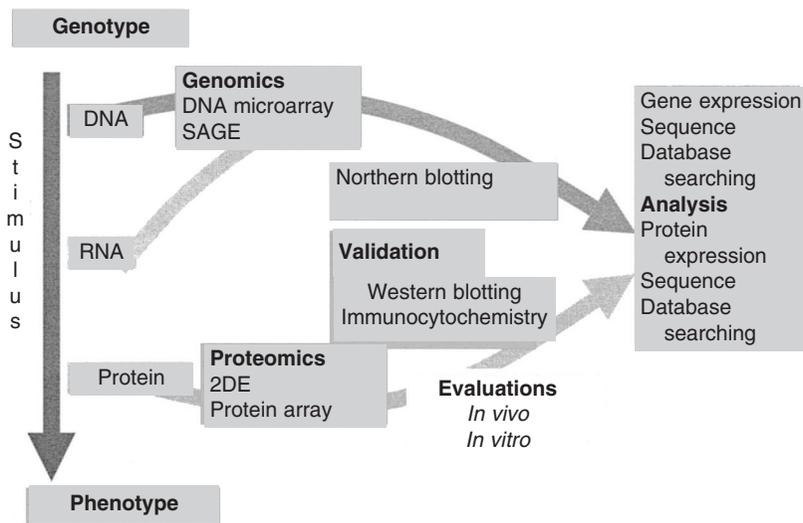


Fig. 13.3. A schematic illustration of the way in which the integration of the ‘-omic’ technologies provide a network of experimental tools for application at various levels to provide data of physiological relevance (adapted from Soulet and Rivest, 2002).

utility is increased. Another considerable benefit of the combined '-omic' approaches is that the functional aspect of the techniques facilitates the elucidation of gene networks and signalling pathways. Thus the potential for both prediction and detection of genes with relevance to livestock production is relatively high. Even so, a substantial effort will be required to establish the correct resources to utilize these tools.

In order to explore the possibilities of these technologies, an ambitious project known as 'QualityPorkGENES' has been developed (www.qualityporkgenes.com; contract QLRT-2000-01888) with funding from the European Commission (EC) Fifth Framework Programme. This project will create a unique database of samples and data relating to growth and meat quality in five different pig genotypes and a total of 500 animals. In addition samples are collected post-slaughter from two muscles (longissimus dorsi and semimembranosus) for detailed functional genomic and proteomic analyses. Several different cDNA libraries will be utilized, including well-characterized libraries from mouse and human (see above), as well as new porcine muscle libraries, in order to create microarray resources for gene expression analysis. The first priority has been to establish the detailed sample collection protocols to ensure that meaningful comparisons can be made. Great attention has been paid to obtaining reproducible samples within 20 min of slaughter such that results from the different analyses, both phenotypic and '-omic' can be related to each other. This is particularly important for the samples used for RNA and proteome analysis, which includes fibre typing as well as 2DE. A similar project, also funded by the EC, 'New predictors for pork quality derived from gene expression profiles of skeletal muscle during prenatal development' (contract QLK5-2000-01363), is seeking to relate differences in muscle developmental expression with differences in meat quality. These projects offer the possibility of rapidly increasing the number of genes that can be studied for their impact on the quality of muscle and muscle-derived products.

Undoubtedly there are two challenges in the application of these newer technologies: the precise experimental question to be answered and how the new information can be employed to benefit the breeding of livestock. For example, if the target of the study is muscle mass, then this raises the question of whether the aim is to increase muscle fibre number or size or both. The double-muscling resulting from disruption of myostatin seen in cattle is a result of both muscle hypertrophy and hyperplasia. If fibre number is targeted then, since it has not yet been possible to increase primary fibre number (and hence slow oxidative fibres), issues of the desirability of increasing secondaries and the potential consequences for subsequent meat quality need to be addressed.

Up until now the application of genomics to improve the quality of livestock muscle has primarily been the diagnosis of gene variants (polymorphisms) associated with variation in meat quality (see Table 13.1). The new approaches described here will generate new candidate genes to be tested for marker-assisted selection. Gene sequences are determined in target animals or groups based on phenotype in order to identify polymorphisms that are then used for association analysis. This approach is illustrated for meat quality by the analysis of the *PRKAG3* gene associated with the *RN* mutation in pigs (Milan *et al.*, 2000; Ciobanu *et al.*, 2001). The Rendement Napole (RN) test predicts cooking yield of ham, and a major dominant gene was identified that resulted in approximately 5% loss in cooked ham processing in the Hampshire breed by segregation analysis. The effect was due to an increase in

muscle glycogen in affected animals that results in a faster rate of acidification of the muscle post mortem leading to increased protein degradation and reduced water-holding capacity in the muscle. The gene was mapped to chromosome 15 and initial DNA tests were developed using linked markers. These tests enabled breeders to begin to eliminate the gene from the Hampshire breed and they were particularly useful for sorting crossbred boars who were carriers or free from the mutation (de Vries *et al.*, 1997). This latter use illustrates another benefit of DNA marker testing, the potential to increase uniformity of product. Eventually an exhaustive search of the chromosomal region was made by sequencing of a BAC contig revealing the existence of a new candidate gene, *PRKAG3*, which codes for a subunit (Gamma 3) of adenosine monophosphate-activated protein kinase (AMPK), which plays a key role in regulating energy homeostasis (Hardie *et al.*, 1998). Milan *et al.* (2000) identified a non-conserved substitution in the *PRKAG3* gene that was a strong candidate for the causative mutation. This polymorphism is used as a diagnostic test (under licence) to remove the *RN⁻* mutation from the Hampshire breed. Interestingly further work identified additional alleles of the *PRKAG3* gene that are associated with variation in measures of meat quality such as pH and colour and ultimately drip loss in pork (Ciobanu *et al.*, 2001). Tests for these polymorphisms, which, unlike *RN⁻*, segregate in most of the common pig breeds, can be included in breeding programmes designed to improve meat quality and to select parents or slaughter pigs to reduce variation in the meat produced by these animals. As indicated above this is an important aspect of marker-assisted selection and new DNA tests for marbling and tenderness in cattle are being utilized in this way (Barendse, 1997, 2002a,b).

The use of functional genomic and proteomic technologies to identify the gene cascades controlling cell commitment, determination and proliferation will also go towards the construction of intervention strategies to achieve the desired increase in muscle mass. It might even be considered that output of these studies could be applied to livestock breeding through transgenesis. For example, the disruption of myostatin in sheep, pigs and poultry to increase the yield of muscle has been proposed (see, for example, articles cited by Turner, 2002, and prolinia.com). Pursel and colleagues (Pursel *et al.*, 1999) succeeded in increasing the expression of IGF1 in muscle using an actin promoter, which resulted in a higher yield with a lack of the negative side effects seen with the original Beltsville Growth Hormone pigs (Pursel *et al.*, 1989). However, there is considerable evidence that this would present some challenges for consumer acceptability especially in Europe (Turner, 2002; Garnier *et al.*, 2003). Even so, the UK Agriculture and Environment Biotechnology Commission noted that there seems to be little outright rejection of applying genetic modification and cloning to animals, although there is concern about the speed of the developments and the potential for mistakes to be made (Anon., 2002). In relation to fibre number regulation, studies based on embryo manipulation (Maxfield *et al.*, 1998a,b; Crosier *et al.*, 2002) suggest that there may be opportunities to manipulate fibre number through the manipulation of the environment of the early embryo (see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume). Although the manipulation of preimplantation embryos can be problematical (Sinclair *et al.*, 2000; McEvoy *et al.*, 2001a), the application of the newer technologies to these studies will allow the identification of the cascade of genes that regulate myogenesis. Through this approach it may be possible to design strategies offering the opportunity to manipulate myogenesis without the use of *in vitro* manipulations or

growth factors (Rehfeldt *et al.*, 1993), but rather employing more ‘natural’ nutritional strategies in the dam to affect fetal myogenic outcome (McEvoy *et al.*, 2001b).

13.6 Conclusion

Genomic technology offers great potential for the improvement of meat quality as well as the development of new products for specific markets. Initial results from the first DNA markers demonstrate the possibilities. However, the application of the newer ‘-omics’ should accelerate this process by increasing our knowledge of biochemical pathways and interactions to reassess existing knowledge of muscle biology. For example, if fibre size is the targeted outcome, then the cascade of genes/gene products that regulate protein turnover and satellite cell inclusion must be identified to allow the development of genetic selection and detection strategies. As indicated in this last section exploitation is potentially wider than just DNA markers. Additional possibilities include the identification of new biochemical or physiological markers as well as new target-based treatments and even the possibility of genetic modification if this should ever be acceptable to consumers. The next few years promise an explosion of information that scientists and technicians will be able to exploit to continue the improvement of meat quality and efficiency of meat production.

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14 Role of Myostatin in Muscle Growth

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14.1 Introduction

Myostatin (GDF-8) is a member of the transforming growth factor beta (TGF- β) superfamily. Loss of functional myostatin in various cattle breeds is known to result in the 'double-muscling' condition with generalized hyperplasia and hypertrophy of most of the muscle and the trait is inherited as 'partially recessive'. Additionally, targeted deletion of the *myostatin* gene in mice also results in the heavy muscling condi-

tion due to hyperplasia and hypertrophy. Hence, myostatin is regarded as a potent negative regulator of muscle growth.

Myostatin protein is predominantly synthesized in skeletal muscle as a 375 amino acid protein, proteolytically processed and mature C-terminal myostatin is secreted. Mature myostatin binds to activin type IIB receptor and mainly transduces the signal via the Smad pathway. Functionally, myostatin appears to regulate both prenatal and postnatal myogenesis. Recent research shows that myostatin regulates the muscle fibre number by controlling the myoblast proliferation and differentiation during early myogenesis, while postnatally myostatin could function by regulating satellite cell activation. Thus myostatin appears to be a target for therapeutic interventions for compensatory muscle growth in farm animals and for old age muscle wasting and cachexia in humans. Here we have reviewed the mechanism of function and the role of myostatin during muscle growth.

14.2 Double-muscling in Cattle

The phenomenon of double-muscling or muscular hypertrophy is an inherited condition occurring in cattle. The condition was first described by Culley (1807) and occurs in several cattle breeds. The 'double-muscled' phenotype results from increased muscle mass. The distribution of the increased muscle mass is not uniform, but most prominent in the more superficial muscles, particularly in the proximal fore- and hindquarters of the animal (Arthur, 1995). The increased muscling results from both hyperplasia (increase in number) and hypertrophy (enlargement) of the muscle fibres (Swatland and Kieffer, 1974), albeit mostly due to hyperplasia (Hanset *et al.*, 1982; see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume). The double-muscled phenotype is apparent early in the development of the fetus. At birth, double-muscled calves possess a higher number of muscle fibres compared with normal-muscled calves (Holmes and Ashmore, 1972; Gerrard *et al.*, 1991) and have a significantly higher birth weight than normal-muscled cattle by as much as 30% (Arthur, 1995). Mature double-muscled cattle have 20% more muscle mass than normal-muscled cattle (Hanset, 1982; Charlier *et al.*, 1995). The degree of muscling varies with genetic background, environment, nutrition, sex and the age of the animal (Arthur, 1995).

Along with increased muscling, 'double-muscled' animals also possess a number of other characteristics. These characteristics include reduced fat deposits, thin skin and fine limb bones (Mason, 1963; Arthur, 1995). Moreover, meat from double-muscled cattle is also reported to be more tender and slightly paler than that from normal-muscled cattle because there is less connective tissue (Bailey *et al.*, 1982) and a higher proportion of white muscle (type II B fibres) coupled with a lower myoglobin content of the muscles (Holmes and Ashmore, 1972; West, 1974) (for more information about muscle fibre types see Reggiani and Mascarello, Chapter 2, this volume; for more information about meat tenderness see Hopkins and Taylor, Chapter 17, this volume). It is due to these traits that cattle with 'double-muscling' are known to have superior carcass characteristics including a higher dressing percentage, a larger proportion of muscle, and lower proportions of fat and bone in the carcass (Shahin and Berg, 1985; Arthur, 1995) resulting in an increased amount of 'high priced' cuts. However, there are drawbacks to these cattle breeds. Double-

muscled cattle have poor reproductive characteristics relative to normal cattle, often due to poor sexual behaviour, delayed sexual maturity in both sexes, reduced size of the reproductive tract in females, calving difficulty, poor maternal performance (Arthur, 1995) and reduced testicular size in males (Michaux and Hanset, 1981). The birthing difficulties associated with double-muscled cattle breeds are due to a combination of an underdeveloped maternal reproductive tract and the excessive muscular development of the calves, particularly the enlarged muscular shoulders and hips. Because of these characteristics there is a frequent requirement for caesarean births to circumvent the high incidence of dystocia (Hanset and Jandrain, 1979).

Although the hereditary nature of the double-muscled condition was recognized early on, the precise mode of inheritance was a cause of dispute. Several models have been proposed including monogenic (dominant and recessive), oligogenic and polygenic models. However, the most commonly accepted model was that of a single major autosomal gene controlling muscular hypertrophy (Rollins *et al.*, 1972; Arthur, 1995). In the Belgian Blue cattle breed, segregation analysis performed in both experimental crosses and in outbred populations suggested an autosomal recessive inheritance (Hanset and Michaux, 1985). This was confirmed when the muscular hypertrophy (*mh*) locus was mapped 3.1 cM from microsatellite TGLA44 on the centromeric end of bovine chromosome 2 (Charlier *et al.*, 1995). Subsequently, *myostatin*, a TGF- β superfamily member, was mapped at the same intervals on chromosome 2 (Smith *et al.*, 1997) and expression of several *myostatin* alleles were described in double-muscled cattle (Grobet *et al.*, 1998).

14.3 Myostatin: a Negative Regulator of Muscle Mass

Myostatin or growth and differentiation factor-8 (GDF-8) was first identified in mice. *Myostatin*-null mice were viable and fertile, but about 30% larger than their wild type littermates. This increase in size was the result of an increase in skeletal muscle mass. The increased skeletal muscle mass was a result of both hyperplasia and hypertrophy (McPherron *et al.*, 1997). Subsequently, several groups discovered that the 'double-muscling' in Belgian Blue and Piedmontese cattle breeds were the result of two distinct mutations in the coding sequence of the *myostatin* gene (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron and Lee, 1997). Since this initial work, several other mutations have been discovered in the coding region of the *myostatin* gene (Table 14.1).

14.4 Structure and Function of Myostatin

The *myostatin* gene appears to be highly conserved. Analysis of the genomic structure of the bovine *myostatin* gene shows that it is organized in three exons and two introns. The first and second exons are 506 and 374 base pairs (bp), respectively, while the size of the third exon is dependent on the polyadenylation site, varying between 1701 or 1812 or 1887 nucleotides. The two introns are 1840 and 2033 bp (Jeanplong *et al.*, 2001).

In order to understand how mutations in the coding region of the *myostatin* gene will affect the functionality of the gene product, it is important to understand how

Table 14.1. Mutations found in the *myostatin* gene of different cattle breeds and the comparison of these mutations with the wild-type bovine sequence.

Mutation	Position	Comparison to wild-type sequence	Cattle breed
F94L	nt282 (aa94)	C to A transition resulting in the substitution of Phe with Leu in the N-terminal LAP	Limousin
nt414(C-T)	nt414 (aa138)	Silent C to T transition in the second exon	Maine-Anjou Limousin Charolais
nt419 (del7-ins10)	nt419	Insertion/deletion that replaces 7 bp with an unrelated 10 bp resulting in a premature stop codon in the N-terminal LAP	Maine-Anjou
Q204X	nt610 (aa204)	C to T transition resulting in a premature stop codon in the N-terminal LAP	Charolais Limousin
E226X	nt676 (aa226)	G to T transition resulting in a premature stop codon in the N-terminal LAP	Maine-Anjou
nt821(del11)	nt821	11 bp deletion resulting in a premature stop codon in the bioactive C-terminal region	Belgian Blue Blonde d'Aquitaine Limousin Parthenaise Asturiana Rubea Gallega
E291X	nt874 (aa291)	G to T transition resulting in a premature stop codon in the bioactive C-terminal region	Marchigiana
C313Y	nt938 (aa313)	G to A transition resulting in the substitution of a highly conserved Cys with Tyr	Gasconne Piedmontese

myostatin and other TGF- β superfamily members function. TGF- β superfamily members characteristically contain several common structural features. One feature is a hydrophobic core of amino acids near the N-terminus that functions as a secretory signal sequence allowing secretion of TGF- β family members (McPherron and Lee, 1996). TGF- β superfamily members also possess a proteolytic processing site in the C-terminal half of the precursor protein (McPherron and Lee, 1996). This proteolytic processing site possesses an RXXR amino acid sequence that is recognized by members of the subtilisin-like proprotein convertase (PC) family (Dubois *et al.*, 1995, 2001; Constam and Robertson, 1999; Leitlein *et al.*, 2001). It is at this position that the large precursor molecule is cleaved to produce the mature active portion and the latency associated peptide (LAP). Myostatin is a secreted factor that meets all of the criteria of a TGF- β superfamily member. Myostatin has a core of hydrophobic amino acids near the N-terminus that functions as a signal for secretion and the dibasic RSRR site for cleavage to release the mature C-terminal signalling molecule. It also shows some degree of homology with the other family members in the C-terminal portion, containing the conserved pattern of nine cysteines for cystine knot formation and homodimerization (McPherron and Lee, 1996; McPherron *et al.*, 1997). Myostatin is most closely related to the TGF- β superfamily member GDF-11, with which myostatin shares 90% homology in the C-terminal region. Myostatin protein appears to be synthesized and processed in myoblasts since all three forms of

myostatin, that is, full length (50–52 kDa), LAP (39 kDa) and mature (26 kDa), are detected in myoblast protein extracts with myostatin-specific antibodies in a Western blot analysis (Thomas *et al.*, 2000). There is some controversy over the molecular masses of the different forms of myostatin, which may be a result of different glycosylation and post-translational modifications in different cell types; for example mouse myoblasts versus Chinese hamster ovary (CHO) cells (Gonzalez-Cadavid *et al.*, 1998). Regardless of the controversy over the sizes of myostatin it is clear that, like other TGF- β superfamily members, myostatin is transcribed as a precursor molecule which is cleaved into the smaller active C-terminal mature myostatin and the N-terminal LAP.

The importance of the ability of myostatin to be cleaved to form the LAP and mature portions was emphasized in a study in which a modified myostatin lacking the RSRR site was overexpressed in the skeletal muscle of mice (Zhu *et al.*, 2000). In this study, the putative proteolytic processing site of myostatin was mutated from RSRR to GLDG. This mutation resulted in a dominant negative myostatin protein which was not cleaved as expected due to the removal of the RSRR site. The overexpression of the mutated myostatin caused a decrease in the proteolytic processing of the endogenous (wild-type) myostatin resulting in muscle hypertrophy in the mice, suggesting the biological activities of myostatin can be regulated by processing. Regulation of myostatin activity by altering the proteolytic processing of the precursor protein has also been suggested to account for the skeletal muscle hypertrophy induced by inhibitors of metalloproteases such as HIMPs (hydroxamate-based inhibitors of metalloproteases) (Huet *et al.*, 2001). However, the link between HIMP inhibition of metalloproteases, and the reduction in myostatin processing is not known.

Another feature of TGF- β superfamily members is the conservation of nine cysteine residues in the C-terminal region of the protein. These cysteine residues facilitate the formation of a cystine-knot dimer structure between two C-terminal monomers after cleavage at the processing site (McPherron and Lee, 1996). Owing to these features, the mature portion forms the cystine-knot dimer structure and associates with the LAP portion of the protein to form a latent TGF- β complex. Similarly, out of the nine cysteine residues in mature myostatin, six cysteine residues are positioned in a proper spacing to form a cystine-knot structure that is seen in TGF- β . Like TGF- β it is also established that mature myostatin forms a homodimer and is secreted. In the Piedmontese cattle the third cysteine of the cystine knot (C₃ amino acid 313) of myostatin is substituted with tyrosine, thus leading to the inactivation of myostatin and double-muscling phenotype.

14.4.1 Truncations of the *myostatin* gene product

Several of the known mutations in the bovine *myostatin* gene result in a truncated gene product. Mutations nt4189(del7-ins10), Q204X and E226X result in a premature stop codon within the LAP portion of the *myostatin* gene; this produces a truncated protein that does not have the ability to produce the mature active myostatin peptide (Grobet *et al.*, 1998). E219X results in a premature stop codon that produces a truncation of the *myostatin* gene within the bioactive C-terminal region (Grobet *et al.*,

1998). nt821(dell1) mutation where 11 bp are deleted from the coding region of myostatin also introduces a premature stop codon, which terminates transcription 14 codons downstream of the site of mutation (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; McPherron and Lee, 1997).

14.4.2 Mutations disrupting myostatin function

The C313Y mutation was first discovered in Piedmontese cattle (Kambadur *et al.*, 1997; McPherron and Lee, 1997). The C313Y mutation is a guanidine to adenine transition at position 938 (G938A) causing the substitution of a cysteine with a tyrosine residue within the bioactive C-terminal region of myostatin. This mutation substitutes a conserved cysteine residue among TGF- β superfamily members with a tyrosine. It has been shown previously that cysteine mutations in TGF- β superfamily members can lead to aberrant signalling. These cysteine residues are required to form the disulphide bonds necessary for the formation of the characteristic cystine-knot structure of TGF- β superfamily members. This mutation affects the function of Piedmontese myostatin and the mutated protein has been shown to inhibit wild-type myostatin (Berry *et al.*, 2002).

Aside from cattle, naturally occurring allelic variations within the *myostatin* gene have been reported in other species. Sequence analysis of the hypermuscular mutation resulting in the 'compact mouse' (*Cmpt*), produced by selective breeding for high carcass protein content, has revealed a 12-base pair deletion in the mouse *myostatin* gene (Szabo *et al.*, 1998). This mutation, unlike the 11-base pair deletion in Belgian Blue cattle, does not truncate the open reading frame of the mRNA and the resulting myostatin molecule is only partially active.

14.4.3 Polymorphisms in the *myostatin* gene

Four DNA sequence variants have been found within the introns of the bovine *myostatin* gene, which occur at nt374-51(T-C), nt374-50(G-A) and nt374-16(dell) in intron 1, and nt748-78(dell) in intron 2. These mutations are probably neutral (Grobet *et al.*, 1998). Polymorphisms in the *myostatin* gene in pig have also been detected. Originally, a single T-A substitution was observed in the *myostatin* promoter region in the Meishan breed, when all other breeds studied were monomorphic for the T allele. C-T substitutions in exon 3 of the Landrace breed were also observed, but did not result in an amino acid substitution. Once again all other breeds tested were homozygous for allele C (Stratil and Kopečný, 1999). Later, another substitution (G-A) was detected in intron 1 (Jiang *et al.*, 2001). Interestingly, the frequency of A alleles in the promoter region and intron 1 was higher for 'double-muscled' Yorkshire than for normal Yorkshire breeds and linkage of these two mutation sites was also observed (Jiang *et al.*, 2001). In other studies, polymorphisms in exon 3 have been related to back fat thickness ($P < 0.05$) and lean meat percentage ($P > 0.05$) (Li *et al.*, 2002), while the presence of allele A in the promoter region (T/A genotype) resulted in an increase in average daily weight gain from 60 to 100 kg in developing animals when compared with the T/T genotype (Jiang *et al.*, 2002). Together these results suggested a strong relationship between polymor-

phisms in the 5' untranslated region (UTR) of the porcine *myostatin* promoter, especially in relation to the presence of allele A, and muscle gain.

In chickens, five single nucleotide polymorphisms (SNPs) have been identified in the *myostatin* gene, three in the 5' regulatory region, and two in the 3' regulatory region. Although the genotype frequencies for these polymorphisms seem to vary significantly in different breeds, no relationship between genotype and phenotype has been established yet (Gu *et al.*, 2002).

The variability of the human *myostatin* gene was originally characterized by Ferrel *et al.* (1999), who determined the nucleotide sequence for human *myostatin* in 40 individuals. This revealed five missense substitutions in conserved amino acid residues in exons 1 and 2 and one substitution mutation in intron 1. The intronic mutation and one of the exon 2 mutations (P198A) were found in only one individual, while two other exon 2 mutations (I225T and E164K) were found in two individuals. The remaining exon 2 (K153R) and exon 1 (A55T) mutations were present in the general population; allele frequencies were significantly different between Caucasians and African-Americans (Ferrel *et al.*, 1999). However, neither of the two mutations showed any significant effect on muscle mass in response to strength training. A subsequent study by Seibert *et al.* (2001), with 286 women aged 70 to 79, identified two previously unseen polymorphisms in the 5' UTR, including a 4-bp deletion, and one new missense substitution in exon 1. Additionally, they confirmed the presence of mutation A55T in exon 1 and I225T and K153R in exon 2 previously described by Ferrel *et al.* (1999). The two mutations detected in the 5' UTR and the one in exon 1 were very uncommon in the Caucasian population, and a higher frequency of these polymorphisms can be seen in African-Americans. When tested for muscle strength, individuals carrying the R genotype (mutation K153R), either homozygous (R/R) or heterozygous (K/R), displayed a decrease of 5 kg in overall strength when compared with wild type homozygous (K/K) for this mutation (Seibert *et al.*, 2001). Similar results were obtained in a study with 450 older Italian men. Once again, the presence of a 153R allele was associated with lower muscle strength (Corsi *et al.*, 2002), suggesting that the R genotype might be associated with accelerated sarcopenia.

14.5 Receptor Binding and Downstream Signalling by Myostatin

TGF- β superfamily members are secreted growth factors and therefore must bind to a receptor to elicit their effect. The TGF- β superfamily members bind to a type II receptor, which binds to and phosphorylates the type I receptor, which in turn phosphorylates the receptor-mediated Smad proteins (R-Smads) that translocate to the nucleus, associated with the co-Smad Smad4, to regulate transcription. The inhibitory Smads (I-Smads) known as Smad6 and Smad7 abrogate the signalling of the R-Smads. The type I and II receptors are specific to the superfamily member. TGF- β s have been shown to bind TGF- β receptor type II (T β RII) activating activin receptor-like kinase-5 (ALK5/T β RI) and in some cases ALK1; activins and nodals have been shown to bind the activin receptor type IIa and type IIb, which activate ALK4 (ActRIB) and possibly ALK7; the bone morphogenetic proteins (BMPs) and GDFs have been shown to bind BMP receptor type II (BMPRII), ActRIIa and ActRIIb, which in turn activate ALK2, ALK3 and ALK6. ALK4 and ALK5

activate R-Smads Smad2 and Smad3, while the remaining ALKs ALK1, 2, 3, 6 and 7 stimulate the phosphorylation of Smad1, 5 and 8 (reviewed in Massague and Chen, 2000; Massague *et al.*, 2000; Moustakas *et al.*, 2001). Recently, Lee and McPherron (2001) have demonstrated that a recombinant mature C-terminal myostatin dimer is capable of binding to activin receptor type IIb (ActRIIb) and to a lesser extent ActRIIa. In support of this finding, Lee and McPherron have also created transgenic mice that specifically overexpress a dominant-negative form of ActRIIB. These mice displayed a dramatic increase in skeletal muscle, comparable to those seen in the *myostatin*-null mice. The type I TGF- β receptor that is activated by Act RIIB in response to myostatin binding has not yet been published. However, in a conference report, the type I receptor is suggested to be the ALK5 (T β RI) receptor (Federman *et al.*, 2000). This report, in agreement with Lee and McPherron (2001), also defined ActRIIB as the receptor for myostatin binding. Recent results from our laboratory showed that myostatin treatment of the C2C12s myoblast cell line resulted in an increase in the phosphorylation of Smad3 (Langley *et al.*, 2002), suggesting that Smad3 is involved in the myostatin signalling.

14.6 Modifiers of Myostatin Function

Several other factors have been reported to regulate the activity of myostatin protein. The N-terminal LAP portion of myostatin, concurrent with other TGF- β superfamily members, has been shown by several groups to bind and suppress the activity of mature myostatin. This latency was demonstrated by inhibition of receptor binding capacity (Lee and McPherron, 2001) and generation of LAP overexpressing transgenic mice, which result in increased muscling (Yang *et al.*, 2001). Follistatin, which has been determined to bind to activins and BMPs and inhibit their activity (de Winter *et al.*, 1996; Iemura *et al.*, 1998), has also been reported to bind myostatin and block receptor binding (Lee and McPherron, 2001). Furthermore, in the same report, the functional significance of this interaction is demonstrated by an increase in muscle mass of ~20–110% in transgenic mice overexpressing follistatin. The authors do not rule out, however, the possibility that other follistatin-sensitive molecules may be involved in this hypermuscularity. The inhibition of myostatin by follistatin is corroborated by earlier reports that follistatin knockout mice show a reduction of muscle mass at birth (Matzuk *et al.*, 1995). In addition to follistatin, two additional proteins, the LAP and the follistatin-related gene (FLRG), are shown to bind to circulating mature myostatin *in vivo* and *in vitro* and inactivate the biological function of myostatin (Hill *et al.*, 2002). Recently a novel protein GASP-1 has been shown to associate with myostatin in normal mouse and human serum (Hill *et al.*, 2003). GASP-1 contains multiple domains associated with protease inhibitory proteins and binds independently to both mature myostatin and myostatin propeptide. GASP-1 has been shown to inhibit the biological activity of mature myostatin possibly by either inhibiting the activation of a latent myostatin complex or regulating furin-mediated myostatin processing (Hill *et al.*, 2003).

Using a yeast two-hybrid system, our laboratory recently demonstrated that one of the sarcomeric proteins, titin cap, specifically interacts with myostatin to regulate the secretion of myostatin (Nicholas *et al.*, 2002). It is interesting that in some of the limb-girdle muscular dystrophy 2G (LGMD 2G) patients, mutations have been

detected in the *titin cap* gene that led to premature stop codons resulting in truncated titin cap protein (Moreira *et al.*, 2000; Schroder *et al.*, 2001). It is conceivable that this could lead to increased circulatory levels of myostatin and hence muscle wasting in these patients (Schroder *et al.*, 2001; Nicholas *et al.*, 2002).

14.7 Myostatin Expression

Originally Lee and McPherron described that, while high levels of myostatin were present in skeletal muscle, low levels of myostatin could also be detected in adipose tissue (McPherron *et al.*, 1997). Since then *myostatin* expression in mammals and birds has been reported to be predominantly restricted to skeletal muscle tissue (Kambadur *et al.*, 1997; McPherron and Lee, 1997; Ji *et al.*, 1998; Carlson *et al.*, 1999; Kocamis *et al.*, 1999; Jeanplong *et al.*, 2001; Oldham *et al.*, 2001). But recent reports have shown that *myostatin* is expressed in a variety of other tissues. *Myostatin* mRNA is also detected in the tubuloalveolar secretory lobules of the lactating mammary gland (Ji *et al.*, 1998). In a report using myostatin-specific antibodies, myostatin protein was also shown to be present in the cardiomyocytes and Purkinje fibres of the heart (Sharma *et al.*, 1999). The role myostatin plays in these tissues is at present largely unknown.

The pattern of *myostatin* expression in muscle has also been analysed in the various vertebrate species. In mice, expression is first detected prenatally in the most mature rostral somites during embryonic myogenesis in day 9.5 post-coitum mouse embryos. By day 10.5 post coitum, myostatin is evident in almost every somite (McPherron *et al.*, 1997). Comparable to this, in cattle, very low levels of *myostatin* mRNA are detected in day 15 to day 29 embryos and increased expression is detected from day 31 onwards (Kambadur *et al.*, 1997; Bass *et al.*, 1999; Oldham *et al.*, 2001). Similarly in the whole pig fetus, *myostatin* mRNA abundance was low at days 21 and 35 of gestation and expression increased markedly by day 49 (Ji *et al.*, 1998). The increase of *myostatin* expression in the bovine and pig fetuses is suggested to directly relate to the gestational stage when primary myoblasts are starting to fuse and differentiate into myofibres and the secondary myoblasts are initially proliferating and then fusing (Ji *et al.*, 1998; Bass *et al.*, 1999; Oldham *et al.*, 2001). In chickens *myostatin* expression is first detected in embryos as early as the blastoderm stage (embryonic day 0), it declines fivefold at day 2 and remains low until day 6. At day 7, levels increase threefold and plateau by day 16 (Kocamis *et al.*, 1999). *Myostatin* continues to be expressed in adult axial and paraxial muscles although the expression levels vary among individual muscles (Kambadur *et al.*, 1997; McPherron and Lee, 1997). In addition, *myostatin* expression is also muscle fibre-type specific. In adult mouse skeletal muscle *myostatin* expression is higher in myosin heavy chain IIb (MHCIIb)-expressing fibres, also known as fast-twitch fibres, indicating that the expression of *myostatin* can be related to specific fibre function (Carlson *et al.*, 1999).

Myostatin has also been detected in lower vertebrates. Several species of fish have been shown to express *myostatin* including brook trout (*Salvelinus fontinalis*), yellow perch (*Perca flavescens*), mahi-mahi (*Coryphaena hippurus*), little tunny (*Euthynnus alletteratus*), king mackerel (*Scomberomorus cavalla*) (Roberts and Goetz, 2001), tilapia (*Oreochromis mossambicus*), white bass (*Morone chryops*) (Rodgers *et al.*, 2001), striped bass (*Morone saxatilis*), white perch (*Morone americana*) (Rodgers and Weber, 2001), Atlantic

salmon (*Salmo salar*) (Ostbye *et al.*, 2001), rainbow trout (*Oncorhynchus mykiss*) (Rescan *et al.*, 2001), gilthead seabream (*Sparus aurata*) (Maccatrozzo *et al.*, 2001) and channel catfish (*Ictalurus punctatus*) (Kocabas *et al.*, 2002). In many of the fish examined, two highly homologous isoforms have been identified. In fish the expression of the two isoforms is not restricted to skeletal muscle and is reported to be expressed also in the brain, ovaries and other tissues. However *myostatin* expression does coincide with myogenesis in fish (Roberts and Goetz, 2001), and there is differential expression of fish *myostatin* in different fibre types (Ostbye *et al.*, 2001). It is also possible that *myostatin* activity in fish is not restricted to regulating muscle mass but may have additional functions similar to GDF-11 in mammals (Ostbye *et al.*, 2001; Rescan *et al.*, 2001; Roberts and Goetz, 2001; Rodgers *et al.*, 2001).

14.8 Transcriptional Regulation of *Myostatin* Gene Expression

Compared to the biology of *myostatin*, little is known about the regulation of the *myostatin* gene. However, recently two studies have demonstrated that MRFs and MEF2 factors, as well as glucocorticoids, do play a role in the regulation of the *myostatin* gene expression.

In the first study by Ma *et al.* (2001) a 3.3 kb 5' region of the human *myostatin* gene was isolated and analysed. Analogous to the case of a typical mammalian basal promoter, three TATA boxes and one CAAT box were found in the first 400 bp from the transcriptional start site. The sequence analysis also revealed a series of potential binding sites for known transcription factors such as POU homeodomain proteins and NF- κ B, two regions homologous to a cAMP response element, a series of E-boxes and two regions homologous to the MEF2 binding site. A number of sites corresponding to the consensus sequences of various hormone-binding sites were also found, including an androgen response element (ARE), five glucocorticoid response elements (GRE) and three thyroid hormone response elements (TRE) (Ma *et al.*, 2001). The results of this study also demonstrate that a glucocorticoid, dexamethasone, can upregulate *myostatin* gene transcription in C2C12 cells and this effect can be antagonized by the glucocorticoid receptor antagonist, RU-486. In line with these results, Lang *et al.* (2001) reported that sustained elevations in circulatory glucocorticoids are able to increase *myostatin* mRNA levels in response to thermal injury in gastrocnemius muscle. Thus, it can be speculated that glucocorticoids may induce muscle atrophy through the upregulation of *myostatin* gene expression.

The sequence analysis of the -1.6 kbp promoter region of the bovine *myostatin* gene revealed the presence of a series of putative binding sites for known transcription factors as well as sites for muscle-specific transcription factors (Spiller *et al.*, 2002). Among the muscle-specific factors were a MEF2 site and ten E-boxes. The E-boxes present in the bovine *myostatin* promoter appear to be arranged in three clusters. The first cluster contains E-boxes E1 and E2, which are located very close to the TATA boxes and the transcription initiation site. The second cluster consists of the so-called proximal E-boxes (E3 to E6) and the third contains the distal E-boxes E7 to E10. There is a considerable homology in the sequence and distribution of E-boxes in the *myostatin* promoter among different species (Spiller *et al.*, 2002).

Analysis of the 5' truncation constructs of the bovine *myostatin* promoter suggests that the proximal cluster of E-boxes is sufficient to drive the reporter gene activity in

C2C12 cells. An internal deletion of only the two very close positioned E-boxes of the proximal cluster, E5 and E6, with all remaining E-boxes intact, lowered the promoter activity sixfold, implying that these E-boxes were important for the promoter activity. Furthermore the mutation of E6 alone decreased the promoter activity to 60% of the wild-type construct activity suggesting that E6 was critical for the promoter activity. In addition, a chromatin immunoprecipitation (ChIP) assay using the chromatin of C2C12 cells stably transfected with the 1.6 kbp construct and immunoprecipitated with antibody against MyoD confirmed that the E6 E-box is occupied by MyoD *in vivo* (Spiller *et al.*, 2002).

The importance of MRFs and MEF2 for the activation of the bovine *myostatin* promoter was demonstrated in assays where the -1.6 kbp construct was co-transfected with vectors overexpressing *MyoD*, *Myf5* or *MEF2C*. While *Myf5* and *MEF2C* enhanced the promoter activity by two- to threefold, respectively, *MyoD* was able to enhance the promoter activity by 15-fold, suggesting that *MyoD* preferentially regulates the *myostatin* promoter. In addition the differentiating myotubes, which express higher levels of *MyoD*, also showed higher levels of *myostatin* promoter activity compared with the quiescent reserve cells, which express low levels of *MyoD* (Spiller *et al.*, 2002). These results are consistent with the earlier study which showed that *myostatin* mRNA expression overlaps with the expression of *MyoD* at day 90 of gestation in cattle (Oldham *et al.*, 2001). Like MyoD, *myostatin* is preferentially expressed in fast fibres and higher levels of myostatin have been seen in the fast glycolytic fibres of cattle (Bass *et al.*, 1999), pig (Ji *et al.*, 1998) and rat (Wehling *et al.*, 2000). Based on these results a genetic hierarchy can be suggested in which *MyoD* controls myogenesis by regulating *myostatin* gene expression during embryonic, fetal and postnatal stages (for more information about the *MyoD* genes see Houba and te Pas, Chapter 10, this volume).

14.9 Physiological Role of Myostatin

14.9.1 Myostatin and muscle atrophy

Several studies have implicated myostatin in playing some role in loss of muscle mass due to various forms of misuse. Studies examining a mouse model of muscle atrophy induced by muscle unloading have revealed that *myostatin* mRNA levels are transiently increased in the fast-twitch fibres though not in more atrophied slow-twitch fibres (Carlson *et al.*, 1999). In a similar experiment with rats, the atrophy of rat hind limb muscles induced by 10 days of unloading resulted in a 16% decrease in plantaris mass, a 110% increase in *myostatin* mRNA and a 37% increase in myostatin protein (Wehling *et al.*, 2000). However, in this study, the increase in myostatin levels was not consistent with the loss of muscle mass; animals subjected to long periods of muscle unloading with intermittent muscle loading experienced no significant loss of muscle mass, but showed increased levels of myostatin. In addition to these studies, rats exposed to a microgravity environment during space shuttle flight also showed loss of skeletal muscle mass accompanied by increased *myostatin* mRNA and protein levels in the skeletal muscle (Lalani *et al.*, 2000). An explanation for elevated levels of myostatin during muscle unloading is that myostatin may function as an inhibitor of satellite cell proliferation (Carlson *et al.*, 1999). Indeed, this is supported by the exhi-

bition of muscle hypertrophy in *myostatin*-null mice. Furthermore hind limb unloading has been shown to inhibit satellite cell proliferation (Darr and Schultz, 1989).

Myostatin may also play a role in muscle wasting associated with disease or ageing. Serum taken from HIV-infected men with weight loss showed increased levels of myostatin indicating that myostatin may play a role in the pathophysiology of muscle wasting associated with HIV infection (Gonzalez-Cadavid *et al.*, 1998). Myostatin may also play a role in age-associated muscle wasting since a correlation between age-associated atrophy of quadriceps muscle group and higher *myostatin* mRNA levels in rats has been seen (Mallidis *et al.*, 1999). Systemic overexpression of *myostatin* in adult mice, by injection of Chinese hamster ovary (CHO) cells producing murine myostatin, induced on average a 33% loss of body weight, due to a combination of loss of muscle and fat. The loss in muscle mass resulted from a decrease in muscle fibre size; CHO-myostatin samples showed a 25% smaller mean muscle diameter than CHO-control samples. The myostatin-induced weight loss was counteracted by the presence of follistatin and myostatin propeptide-expressing CHO cells (Zimmers *et al.*, 2002). The evidence so far indicates that excess myostatin causes muscle wasting thus increasing the possibility that myostatin may be involved in human cachexia.

14.9.2 Myostatin in postnatal muscle growth and regeneration

Muscle satellite cells are a distinct lineage of myogenic progenitors responsible for postnatal muscle growth. A satellite cell is juxtaposed to the surface of myofibres such that the basal lamina surrounds the satellite cell and the associated muscle. Satellite cells are normally mitotically quiescent and when stimulated by the damage to the muscle or by explant and culture manipulations, a proportion of satellite cells are activated to re-enter the cell cycle and express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to contribute to existing fibre or to form new myofibres (Bischoff, 1989). Satellite cells are known to express several myogenic markers including m-Cadherin (Irintchev *et al.*, 1994), Myf-5, CD34 (Beauchamp *et al.*, 2000), myocyte nuclear factor (Foxk1) (Garry *et al.*, 1997), c-Met (Cornelison *et al.*, 2000), Syndecan 3 and 4 (Cornelison *et al.*, 2001) and Pax7 (Seale *et al.*, 2000), suggesting that satellite cells are already committed to the myogenic lineage prior to activation. Using a single quiescent satellite cell for RT-PCR, Cornelison *et al.* (2001) demonstrated the expression of *myostatin* in quiescent satellite cells. Recently myostatin has been detected in satellite cells isolated from chicken pectoralis major and biceps femoris muscles during proliferation and was upregulated during differentiation (Kocamis *et al.*, 2001). Furthermore, Kocamis *et al.* (2002) observed an increase in IGF-II levels in the soleus of myostatin knockout mice, which is believed to originate from activated satellite cells. Based on the available data it can be suggested that myostatin maintains the quiescent state of satellite cells and regulates the transition to the activated state thus controlling postnatal muscle growth. Furthermore when myostatin-specific antibodies were administered to adult mice, increased skeletal muscle mass and increased grip strength were observed (Whittemore *et al.*, 2003). This increase in muscularity could be accounted for by the increased activation of satellite cells, and their contribution to the hypertrophy of muscle fibres.

Myostatin has also been implicated in the process of muscle regeneration. Skeletal muscle regeneration occurs following an injury. First, there is an influx of neutrophils and inflammation occurs. This is followed by the phagocytosis of necrotic debris by macrophages that have invaded the damaged region (Tidball, 1995; McLennan, 1996). Normally quiescent satellite cells are activated to proliferate and migrate to the site of injury where they fuse to form myotubes and then mature to replace and repair damaged skeletal muscle fibres (Bischoff, 1994; Grounds, 1998; Seale and Rudnicki, 2000). Kirk *et al.* (2000) showed high levels of myostatin protein in necrotic fibres and connective tissue of muscle damaged by injection of the notexin. The simplest interpretation of these findings is that myostatin may be inhibiting satellite cell activation, proliferation and differentiation in the necrotic tissue while the degeneration process takes place. Kirk *et al.* (2000) suggest that myostatin may also be acting as a chemoattractant for phagocytes and inflammatory cells, a role that has been ascribed to the related TGF- β 1 (Wahl *et al.*, 1989). In contrast to the high *myostatin* expression seen during the necrotic fibre stage, no myostatin was found in the mononucleate cells located in regenerating areas where activated satellite cells are most abundant. Similarly, no *myostatin* expression was found in nascent myotubes. Low levels of myostatin were detected later in the regenerating myotubes (Kirk *et al.*, 2000). These findings are consistent with myostatin acting as an inhibitor of myoblast proliferation whereby the absence of myostatin permits myoblast cell cycle progression (Thomas *et al.*, 2000). Interestingly, in growth hormone (GH)-deficient *dw/dw* rats, where muscle regeneration is delayed relative to that of normal rats, the increase in *myostatin* directly following injury did not occur; however, the subsequent *myostatin* expression was comparable between *dw/dw* and wt mice (Kirk *et al.*, 2000). Other evidence that myostatin may play a role in muscle regeneration comes from the examination of *myostatin* expression in muscle in mouse models of degenerative disorders such as muscular dystrophy. In *mdx* mice, which lack dystrophin and are a model for Duchenne's muscular dystrophy (Sicinski *et al.*, 1989), and *gsg*^{-/-} mice, which lack γ -sarcoglycan and are a model for limb girdle muscular dystrophy (Hack *et al.*, 1998), a significant degree of regeneration takes place after repetitive cycles of degeneration. Consistent with this, *myostatin* expression is significantly lower in *mdx* and *gsg*^{-/-} mice compared with wild-type mice (Zhu *et al.*, 2000).

In addition to skeletal muscle regeneration, myostatin has also been proposed to play a role in cardiac tissue after injury. *Myostatin* is expressed in the Purkinje fibres and cardiomyocytes in heart tissue. Furthermore, following myocardial infarct, myostatin is upregulated in the cardiomyocytes surrounding areas of myocardial infarct (Sharma *et al.*, 1999). In these areas, it is speculated that myostatin may be functioning to induce migration of monocytes to ischaemic myocardium and promote healing as described for TGF- β 1 (Birdsall *et al.*, 1997; Sharma *et al.*, 1999). Another possibility is that myostatin serves to resist apoptosis in a muscle type that does not contain satellite cells (Sharma *et al.*, 1999). Indeed the upregulation of p21 in myoblasts by myostatin has been suggested to offer protection from apoptosis during myoblast differentiation (Rios *et al.*, 2001), and survivor fibres in damaged muscle after notexin treatment have been found to have increased *myostatin* expression (Kirk *et al.*, 2000).

14.10 Mechanism of Myostatin Function

The phenotypes of myostatin-mutant cattle and mice have clearly established myostatin as a negative regulator of skeletal muscle growth. In order to understand how myostatin regulates muscle mass as a whole, it is important to understand how myostatin acts at a cellular level. It has been found that the growth rate of myoblasts cultured in serum from double-muscling fetuses was higher than myoblasts in normal-muscling serum (Gerrard and Judge, 1993). Furthermore, addition of myostatin to culture medium inhibits the proliferation of myoblasts (Thomas *et al.*, 2000; Rios *et al.*, 2001; Taylor *et al.*, 2001). Moreover, the degree of inhibition is dose dependent and reversible (Thomas *et al.*, 2000).

14.10.1 Myoblast proliferation

Myostatin affects the rate of myoblast proliferation by affecting cell cycle progression. In cell culture studies, the myostatin regulation of myoblast proliferation occurred through cell-cycle arrest in the G₁ and G₂ phases not allowing cells to make the transition to S phase where DNA synthesis occurs and M phase where cell division occurs, respectively. This cell cycle arrest is mediated through the up-regulation of one of the cyclin-dependent kinase inhibitors (CKI), p21. In addition, myostatin treatment also slightly reduced levels of cyclin dependent kinase-2 (Cdk2). It is the combination of the increased CKI, p21, and the decreased Cdk2 that results in a marked reduction in Cdk2 activity. Consistent with this, Rb, a substrate of Cdk2, accumulates in an active hypophosphorylated state resulting in inhibition of cell cycle progression at the G₁ phase (Thomas *et al.*, 2000). Rios *et al.* (2002) also reported an increase in the expression of p21 in the presence of ectopically overexpressed *myostatin*. They attributed this also to the increased cell survival seen in the presence of myostatin, deduced by the fact that an adenoviral construct expressing p21 efficiently inhibited apoptosis in differentiating myocytes (Wang and Walsh, 1996).

14.10.2 Myoblast differentiation

In C2C12 myoblasts, *myostatin* expression increases during differentiation (Rios *et al.*, 2001). In addition myostatin has also been shown to inhibit the differentiation of myoblasts (Rios *et al.*, 2002) and this inhibition is reversible (Langley *et al.*, 2002). Myostatin inhibition of differentiation was associated with an inhibition of the expression of the MRFs, *MyoD*, *myogenin* and *Myf5*, as well as the expression of the late myogenic differentiation marker, myosin heavy chain (MHC) (Langley *et al.*, 2002; Rios *et al.*, 2002). Interestingly, the expression of p21, in contrast to proliferating myoblasts, is also down-regulated by myostatin in differentiation conditions (Langley *et al.*, 2002). The mechanism by which myostatin regulates *MyoD* has been partially elucidated. Langley *et al.* (2002) showed that the presence of myostatin increased the phosphorylation of Smad 3, and increased the association of *MyoD* and *Smad 3*, which is known to repress the activity of *MyoD* (Liu *et al.*, 2001). In addition a *MyoD* promoter–luciferase reporter construct was down-regulated by the presence

of myostatin and this down-regulation was partially rescued by the presence of a dominant-negative Smad3 construct. However, it is not the regulation of *MyoD* alone that results in the myostatin inhibition of differentiation, as overexpression of *MyoD* does not overcome the effect of myostatin treatment (Langley *et al.*, 2002). It has also been shown that cells made quiescent by myostatin are not the same as reserve cells. During myoblast differentiation, a subset of myoblasts, termed reserve cells, remain quiescent and undifferentiated but retain the capacity to proliferate and differentiate. Reserve cells are characterized by a high expression of *p130* and *Myf5* and low *MyoD* expression. Myostatin-induced quiescent cells, however, do not express high levels of *p130* or *Myf5*, although they do have decreased *MyoD* expression as well as the other MRFs (Langley *et al.*, 2002).

During myogenesis, myoblasts first proliferate and then withdraw from the cell cycle to commit to a differentiation pathway where they fuse to form myotubes. Because the amount of myostatin present controls the myoblast proliferation, via the regulation of *cdk2* and *p21* and therefore the phosphorylation state of *Rb*, this in turn determines the number of committed myoblasts. Myostatin then regulates the differentiation of these muscle precursor cells via the regulation of the MRFs. In *myostatin*-null mice and double-muscling cattle breeds where there is no myostatin present, there is an increase in muscle fibre number (Holmes and Ashmore, 1972; Gerrard *et al.*, 1991; McPherron *et al.*, 1997); this can be accounted for by the lack of inhibition of myoblast proliferation and differentiation by myostatin.

14.11 Perspectives and Conclusion

Recent research on myostatin clearly indicates that myostatin not only functions during early embryonic myogenesis but also regulates postnatal muscle growth. Passive immunization against myostatin or genetic crossing of the *mdx* phenotype into a *myostatin*-null background resulted in restoration of functional muscle in adult mice. Acute muscle-wasting conditions such as cachexia can be induced by the administration of excess systemic myostatin. Thus, antagonists to myostatin could be used as therapeutic agents for muscle growth during muscle-wasting situations. Further investigations into the precise mechanism by which myostatin induces muscle wasting would help in the rational design of such therapies. These myostatin-based therapies could be useful in farm animals to promote compensatory muscle growth in animals that are undergoing muscle wasting due to sub-clinical infections and sepsis.

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15 The *Callipyge* Mutation for Sheep Muscular Hypertrophy – Genetics, Physiology and Meat Quality

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15.1 Introduction

Advances in genomic technology led to the discovery of the genetic basis for several naturally occurring mutations that alter muscle growth phenotypes in mammals

(Zhang *et al.*, 1994; Kambadur *et al.*, 1997; Horvat and Medrano, 2001). These discoveries have presented scientists in the animal and human health fields with opportunities to manipulate pathways that regulate muscle growth (Bogdanovich *et al.*, 2002). One such opportunity is a mutation that occurred in a single Dorset ram causing dramatic effects on muscle development. Jackson and Green (1993) first reported scientific evidence of this heritable phenotype, and its genetic location was subsequently mapped to a region of distal chromosome 18 (Cockett *et al.*, 1994). The genetic locus was given the descriptive name *callipyge* (*CLPG*), derived from Greek for 'beautiful buttocks'. The phenotype is characterized by extreme muscle development due to hypertrophy during early postnatal (3–8 weeks of age) growth. The most obvious of these changes occur in the longissimus dorsi and biceps femoris muscles. Large favourable effects of this hypertrophy condition on slaughter and carcass composition traits present a tremendous opportunity to improve production of lean meat. However, the associated adverse effects on meat quality traits have prevented utilization of this mutation by the US sheep industry.

Several institutions developed research projects from descendants of the progenitor ram named Solid Gold, born in 1983 into the Moffat flock from Piedmond, Oklahoma. Much has been learned during the subsequent decade about the genetic basis of this mutation. A single nucleotide change was recently identified as the causative polymorphism (Freking *et al.*, 2002) making a direct genetic test possible. In this chapter we will summarize research investigating the genetic mechanism of the *CLPG* locus and the associated effects on carcass composition and meat quality traits. We will identify current gaps in research knowledge and how this information could be used to capture the benefits and mitigate the antagonisms associated with this mutation.

15.2 *CLPG* Map Location and Muscle Hypertrophy Phenotype Gene Action

Empirical evidence from sheep producers using Solid Gold and his descendants as parents indicated an unusual interaction of the muscle hypertrophy phenotype of progeny with sex of the affected parent. A male parent with muscle hypertrophy, mated to unrelated females would pass on the condition to one-half of its progeny suggesting a heritable, autosomal mutation with non-recessive gene action. However, a female parent with muscle hypertrophy would never pass the same phenotype to its progeny unless mated to a male that also displayed hypertrophy. This unusual parent-of-origin effect indicated potential epigenetic regulation of gene expression near the mutation.

Experimental designs from initial studies (see Table 15.1) used matings of affected rams to normal ewes to produce only two of the four genotypic classes at the *CLPG* locus (Jackson and Green, 1993; Cockett *et al.*, 1994; Jackson *et al.*, 1997). This simple design was suitable and efficient for mapping the chromosomal location of the mutation, but limited hypothesis testing of gene action. Preliminary mapping to the distal portion of chromosome 18 (Cockett *et al.*, 1994) allowed identification of animals representing all genotypes at a single locus with two alternative segregating alleles, using flanking markers. A more precise map location was reported in Freking *et al.* (1998a) and used recombinant individuals to limit the region containing the

Table 15.1. Predicted percentages of offspring that show the callipyge phenotype for each mating combination under the polar overdominance genetic model.

Ewe		Ram			
Genotype ^a	Phenotype	CC Normal	CN Callipyge	NC Normal	NN Normal
CC	Normal	0	0	0	0
CN	Callipyge	50	25 ^b	25	0 ^b
NC	Normal	50	25	25	0
NN	Normal	100 ^b	50 ^b	50 ^b	0 ^b

^a C represents the mutated *CLPG* allele and N represents the normal allele with the paternal allele listed first.

^b Mating combinations where estimates have been published to compare with this expectation.

CLPG locus to a 3.9 centimorgan (cM) genetic region between the microsatellite markers CSSM18 and OY5.

Two independent research efforts in the US developed pedigrees to test more complete hypotheses of gene action (Cockett *et al.*, 1996; Freking *et al.*, 1998a). To complement the initial experiments, four additional types of matings contributed new information: (i) homozygous normal males mated to paternally derived heterozygous females; (ii) *inter se* matings among paternally derived heterozygous males and females; (iii) maternally derived heterozygous males mated to homozygous normal females; and (iv) homozygous mutant males mated to homozygous normal females. The genetic expectation for progeny resulting from matings described in (i) would be two equally represented genotypic classes with segregation of the mutant *CLPG* allele occurring on the maternally derived chromosome. Cockett *et al.* (1996) reported 21 progeny from this mating type, distributed approximately equally between the *CLPG* genotypes inferred by flanking markers, with none of these lambs displaying the muscle hypertrophy phenotype. In contrast to the initial experiments using carrier males (segregation on the paternal chromosome), the muscle hypertrophy phenotype is normal when the mutated allele is inherited from the maternal gamete. Thus, the phenotypes of reciprocal heterozygous genotypes are not equivalent and exhibit a polarity in expression.

Progeny from matings described in (iii) and (iv) were evaluated to examine the reversible nature of the polarity at the *CLPG* locus. Phenotypically normal rams carrying either one (maternally derived) or two copies of the mutation were mated to homozygous normal ewes. An approximately 50:50 segregation of the muscle hypertrophy phenotype in lambs sired by the heterozygous rams indicated the reversible nature of the phenotype after the mutation had passed through a male germline. Consistent with this, the progeny sired by homozygous rams mated to normal ewes were nearly all classified as having the muscle hypertrophy phenotype. The approach taken by Cockett *et al.* (1996) was to assign a specific *CLPG* genotype to each animal based on flanking marker information and compare the subjective phenotypic classification with the expected phenotype based on the polar overdominance model (Table 15.1). Inconsistencies between the expected and subjective phenotype classification can be attributed to genetic recombinants in the interval, genotyping errors or phenotypic misclassification.

Polar overdominance as a genetic model was validated more rigorously by Freking *et al.* (1998a) where several objectively measured traits in a segregating F_2 population were regressed on orthogonal contrasts of genotypic probabilities calculated within each animal. Probabilities for each genotype of each animal were based on information at flanking markers. For brevity, the mutant allele at the *CLPG* locus will be abbreviated as *C* and the normal allele as *N*, with the paternal allele of a genotype listed first. A set of orthogonal contrasts tested traditional additive (*CC-NN*), dominance (*CN+NC-CC-NN*) and reciprocal heterozygote (*CN-NC*) effects. If dominance and reciprocal heterozygote effects were significant, a second set of orthogonal contrasts was applied to clarify gene action. The second set included the polar overdominance (*CN-CC-NC-NN*) effect, and additive (*CC-NN*) and maternal dominance (*NC-CC-NN*) effects. Each contrast was calculated within animal with values ranging from 1 to -1. Several key traits used as indicators of muscle size, carcass shape and carcass composition established significance of dominance and reciprocal heterozygote effects in the set of traditional contrasts. When investigating the second set of contrasts, the additive and maternal dominance genetic effects were not statistically significant for the key traits, leaving the polar overdominance contrast as the only relevant genetic effect.

Qualitative and quantitative methods established polar overdominance as a new genetic model defined by reciprocal heterozygotes expressing different phenotypes (polar), while alternate homozygous genotypes produce similar, normal phenotypes (overdominance). The expectations from this genetic model are displayed in Table 15.1. Callipyge is the only known example of this interaction of phenotype and genotype in mammals and the mutation is clearly associated with a unique form of genomic imprinting regulation.

15.3 Gene Action Associated with Meat Quality Phenotypes

Polar overdominance has been established as the genetic model to describe the interaction of genotypes with the callipyge muscle hypertrophy phenotype. However, genetic models for meat quality traits influenced by the *CLPG* locus have not been as widely investigated. Estimates of *CLPG* effects on meat quality traits have been primarily limited to data collected on *CN* and *NN* lambs (see Table 15.2), thus are not adequate to test hypotheses of gene action for these traits. A single experiment was designed to estimate effects involving all four *CLPG* genotypes, inferred from flanking DNA-based markers, on meat quality traits (Freking *et al.*, 1999). Meat quality traits recorded during the serial slaughter experiment included longissimus dorsi muscle Warner-Bratzler shear force, calpastatin activity and level of intramuscular fat (marbling) (see also Gerbens, Chapter 16, and Hopkins and Taylor, Chapter 17, this volume). A more detailed description of the impact of the *CLPG* locus on meat quality traits follows later in the chapter.

Analyses of slaughter and carcass composition traits by Freking *et al.* (1998b) indicated no additive and maternal dominance effects, leaving polar overdominance as the only relevant genetic effect (Fig. 15.1B). Therefore, polar overdominance as a genetic model predicts that two distributions completely describe all four genotypes. However, analysis of meat quality traits reveals additional complexity in regulation of physiology by the *CLPG* locus. Meat quality traits also displayed significant addi-

tive and maternal dominance effects in addition to significant polar overdominance genetic effects. Thus, meat quality traits required more than two distributions to adequately describe the variation associated with the four genotypes at the *CLPG* locus (Fig. 15.1C and D). *CN* genotypes exhibited the most extreme values from *NN* genotypes while *CC* animals exhibited intermediate values, thus the significant additive effect. For shear force and calpastatin activity, the deviation of the mean value of the *NC* genotype from the mean of alternative homozygous classes was also significant. This deviation was not consistent between the two traits. Mean shear force value of the *NC* class was similar to the mean value of the *NN* genotype (Fig. 15.1C), while for calpastatin activity the mean value was similar to the *CC* genotype (Fig. 15.1D). Therefore, a single genetic model cannot describe the complex genotype-specific distributions for all traits influenced by the *CLPG* locus.

Investigating the novel interaction of genotypes and phenotypes associated with the *CLPG* locus will offer opportunities to improve understanding of the complex relationships involving muscle growth and meat quality traits. Future research in this area should use all four *CLPG* genotypes to evaluate these unique forms of gene action.

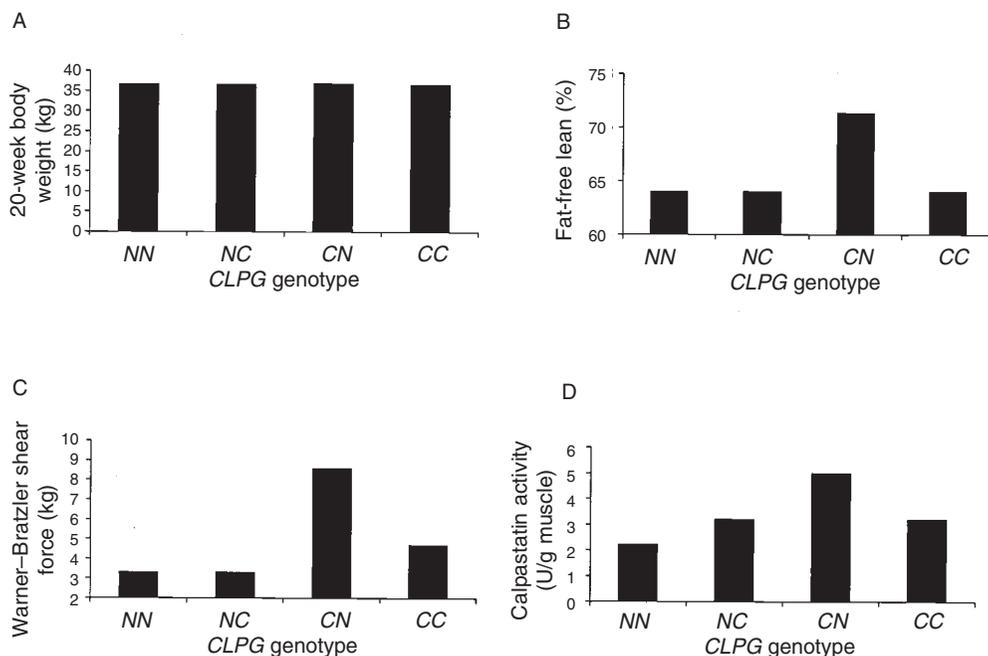


Fig. 15.1. Types of gene action at *CLPG* associated with different phenotypes. The mutant *CLPG* allele is represented as *C*, the normal allele as *N*, and the paternal allele of a genotype combination is given first. (A) Gene action associated with growth and body weight traits. (B) Gene action associated with slaughter and carcass composition traits. (C) Gene action associated with Warner-Bratzler shear force (day 14 post mortem) of longissimus muscle. (D) Gene action associated with longissimus muscle calpastatin activity (day 7 post mortem).

Table 15.2. Characteristics of studies evaluating meat quality aspects of callipyge lamb.

Reference	Experimental objective	Genetic background	Number of observations	Genotype classes represented	Primary outcome
Koohmaraie <i>et al.</i> (1995)	Estimate effects of phenotype on muscle growth and meat quality	Dorset	40	2	Documented muscle-specific hypertrophy, altered fibre type distribution, and correlation of increased shear force and calpastatin activity
Carpenter <i>et al.</i> (1996)	Estimate effects of phenotype on histology and composition of muscles	Dorset–Rambouillet	21	2	Described altered fibre type distribution contribution to hypertrophy
Field <i>et al.</i> (1996)	Estimate role of collagen content and maturation of collagen crosslinks	Dorset–Columbia	20	2	Tenderness problem was not due to altered collagen content or degree of crosslinking (maturation)
Koohmaraie <i>et al.</i> (1996)	Estimate effects of sex class and β -agonist on phenotype	Dorset–Romanov	29	1	Muscle growth was not enhanced further by dietary β -agonist treatment
Shackelford <i>et al.</i> (1997)	Estimate effects of phenotype by various cooking methods on tenderness	Dorset and Dorset–Romanov	39 and 36	2	Phenotype will decrease consumer satisfaction of longissimus chops, but leg muscles may be oven roasted to satisfaction
Duckett <i>et al.</i> (1998a)	Estimate effects of freezing on calpastatin activity and tenderness	Dorset–Columbia	12	2	Freezing carcasses for 8 days prior to normal ageing can improve tenderness
Koohmaraie <i>et al.</i> (1998)	Estimate effect of rapid pre-rigor freezing and post-rigor CaCl_2 injection on tenderness	Dorset–Romanov	49	2	Rapid pre-rigor freezing plus CaCl_2 injection and 14 days of ageing can alleviate the tenderness problem
Taylor and Koohmaraie (1998)	Examine ultrastructural changes during 14 days of ageing for each phenotype	Dorset–Romanov	6	2	Endomysium changes were similar between phenotypes, implicating post-mortem stability of myofibrillar proteins as the cause of tenderness problem
Freking <i>et al.</i> (1999)	Test models of gene action and estimate genotype-specific effects on meat quality	Dorset–Romanov	362	4	Unique forms of gene action specific to meat quality traits were detected and the distributions within genotypes were described for meat quality traits
Solomon <i>et al.</i> (1998)	Estimate effects of electrical stimulation and Hydrolyne treatment	Dorset–Columbia	32	2	Hydrolyne process can tenderize meat from lamb carcasses with the phenotype
Geesink and Koohmaraie (1999)	Compare post-mortem proteolysis and activities of calpains and calpastatin between phenotypes	Dorset–Romanov	12	2	Calpastatin inhibits both the rate and extent of post-mortem proteolysis

Kerth <i>et al.</i> (1999)	Estimate effect of electrical stimulation on meat tenderness	Dorset–Hampshire–Rambouillet	12	1	Tenderness was improved slightly by electrical stimulation
Duckett <i>et al.</i> (2000)	Estimate changes in muscle growth, calpastatin activity and tenderness from birth to slaughter	Dorset–Columbia	40	2	Attempts to reduce the tenderness problem by slaughtering at very low or very high live weights will not be successful
Lorenzen <i>et al.</i> (2000)	Determine the relative roles of muscle protein synthesis and degradation in muscle growth	Dorset–Romanov	37	2	Reduced protein degradation rather than increased protein synthesis maintain enhanced muscle growth of phenotype
Delgado <i>et al.</i> (2001a)	Estimate μ - and m-calpain and calpastatin activities at four post-mortem times and in three muscles	Dorset	12	2	Activity of μ - and m-calpain is not lowered in carcasses with the phenotype
Delgado <i>et al.</i> (2001b)	Estimate differences in myofibril-bound calpain between the phenotypes	Dorset	10	2	Activities and properties of the myofibril-bound calpain were similar between phenotypes
Weigand <i>et al.</i> (2001)	Estimate effect of dietary dosing with vitamin D ₃ on post-mortem muscle [Ca ²⁺] and tenderness	CIII ^a –Finn–Dorset–Polypay	32	2	Feeding for 7 days 2 × 10 ⁶ IU/day did not increase muscle [Ca ²⁺] or alter tenderness

^a The CIII population contains the introgressed mutant form of *CLPG* allele from Dorset into the terminal sire composite population (1/2 Columbia, 1/4 Hampshire, 1/4 Suffolk). The new composite population is on average 1/16 Dorset germplasm.

15.4 Fine Mapping and Positional Cloning of the *CLPG* Mutation

Positional cloning of an unknown gene or mutation in livestock species has typically been conducted in three phases. Reduction of the genetic interval containing the mutation is attained in the first phase using markers within the interval and animals that exhibit a recombination event on informative (heterozygous) gametes. These recombinant animals must be of known quantitative trait loci (QTL) genotype based on phenotype or progeny test information. Integrated linkage, physical and comparative maps are developed in the second phase to facilitate selection of positional candidate genes from gene-rich human and/or mouse maps. Sequencing of candidate genes among animals of different known QTL genotypes is conducted in the third phase, and differences evaluated for causality. Verification of causality may require further and substantial functional analysis. The traditional paradigm of sequencing within selected candidate genes has limitations because all mammalian genes have not been recognized previously in the human or mouse genomes and mutations may also exist within unknown regulatory elements rather than protein coding regions.

Location of the *CLPG* locus was described first as just centromeric of microsatellite marker CSSM18 on the distal region of chromosome 18 (Cockett *et al.*, 1994). Due to the paucity of markers available on the sheep map at the time, bovine-derived microsatellite markers were used to initiate linkage studies. The CSSM18 marker had been previously mapped to the distal region of bovine chromosome 21, a previously recognized evolutionary homologue of ovine chromosome 18. This genetic interval displayed conserved synteny with the telomeric portions of human chromosome 14 and mouse chromosome 12. Interestingly, these regions had been shown previously to be subject to epigenetic regulation from uniparental disomy studies suggesting imprinting regulation (Georgiades *et al.*, 1998, 2000).

A lack of highly informative markers telomeric to the *CLPG* position stimulated efforts to develop new markers in this region. Three new microsatellite markers (OY3, OY5, OY15) were derived from an ovine yeast artificial chromosome (YAC) clone (Broom and Hill, 1994) positive for the bovine marker BMS1561 and defined a new telomeric boundary (Freking *et al.*, 1998a). This study identified definitive recombinant individuals that repositioned the *CLPG* locus to the interval telomeric of CSSM18. Genetic distance between CSSM18 and the OY3, OY5, OY15 haplotype was an estimated 3.9 cM, an interval that could be physically walked with a reasonable number of overlapping large insert genomic bacterial artificial chromosome (BAC) clones. Development of an integrated linkage and physical map of the region was reported in Fahrenkrug *et al.* (2000) and first identified two positional and biologically plausible candidate genes, *DLK1* and *MEG3*.

Construction and characterization of BAC contigs spanning the genetic region in ovine (Segers *et al.*, 2000) and bovine (Shay *et al.*, 2001) provided reagents to establish the physical distance of the genetic interval and facilitate genomic sequence acquisition. Several new microsatellite markers were detected within this BAC contig and used to refine the map position of *CLPG* to a 450 kilobase pair (kbp) chromosome segment flanked by MULGE5 and OY3 markers (Berghmans *et al.*, 2001). This physical distance was less than might be expected from the genetic distance, making sequencing of the entire region a more reasonable approach. Two of the ovine BAC clones (359E3 and 229G11 from Vaiman *et al.*, 1999) were subjected to shotgun

sequencing at threefold redundancy. A single long-range PCR product of 7.5 kbp connected the two BAC contigs which cover the central 250 kbp of the MULGE5–OY3 interval. In addition to the *DLK1* and *MEG3* genes, four novel transcripts named *DAT*, *PEG11*, *antiPEG11* and *MEG8* were identified within the genomic sequence (Charlier *et al.*, 2001a; GenBank accession AF354168).

Identification of positional candidate genes *DLK1* and *MEG3* generated great interest due to reports that these two loci were reciprocally imprinted and expressed only from the maternal (*MEG3*) or paternal (*DLK1*) alleles in humans and mice (Schmidt *et al.*, 2000; Takada *et al.*, 2000; Wylie *et al.*, 2000). The human *DLK1–MEG3* region also has a conserved spatial, structural and epigenetic organization comparable to the more intensely studied *IGF2–H19* imprinted region (Wylie *et al.*, 2000), indicating that this structural relationship may be important for imprinting regulation. Sequencing animals of known *CLPG* genotypes within both of these candidate gene regions failed to identify polymorphisms that uniquely differentiated the *CLPG* alleles. It was then determined that a different approach was necessary to identify the causative mutation.

Availability of ovine genomic sequence (GenBank accession AF354168) within the candidate region accelerated research to discover the *CLPG* mutation. A strategy of PCR amplification and direct sequencing of the overlapping products spanning the candidate interval to detect nucleotide sequence differences among animals of known *CLPG* genotypes was used in Freking *et al.* (2002). A total of 388 unique primer pairs were developed to span and interrogate the candidate region. A panel of animals for single nucleotide polymorphism (SNP) discovery was selected that included six progeny-tested heterozygous Dorset rams, two heterozygous Dorset–Romanov F₁ rams, two homozygous normal Romanov ewes, and two rams from an introgression flock progeny-tested homozygous for the mutation.

Detection of the causative mutation focused on an inbred ram identified with the muscle hypertrophy phenotype, thus heterozygous at the *CLPG* locus, yet homozygous for all known markers in the vicinity. It was hypothesized that this ram was identical-by-descent in the candidate region with the exception of the mutated position (see inbreeding path displayed in Freking *et al.*, 2002). In support of this hypothesis, this ram was homozygous over 210 kbp of sequence and 616 polymorphisms identified in the panel, except for a single heterozygous base position. A single A to G transition polymorphism was detected at position 267 of GenBank accession AF401294 (Fig. 15.2). This SNP was identified between the *DLK1* and *MEG3* genes and outside of any known coding or recognized regulatory element. Genotyping of this SNP in several segregating families demonstrated complete concordance of the *G* allele at the SNP, which was uniquely associated with the mutant allele at the *CLPG* locus. Genotypic data generated from a breed diversity panel of 90 rams from nine breeds (Freking *et al.*, 2002) provided further evidence that this SNP was specific to the descendants of the progenitor Dorset ram Solid Gold, as the *G* allele was not observed in any animal of any breed. The causative nature of this SNP was validated in Smit *et al.* (2003). DNA from Solid Gold was demonstrated to be mosaic for this SNP, indicating that the *G* allele mutation occurred during early embryonic development.

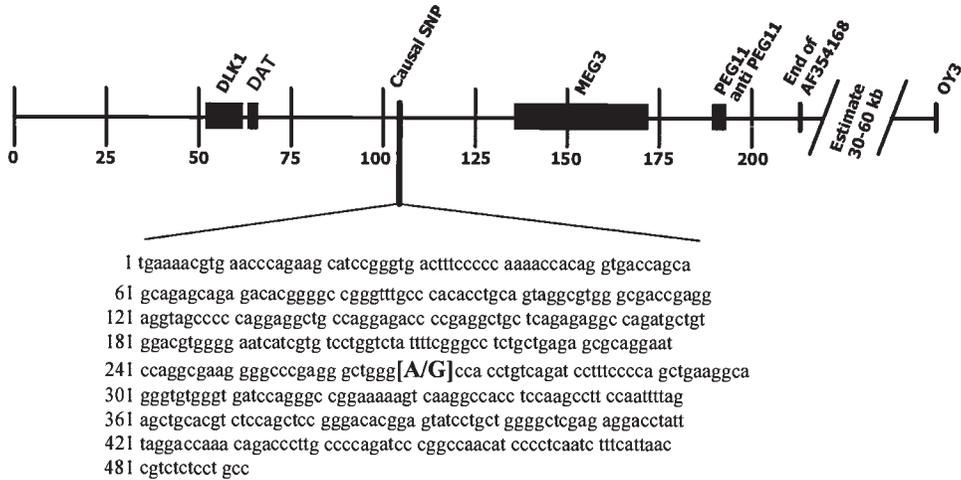


Fig. 15.2. Identification of the causal base change (SNP) for *CLPG*. Position of the identified SNP relative to previously identified candidate genes (*DLKI*, *DAT*, *MEG3*, *PEG11* and *antiPEG11*). Microsatellite marker *OY3* was the previously defined telomeric boundary. Scale is set to 1 = 1000 bp of the sheep genomic sequence in GenBank accession AF354168. The SNP is at position 267 of the sheep STS sequence in accession AF401294.

15.5 Preliminary Functional Evaluation of Mutated Region

Although finding the causative mutation marks the end of a 10-year effort, it represents the starting point for determining the mechanism by which this polymorphism leads to the phenotypic characteristics of callipyge. How the discovered mutation causes muscle hypertrophy is not obvious based on the location of the SNP outside of the boundary of any previously identified transcript. Alignment of 144 bp of ovine sequence centred on the SNP revealed substantial conservation with corresponding bovine, mouse and human sequence. Complete conservation of sequence across these four species was observed for > 74% of nucleotides. This degree of conservation would indicate a sequence with previously unrecognized biological significance. However, *in silico* evaluation of the sequence region containing this SNP failed to reveal conserved open reading frames. The role of this region in gene regulation was also examined by searching for transcription control elements within the sequence. A number of motifs were identified near the SNP that were consistent with binding of muscle-related transcription control factors. Specifically, the SNP alters a sequence motif with homology to a muscle regulatory factor-binding site. However, it does not appear that the mutation acts through altered affinity for the muscle transcription factor MyoD, since the *in vitro* affinity for this complex is similar between the synthesized normal and mutated alleles (Freking *et al.*, 2002).

Epigenetic modifications to the immediate region surrounding the mutation were evaluated for methylation status of cytosine-guanine di-nucleotide (CpG) regions. Differential methylation of CpG regions is a key component of imprinting regulation, and has been invoked as a possible mechanism for explaining polar over-dominance gene action. Methylation status in both fetal and adult muscle tissues indicated that the methylation pattern of the region was not parent-of-origin

dependent, and the degree of methylation did not correlate with the expression of the muscle hypertrophy phenotype (Freking *et al.*, 2002). Thus, altered methylation in the vicinity of the SNP is not the mechanism by which the mutation affects the muscle hypertrophy phenotype.

Preliminary evidence was generated that the genomic region is expressed as an RNA transcript in sheep muscle tissue. A reverse-transcriptase-dependent product with the correct genomic sequence containing the mutation was produced from longissimus muscle RNA. Using random amplification of cDNA ends (RACE) a 547-bp product was produced that presumably defines the 5' end of a transcript. The discovered mutation alters a serine codon to a proline codon within an open reading frame predicting 123 amino acids. However, this open reading frame is not conserved in human and mouse genomic sequence. Methods such as Northern blotting to establish the molecular weight of the entire putative transcript were unsuccessful. This transcript could be a very high molecular weight and/or present in very low copy number. It is possible that the putative transcript actually belongs to a small fraction of RNA molecules generated from a common transcriptional unit similar to the *Air* transcript described for the imprinted *Igf2r* locus (Lyle *et al.*, 2000). The relevance of this genomic region and the impact of the *CLPG* mutation on a putative RNA transcript product are yet to be fully understood and will require substantial functional analyses. This is the first time in livestock that a potential novel transcript has been identified subsequent to, and the direct result of, discovery of the causal mutation associated with a phenotype. This discovery will focus new investigations on both genetic and epigenetic aspects of this important genomic region.

15.6 Expression Profiles and Imprint Status of Genes Near the Mutation

Evidence is evolving from several imprinting anomalies in multiple species that indicates this region near the *DLK1* and *MEG3* genes is involved in regulation of growth in addition to *CLPG*. Mouse embryos have growth defects and are not viable when harbouring maternal or paternal uniparental disomy for the homologous chromosome 12 region (Georgiades *et al.*, 2000, 2001). Discovery of *MEG3* (also known as *GTL2*) was the result of a mouse study where insertion of a *lacZ* transgene occurred in the intergenic *DLK1*–*MEG3* region. Mice were growth-retarded when the transgene was paternally inherited (Schuster-Gossler *et al.*, 1996). Retarded growth and accelerated adiposity were observed in a knockout mouse for the *DLK1* gene (Moon *et al.*, 2002). In humans, patients with uniparental disomy for the homologous chromosome 14 region exhibit growth retardation (Georgiades *et al.*, 1998; Sutton and Shaffer, 2000). These examples highlight the importance of this coordinated region in growth and imprinting regulation in mammals.

Sequencing the ovine genomic region encompassing the *CLPG* locus revealed four additional transcripts when aligned with the human orthologous region (*DAT*, *PEG11*, *antiPEG11* and *MEG β*). These novel transcripts have been evaluated for expression and imprinting only in sheep (Charlier *et al.*, 2001a,b) based on small numbers of observations and time points. Imprinting status of a transcript is determined by sequencing both genomic and cDNA templates of heterozygous individuals for an SNP identified within the transcript. Depending on the allelic status of the

parents for the given SNP, the population of RNA transcripts from a tissue can be identified as paternally or maternally derived.

15.6.1 *DLK1*

Altered expression from the *DLK1* gene is considered the most likely candidate to be directly involved in the muscle hypertrophy phenotype for the *CLPG* locus. This molecule is a transmembrane homeotic protein with six epidermal growth factor repeats and is thought to play an important role in differentiation of multiple cell types. *DLK1* expression is suppressed postnatally during adipocyte differentiation while ectopic expression of this protein inhibits adipogenesis (Smas and Sul, 1993). In addition to adipocyte development, *DLK1* expression levels may also impact skeletal muscle development by influencing development, differentiation, function and growth of cells in the pancreas and adrenal gland (Jensen *et al.*, 1993, 1994). Production of catecholamines, corticoids and insulin products by these organs certainly influences nutrient partitioning and protein metabolism. Transcripts for *DLK1* are imprinted and expression was observed to be exclusively from the paternally derived allele in humans and mouse (Schmidt *et al.*, 2000; Wylie *et al.*, 2000).

Elevated postnatal expression levels of *DLK1* in longissimus muscle tissue from sheep exhibiting the *CLPG* muscle hypertrophy phenotype were observed compared with normal phenotypes (Charlier *et al.*, 2001b). Interestingly, *DLK1* expression was also elevated to a lesser extent in this tissue from individuals homozygous (normal hypertrophy phenotype) for the mutation at *CLPG*. Consistent with the human and mouse data, imprinting evaluation of the *DLK1* gene in sheep demonstrated that expressed transcripts were paternally derived, regardless of genotype at the *CLPG* locus (Charlier *et al.*, 2001a).

15.6.2 *MEG3*

Transcripts for the *MEG3* gene are generated in a reciprocal manner compared with *DLK1*. *MEG3* is also imprinted, but exclusively expressed from the maternally derived allele in human and mouse (Schmidt *et al.*, 2000; Wylie *et al.*, 2000). Its physical orientation with *DLK1* is clearly similar to the intensely studied *H19/IGF2* imprinted domain (Wylie *et al.*, 2000). Similar to the *H19* locus, the *MEG3* gene does not encode a protein, instead generating multiple splice variants indicative of a regulatory RNA molecule. Timing of elevated expression of the *MEG3* gene is coordinated with myogenic differentiation in mouse embryos, but its role as a regulator is not well known.

Muscle-specific expression levels of *MEG3* are abundant during prenatal stages of growth and downregulated in postnatal stages. Hypertrophy-responsive muscles of paternally derived heterozygous *CLPG* lambs exhibited higher *MEG3* expression levels at 14 days before parturition than normal genotype lambs (Bidwell *et al.*, 2001). Expression of *MEG3* in 8-week-old lambs remained elevated in longissimus muscle when the mutant *CLPG* allele was inherited from the maternal chromosome (Charlier *et al.*, 2001b). Elevated *MEG3* expression levels were observed in both heterozygous and homozygous mutant genotypes. Only hypertrophy-responsive

muscles exhibited this genotype-specific elevated expression pattern. In the supraspinatus, which does not undergo hypertrophy, expression of *MEG3* remained low in all *CLPG* genotypes and ages (Bidwell *et al.*, 2001). Unfortunately, only two of the four genotypes were evaluated for the prenatal and early postnatal stages in that study.

Evaluation of *MEG3* in sheep demonstrated that expressed transcripts were maternally derived, regardless of genotype at the *CLPG* locus (Charlier *et al.*, 2001a). This is consistent with *MEG3* information from the human and mouse, and also indicates that a polarity shift has not occurred as a consequence of the *CLPG* mutation.

15.6.3 DAT

An evolutionary conserved region represented by a clustering of human expressed sequence tags (ESTs) within 4 kbp of the 3' end of *DLK1* was observed in the genomic sequence comparison between ovine and human, and referred to as *DLK1*-associated-transcripts (DATs). This region was shown by RT-PCR to be preferentially expressed in skeletal muscle and to have a functional polyadenylation signal. This transcript is in the same orientation as *DLK1* but experiments have not yet revealed it as a novel extended *DLK1* transcript (Charlier *et al.*, 2001a). Biological function of these DATs is unknown at the present time, however in sheep the transcript is imprinted and paternally expressed. Genotype status of the *CLPG* locus did not alter this imprinting status.

15.6.4 *PEG11* and *antiPEG11*

At approximately 9–10 kbp telomeric of the 3' end of *MEG3* resides a single exon gene product with a high degree of homology to the gag and pol polyproteins of gypsy-like retrotransposons. However, flanking genomic sequence does not contain the repetitive elements that would be expected for retroviral-like sequences, and the gene appears to be unique in the genome. Expression of the transcript was observed in a 21-day-old conventional sheep fetus, and in skeletal muscle of a 6-week-old lamb with the muscle hypertrophy phenotype only (Charlier *et al.*, 2001a). Imprinting status of this transcript was evaluated and expression was determined to be from the paternally derived allele (Charlier *et al.*, 2001a). This transcript was named *PEG11* (for paternal expressed gene 11) and is transcribed in the opposite direction to *DLK1*, DAT and *MEG3*. Expression of *PEG11* was increased when the *CLPG* mutation existed on the paternal gamete (Charlier *et al.*, 2001b).

Analysis of known human and bovine ESTs mapping to this position also indicated the possibility of a transcript produced on the opposite strand, thus, in the same orientation as *DLK1* and *MEG3*. Experiments using RT-PCR verified the existence of the *antiPEG11* transcript in sheep fetal tissue, skeletal muscle and to a lesser extent kidney, heart and lung tissues (Charlier *et al.*, 2001a). Sequencing of *antiPEG11* transcripts indicated that it was imprinted and expressed exclusively from the maternal allele. Polarity of imprinting for both *PEG11* and *antiPEG11* was not altered by the *CLPG* mutation and their function biologically is not well understood.

15.6.5 *MEG8*

The fourth novel RNA molecule identified based on genomic sequence comparison and EST matches between sheep and human in this region was approximately 10 kbp telomeric to the *PEG11* gene. As seen with *MEG3*, lack of a conserved open reading frame across species and frequent occurrence of frame-shifting mutations suggested that this transcript acts as a regulatory RNA molecule rather than a protein product. Expression products from RT-PCR experiments detected transcripts from sheep skeletal muscle and to a lesser extent kidney (Charlier *et al.*, 2001a). This transcript was imprinted and the RNA populations evaluated from skeletal muscle were maternally derived (Charlier *et al.*, 2001a). Thus, the transcript was named *MEG8* (maternal expressed gene 8). Expression of *MEG8* in skeletal muscle was increased by the presence of the *CLPG* mutation on the maternal gamete (Charlier *et al.*, 2001b). Consistent with the other genes in the region, polarity of imprinting for *MEG8* was not altered by the *CLPG* mutation. How *MEG8* might serve as an RNA effector molecule in muscle tissue is unknown.

15.7 Summary of *CLPG* Domain Expression

Coordinated expression changes of several muscle-specific transcripts near the *CLPG* locus provide evidence of a shared regulatory domain. Expression levels of all six of the transcripts discussed have been impacted by the presence of the *CLPG* mutation. However, polarity of expression has not been altered. Paternally expressed transcripts (*DLK1*, *DAT*, *PEG11*) are upregulated when the mutation is present on the paternal gamete and maternally expressed transcripts (*MEG3*, *antiPEG11*, *MEG8*) are upregulated when the mutation is present on the maternal gamete. Charlier *et al.* (2001b) proposed a model of coordinated expression that may produce the observed polar overdominance gene action at the *CLPG* locus. The model implicates both the upregulating *cis* effects mentioned above and downregulating *trans* effects of the maternally derived mutation on paternal allelic expression of *DLK1* and *PEG11* (Charlier *et al.*, 2001b). This negative influence could be acting at the transcriptional level, or on RNA stability, or even at the protein level.

Cumulative results of these *cis* and *trans* effects are that expression of *DLK1* and *PEG11* in muscle tissue is elevated to abnormally high levels in the absence of increased *MEG3* and *MEG8*. This situation is only observed in muscle tissue during postnatal growth when the mutation is present on the paternal gamete and a normal allele is present on the maternal gamete. This profile parallels the interaction of *CLPG* genotype and the observed muscle hypertrophy phenotype. These gene expression interactions without altering imprinting status are key to understanding how this single base mutation could influence both muscle and fat accretion as well as impact meat quality traits in very different ways.

15.8 Impact of *CLPG* Mutation on Growth, Slaughter and Carcass Traits

A major shift in carcass composition without a change in growth rate is associated with the gene expression profile caused by the *CLPG* mutation. Much of the work

has estimated callipyge phenotypic effects from data collected on small numbers of lambs produced by heterozygous rams and non-carrier ewes. Statistical inferences of these phenotypic effects applied to restricted ranges of slaughter end points and relationships of traits with slaughter age or carcass weight within phenotype were not fitted. Despite the design limitations and imprecise estimates, statistical significance was usually detected simply because the magnitude of the effects was so great. In general, most estimated effects did not deviate substantially from those reported in a large serial slaughter design that included all four genotypes at the *CLPG* locus (Freking *et al.*, 1998b), thus these more precise estimates will primarily be summarized.

Genotypes at the *CLPG* locus did not affect body weights at birth, weaning and post-weaning up to slaughter (Jackson *et al.*, 1997; Freking *et al.*, 1998b). Consistent with this growth pattern is the finding that no systemic hormonal differences have been detected (Whisnant *et al.*, 1998). Appearance of the muscle hypertrophy phenotype becomes apparent in most individuals by 8 weeks of age without altering body weight. However, carcass weights at similar live weights were greater for lambs with the muscle hypertrophy phenotype. The substantial advantage in dressing percentage (55.9% vs. 51.7%) of these lambs can be attributed in part to lower pelt, liver and kidney–pelvic fat weights (Freking *et al.*, 1998b). Lighter mass of metabolically active internal organs is also consistent with the reduced daily feed intake associated with the syndrome (Jackson *et al.*, 1997). Lambs with the muscle hypertrophy phenotype deposited less fat at the 12th rib and fourth sacral vertebrae and produced heavier carcasses than normal lambs before reaching similar levels of subcutaneous fat depth. Even at heavier weights, these carcasses were significantly leaner (Freking *et al.*, 1998b), which should present producers and packers with potential added value.

Several measures of carcass shape indicated that lambs with the muscle hypertrophy phenotype were more compact in skeletal structure and exhibited a more pronounced muscle shape (Freking *et al.*, 1998b). Increased longissimus dorsi surface area at the 12th rib, one of the hallmarks of the muscle hypertrophy syndrome, was increased from 15.1 cm² to 20.5 cm² in Romanov–Dorset crossbred lambs at an average slaughter age of 215 days. Rear leg conformation scores in the same experiment were increased from 10.9 (average choice = 11) to 14.8 (average prime = 14); the polar overdominance contrast accounted for nearly 70% of the variation (Freking *et al.*, 1998b).

Differences between *CLPG* genotypes in carcass composition were established before typical slaughter ages and weights for lamb. These differences were essentially maintained or slightly increased as animals reached older slaughter ages (Figs 15.3–15.5). The curves in Fig. 15.3 represent accrual of carcass tissue components. Accretion rates (slopes of the curves in g/day) of muscle hypertrophy lamb carcass tissues at 215 days of age were 48, 35 and 3 for lean, fat and ash, respectively. Analogous accretion rates from normal lamb carcasses were 41, 42 and 3. Increased lean growth rate and decreased fat growth rate are consequences of the muscle hypertrophy condition. It has been suggested that this increased lean growth rate is the result of reduced protein degradation rather than increased protein synthesis (Lorenzen *et al.*, 2000), although a higher capacity for protein synthesis is evident through increased satellite cell proliferation (Koochmaraie *et al.*, 1995).

At the mean carcass weight, a 1-kg increase in carcass weight from lambs

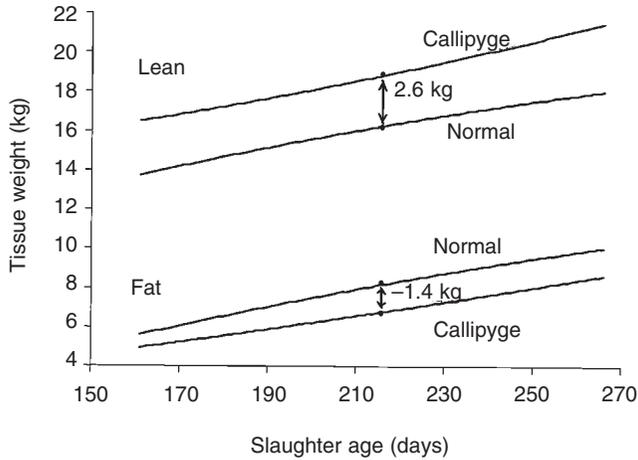


Fig. 15.3. Relationships of carcass fat and fat-free lean tissue with slaughter age of callipyge (CN) and normal (CC, NC, NM) phenotypic (genotypic) groups. Difference between the phenotypic groups at the mean slaughter age of 215 days is indicated.

expressing muscle hypertrophy consisted of 36 g ash, 373 g fat, 148 g protein and 445 g water. The same 1-kg increase in carcass weight from normal phenotype lambs consisted of 37 g ash, 484 g fat, 115 g protein and 365 g water. Increased weight of fat-free lean in the carcasses from lambs with muscle hypertrophy was offset by the same amount of fat (Fig. 15.4). Carcasses from lambs with muscle hypertrophy consisted of 24.3% fat and 71.3% fat-free lean, compared with 31.5% and 64%, respectively, for normal carcasses of the same weight (Fig. 15.5). These effects on carcass composition are much greater than can be generated from breed

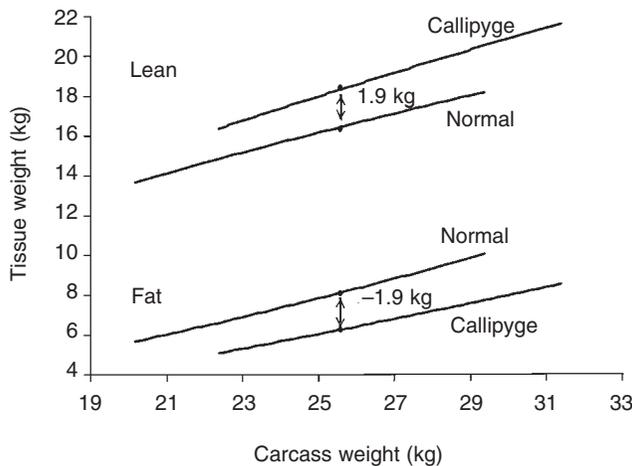


Fig. 15.4. Relationship of carcass fat and fat-free lean tissue with carcass weight of callipyge (CM) and normal (CC, NC, NM) phenotypic (genotypic) groups. Difference between the phenotypic groups at the mean carcass weight of 25.6 kg is indicated.

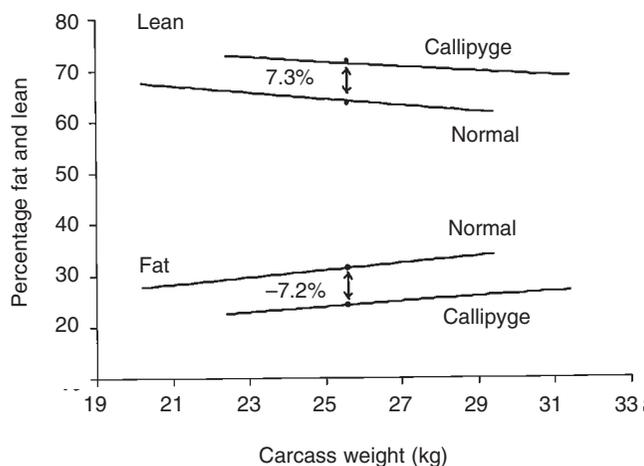


Fig. 15.5. Relationship of percentages of fat and fat-free lean tissue with carcass weight of callipyge (CN) and normal (CC, NC, NM) phenotypic (genotypic) groups. Difference between the phenotypic groups at the mean carcass weight of 25.6 kg is indicated.

substitution effects. The composition advantage was so large that feeding β -adrenergic agonist to Romanov–Dorset crossbred wether and ram lambs with the muscle hypertrophy phenotype had no effect (Koochmaraie *et al.*, 1996).

Polar overdominance gene action favours its use in terminal sire mating systems to consistently generate the resulting phenotype. With 16 mating combinations of *CLPG* genotypes, only one parental genotype combination will generate 100% progeny with the muscle hypertrophy phenotype (Leymaster *et al.*, unpublished data; also see Table 15.1). Specific genotypes at *CLPG* can be used in structured mating systems to make large improvements in dressing percentage, lean growth rate, muscle shape and carcass composition. Lambs with the muscle hypertrophy phenotype can be taken to heavy carcass weights and still produce lean carcasses, thus spreading fixed costs per head over more lean retail product.

15.9 Impact of *CLPG* Mutation on Meat Quality Traits

Although substantial improvement in carcass composition is possible, adverse effects on meat quality traits have been well documented. A solution to this antagonism must be overcome prior to industry adaptation of this genotype. Research in this area has focused on the following general areas (Table 15.2): impact on meat tenderness, impact on fibre histology or structural changes, impact on the calpain/calpastatin enzyme system, ante-mortem treatments and post-mortem treatments. Most of these studies were conducted with small numbers of observations yielding imprecise estimates. In addition, only two *CLPG* genotypes were evaluated, limiting inferences to gene action. However, a severe antagonism with tenderness of longissimus muscle was predominantly reported.

15.9.1 Effect on tenderness

Variation in tenderness of meat has a large impact on consumer satisfaction. Muscles that appear to be affected by *CLPG* both in terms of hypertrophy and tenderness include longissimus dorsi, gluteus medius, semimembranosus, semitendinosus, biceps femoris, adductor, quadriceps femoris and triceps brachii. Chops obtained from longissimus dorsi of lambs exhibiting the muscle hypertrophy phenotype were shown to have substantially greater Warner–Bratzler shear force values indicating a less tender product (Koochmaraie *et al.*, 1995, 1996). This antagonism was substantial, even at 21 days post mortem, when the mean shear force values still exceeded the mean shear force values of normal lambs at 1 day post mortem (Koochmaraie *et al.*, 1995). This result appeared unrelated to pH, temperature or sarcomere length effects, as the two phenotypes have similar rate or pattern of pH and temperature decline as well as similar sarcomere lengths. Degradation of key myofibrillar proteins known to be involved in normal post-mortem tenderization (titin, desmin, nebulin, troponin-T, vinculin) was delayed by as much as 20 days in lambs exhibiting the muscle hypertrophy phenotype. This delayed degradation pattern is linked to a reduction in the calpain proteolytic system due to the high levels of calpastatin activity in muscles affected by the hypertrophy phenotype. Calpastatin is the endogenous inhibitor of the calpain proteolytic system responsible for post-mortem tenderization (see Hopkins and Taylor, Chapter 17, this volume).

Comprehensive genotype-specific effects of the *CLPG* locus on longissimus muscle shear force were reported in Freking *et al.* (1999). Means and variances were greatest for the paternally derived heterozygous genotype, while the homozygous mutant genotype was intermediate compared with homozygous normal and maternally derived heterozygous genotype classes. While the paternally derived heterozygous specific shear force distribution was extreme, it did overlap with the normal phenotypic distribution and the effect of individual sires was significant. Epistatic relationships with other loci may exist, which could alter the impact of the *CLPG* locus on tenderness. Significant sire effects would indicate genetic variance for shear force that could be exploited through selection within flocks homozygous for the mutation. Candidate loci influencing tenderness such as *CAPN1* (μ -calpain) could be suitable targets for evaluation (Page *et al.*, 2002). A genome scan within the sheep resource population described in Freking *et al.* (1998a) is planned with the objective of identifying loci interacting with *CLPG* for tenderness.

A long-term objective is to implement selection schemes for favourable alleles influencing tenderness within flocks homozygous for the mutation. A genetic solution to shift the distribution of shear force to a more acceptable level would be more economical than post-mortem treatment of lamb products. Many of these postharvest methods require additional costs associated with new equipment, extended holding times and increased cooler space.

15.9.2 Fibre histology and structural alterations

Consistent with hypertrophic rather than hyperplastic growth, number of muscle fibres was not altered by the *CLPG* mutation. Overall mean fibre area in hypertrophy-responsive muscles was increased in lambs with the callipyge phenotype

(Koochmaraie *et al.*, 1995; Carpenter *et al.*, 1996). Increased percentage of fast-twitch glycolytic fibres and decreased percentages of both slow-twitch oxidative and fast-twitch oxidative glycolytic fibres contributed to this increased average fibre area. In addition, individual fibre area for the fast-twitch glycolytic and fast-twitch oxidative glycolytic fibres was increased in lambs with the muscle hypertrophy phenotype, while the fibre area for slow-twitch oxidative fibres was decreased. Changes in fibre type distribution and fibre diameter were limited to muscles that displayed a change in mass. Muscles such as the supraspinatus, which does not hypertrophy, did not display this fibre alteration. The increased percentage of calpastatin-rich, fast-twitch glycolytic fibres may decrease post-mortem proteolysis, causing an adverse effect on meat tenderness (for more information on muscle fibre types see Reggiani and Mascarello, Chapter 2, this volume).

Field *et al.* (1996) examined the potential role of connective tissue in the decreased tenderness of meat from callipyge lambs. Collagen is the major component of connective tissue and the less soluble trivalent collagen crosslink, hydroxylsilypyridinoline, increases with age and is an indicator of maturation. Collagen percentage in muscle from lambs with the hypertrophy phenotype was slightly lower but not statistically different from normal lamb muscle. The hydroxylsilypyridinoline crosslink in intramuscular collagen from callipyge lambs was also present in lower concentrations than in normal lamb. Consistent with the delayed maturation of fat deposition, the collagen maturation process is slowed in lambs exhibiting the muscle hypertrophy phenotype. Lack of evidence of changes in collagen or collagen crosslinking indicates that the muscle tenderness antagonism is associated exclusively with the myofibrillar fraction of muscle. Taylor and Koochmaraie (1998) measured the ultrastructural changes in muscle cytoskeleton and myofibrils and also suggested that the key component was the post-mortem stability of the myofibril.

15.9.3 Calpain/calpastatin system (see also Hopkins and Taylor, Chapter 17, this volume)

The calcium-dependent protease μ -calpain has been well established as a major contributor to the post-mortem tenderization of skeletal muscle via degradation of key myofibrillar proteins. Concentrations of calcium activate the different m- and μ -calpain systems. Activity of μ -calpain not bound to the membrane or myofibril is primarily regulated by its endogenous inhibitor calpastatin. Membrane- and myofibril-bound μ -calpain is not inhibited by calpastatin (Boehm *et al.*, 1998). Level of calpastatin activity varies between species, breeds and individual muscles and is highly related to extent of post-mortem tenderization, thus ultimately tenderness (Geesink and Koochmaraie, 1999). Rate of calpastatin degradation in post-mortem muscle is a key regulator of post-mortem tenderization (Delgado *et al.*, 2001a). Therefore, it is not surprising that substantial research effort has focused on this enzyme system when evaluating the antagonism of tenderness with lambs expressing the muscle hypertrophy phenotype associated with *CLPG*.

At death, longissimus muscle μ -calpain activity was similar and m-calpain activity was higher from lambs with the muscle hypertrophy phenotype compared with normal contemporaries (Koochmaraie *et al.*, 1995). However, calpastatin activity was substantially higher in longissimus from lambs exhibiting hypertrophy (Koochmaraie

et al., 1995; Duckett *et al.*, 2000). This high level of calpastatin activity essentially overrides any increased calpain activity. The membrane- or myofibril-bound portion of μ -calpain activity that is not inhibited by calpastatin was reported to be similar in longissimus muscle from both normal and hypertrophied lambs and probably plays no role in the post-mortem tenderization process (Delgado *et al.*, 2001b). Calpastatin activity was not uniformly higher in all muscle tissues. Calpastatin activity differences between the phenotypes seemed proportional to its effect on muscle weight (Koochmaraie *et al.*, 1995). In tissues such as the longissimus where calpastatin activity was increased, the increased activity was maintained even at 21 days post mortem. This increased calpastatin activity parallels the pattern of myofibrillar protein degradation, or lack thereof. Even during extended (56 days) post-mortem storage, less extensive proteolysis has occurred in biceps femoris muscle from lambs with the muscle hypertrophy phenotype (Geesink and Koochmaraie, 1999). At 56 days, both calpastatin and μ -calpain activity were similar between the two phenotypes. However, post-mortem proteolysis proceeds very slowly at this time. Geesink and Koochmaraie (1999) indicated that the increased calpastatin activity inhibited both the rate and total extent of post-mortem proteolysis achieved in skeletal muscle from lambs with the hypertrophy phenotype up to day 56.

Genotype-specific effects of the *CLPG* locus on calpastatin activity from longissimus muscle over a range of slaughter ages and carcass weights were reported in Freking *et al.* (1999). Levels of calpastatin activity were greatest for paternally derived heterozygous genotypes at all ages and carcass weights. Measurements of calpastatin activity of maternally derived heterozygous and homozygous mutant genotypes were intermediate compared with the homozygous normal genotype. In contrast to shear force values of these same carcasses (Fig. 15.1C and D), the calpastatin activity of maternally derived heterozygous genotypes was similar to the homozygous mutant genotype rather than the homozygous normal genotype. Genotype-specific effects on calpastatin activity were not entirely consistent with the magnitude of the effects on shear force (Freking *et al.*, 1999). The significance of these unique forms of gene action for post-mortem traits has yet to be fully realized and offers an opportunity for further study. Evaluation of altered expression of several genes in this region may help explain the unique interactions of phenotypes with *CLPG* genotypes.

Lambs with the muscle hypertrophy phenotype offer an excellent model for meat scientists to study roles of the calpain and calpastatin systems in post-mortem tenderization due to the large differences and unique interactions observed in both calpastatin activity and tenderness of the muscle.

15.9.4 Ante-mortem treatments

There has been a limited attempt to alleviate the tenderness antagonism via ante-mortem treatment of lambs with the muscle hypertrophy phenotype. Wiegand *et al.* (2001) reported a feeding trial with vitamin D₃ where the objective was to increase the free calcium available in the muscle to stimulate calpain activity and subsequent proteolysis and tenderness. Feeding 2×10^6 IU/day of vitamin D₃ for 7 days prior to slaughter increased the serum Ca²⁺ concentration, but did not increase muscle Ca²⁺ concentration. Thus, differences in shear force or degradation of troponin-T were not observed. A higher dosage of vitamin D₃ would be required to improve tenderness.

15.9.5 Post-mortem treatments

Substantial research efforts have been invested into post-mortem intervention strategies to mitigate the tenderness antagonism. Much of the work is targeted at the longissimus muscle due to high market value and because typical cooking methods for chops (broiling) tend to expose tenderness and juiciness problems to a greater extent than other methods. Shackelford *et al.* (1997) reported that oven roasting could prevent reduced tenderness in leg muscles from lambs with the muscle hypertrophy phenotype. Muscles that do not exhibit the muscular hypertrophy condition are essentially unaffected from a tenderness standpoint (Koochmaraie *et al.*, 1995; Shackelford *et al.*, 1997).

Reported post-mortem intervention strategies include carcass electrical stimulation, extensive post-mortem ageing, the combination of freezing and thawing before ageing, calcium chloride injection and hydrodynamic pressure treatment. These strategies are employed to either physically degrade the myofibril or stimulate the activity of the calpain proteolytic system to promote the ageing effect. Various degrees of success have been reported in mitigating the tenderness problem. At this point no single treatment has been established that is cost-effective and readily implemented with a traditional fresh-lamb market system.

Pre-rigor electrical stimulation has been used to cause myofibrillar fractures as well as depolarize cell membranes causing a release of calcium into the cell, which then activates the calcium-dependent proteases. Kerth *et al.* (1999) reported no effect of electrical stimulation on shear force of longissimus muscle from lamb with the muscle hypertrophy phenotype. However, there was an indication of some improved tenderness evaluated by trained sensory panel scores. Electrically stimulated carcasses increased the percentage of chops that were rated in the category of slightly tough to slightly tender. Electrical stimulation alone has not been shown to be effective in reducing the tenderness antagonism, but has been effectively used in conjunction with other treatments.

Based on the relationship of sarcomere length and longissimus tenderness during the first 24 h after slaughter, Koochmaraie *et al.* (1998) suggested rapidly freezing carcasses immediately after harvest to prevent sarcomere shortening. Subsequent storage just below freezing to deplete ATP stores and prevent thaw rigor would ultimately prevent toughness development. Results in Koochmaraie *et al.* (1998) indicated that rapidly freezing carcasses in liquid nitrogen significantly improved expert sensory panel tenderness ratings and shear force values. This treatment used in combination with calcium chloride infusion resulted in longissimus chops that were similar in tenderness to that of normal, untreated lamb after 14 days of post-mortem ageing. Although results seem promising, the reality of establishing this treatment protocol for lambs at a processing plant is a major challenge.

Freezing longissimus chops from lambs with the muscle hypertrophy phenotype at -20°C for 42 days prior to ageing for 14 days was shown to accelerate the rate of post-mortem tenderization and reduce shear force to levels seen in normal lamb (Duckett *et al.*, 1998a). This reduction in shear force proceeded in a quadratic manner. Freezing time required prior to the standard 14-day ageing process, to obtain shear force values similar to normal lamb chops was 8 days (Duckett *et al.*, 1998b). This protocol would seem to be more amenable to industry adaptation but does require significant holding times for carcasses.

Solomon *et al.* (1998) used a different approach to generate myofibrillar fractures and produce more tender longissimus chops from lambs with the muscle hypertrophy phenotype. The Hydrodyne process uses a small amount of high-energy explosive to generate a supersonic-hydrodynamic shock wave in water. This shock wave passes through objects in the water that are an acoustical match with water. Shear force results indicated that the Hydrodyne process was effective at instantaneously tenderizing longissimus muscle from lambs with the muscle hypertrophy phenotype. This improvement in tenderness was further enhanced if used in conjunction with carcass electrical stimulation. Shear force values comparable to aged normal longissimus chops were obtained with this treatment protocol. A multi-institutional study with reasonably large numbers of observations directly compared several post-mortem treatments (Leckie *et al.*, 1997). The most effective methods were calcium chloride injection and the Hydrodyne process. Both methods were able to produce shear force values comparable to normal chops aged for 14 days. Similar to nearly all of the post-mortem intervention strategies, implementation of an online system for use in a packing facility that is cost-effective in a traditional fresh-lamb market is the primary limitation to the Hydrodyne process.

A few additional post-mortem intervention strategies have been attempted, including high temperature conditioning, blade/needle tenderization and tropical plant enzyme applications. Results from these experiments have either not been published or produced inconsistent results and have limited information. Clearly, many treatments have been attempted and much needs to be done to provide a cost-effective and useful treatment.

15.10 Conclusion

The callipyge phenotype in sheep is the result of a naturally occurring mutation producing dramatic effects on muscle development and carcass composition through altered fibre type distribution (increased fast-twitch glycolytic), increased average fibre diameter and decreased fat deposition. This hypertrophy condition was more pronounced in muscles of the hind leg and loin regions. Genetic location of the mutation (*CLPG*) was mapped to the distal end of sheep chromosome 18 and subsequently identified as a single nucleotide polymorphism in an imprinted domain region between the *DLK1* and *MEG3* genes. Imprinting status of several genes in this region was not changed by the mutation but expression levels of several transcripts have been altered. Paternally expressed transcripts are upregulated when the mutation is present on the paternal gamete and maternally expressed transcripts are upregulated when the mutation is present on the maternal gamete. Gene action exhibited at *CLPG* is referred to as paternal polar overdominance, due to the expression of the muscle hypertrophy phenotype only in sheep that receive a mutated allele from the sire and a normal allele from the dam. Large favourable effects of this mutation on slaughter and carcass composition traits have been identified. However, adverse effects on meat tenderness require an intervention strategy to ensure a favourable eating experience. Intervention strategies have been effective at decreasing the tenderness problem but remain difficult to implement on a commercial basis.

Discovery of the causative polymorphism for *CLPG* muscle hypertrophy marks the starting point for future research efforts to determine the mechanism by which

this single base change leads to such marked phenotypic alterations. New insights into basic biology of imprinting regulation in this important region of the genome for livestock and humans will certainly be an outcome of basic research in this area. Functional interactions among gene products and regulatory elements or transcripts from this genetic region will need to be evaluated in greater detail. From a sheep production standpoint, implementation of this mutation in a terminal sire crossbreeding system where the paternal population is fixed for the mutated allele and the maternal population is fixed for the normal allele is now manageable with the availability of a diagnostic test. Evaluation of germplasm resources, both within and between breeds, that could be used in combination with the *CLPG* locus to shift the distribution of longissimus shear force to an acceptable level is a high priority. A genetic solution or an easily adaptable post-mortem intervention strategy that solves the tenderness antagonism would have great impact on lean meat production in sheep. Detection of genomic regions favourably influencing tenderness would allow opportunities to select within specialized sire lines to both capture the benefits and mitigate the antagonisms associated with this mutation.

Disclaimer

Mention of trade names or commercial products in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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16 Genetic Control of Intramuscular Fat Accretion

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16.1 Introduction

Intramuscular fat (IMF) content of the meat is an important attribute in meat quality characteristics such as tenderness and taste (reviewed by Hovenier *et al.*, 1993; Verbeke *et al.*, 1999). Before going into these relationships it should be noted that different definitions of IMF are commonly used in sport physiology and animal sciences. The first defines IMF as the proportion of fat in the myocytic cells alone whereas for the latter IMF is the proportion of fat in a sample of muscle devoid of

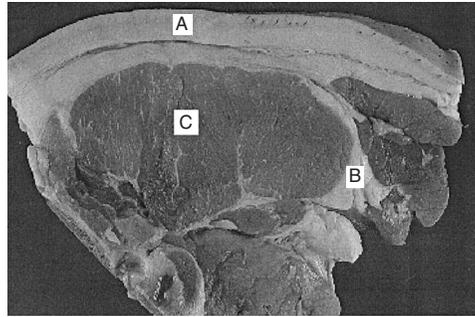


Fig. 16.1. Image of pig muscle and surrounding tissue showing (A) subcutaneous fat, (B) intermuscular fat surrounding the muscle tissue and (C) intramuscular fat between muscle fibres within the muscle tissue.

fat surrounding muscles, i.e. intermuscular fat (Fig. 16.1). Thus, IMF is the chemically extractable fat in a muscle sample, therefore originating predominantly from adipocytes and myocytes. This latter definition will be applied in this chapter.

Most of the research into the relationship of IMF with meat quality has been performed in pigs and less so in beef cattle. Therefore pigs will be the main subject of this chapter unless stated otherwise.

In the first part of this chapter the effects of IMF content on meat quality will be reviewed. In the remaining part different strategies to improve IMF content of meat will be reviewed and discussed.

16.2 Intramuscular Fat Physiology

16.2.1 Fat deposition

Generally, four major fat depots are recognized, the subcutaneous, internal organ-associated, and inter- and intramuscular fat depots. Substantial differences in fat distribution are present across and within species. In pigs, the subcutaneous fat depot is the most pronounced, averaging 60 to 70% of total fatty tissue at slaughter, whereas the internal organ-associated and the intermuscular fat depots average about 10 to 15% and 20 to 35%, respectively (Kuhn *et al.*, 1997; Kouba *et al.*, 1999). In cattle, the intermuscular fat depot is the most important site of fat deposition at slaughter with 45% of total carcass fat; the internal organ-associated and subcutaneous fat depots average 38% and 17%, respectively (Wegner *et al.*, 1997). Obviously, differences in body fat distribution between species are genetically determined but this is also true within species. This is shown by genetic correlations between IMF content and backfat thickness in pigs (0.37; Hovenier *et al.*, 1992) and differences in development of intermuscular fat relative to subcutaneous fat between genetically different pigs (Kouba *et al.*, 1999). Interestingly, even within major fat depots, genetically determined differences can occur. For example, in humans the relative fat patterning between different subcutaneous depots was shown to be recessively inherited (Hasstedt *et al.*, 1989).

Besides body fat distribution, fat deposition in each depot itself is also under genetic control. In humans, mice and also livestock animals the genetic control of fat deposition is of major interest because of the links between obesity and diabetes, coronary artery disease and atherosclerosis. This has resulted in the identification of various genes involved in fat metabolism, in particular those involved in obesity. In this respect, leptin and its receptor have been shown to be important components of fat deposition regulation (reviewed by Friedman and Halaas, 1998).

We will continue by focusing on the subject of this book, muscle physiology, and hence the intramuscular fat depot.

16.2.2 Intramuscular fat

Morphologically, IMF is the total of lipids associated with all cells present in a meat sample, mainly myocytes and adipocytes, but excluding adipocytes from the intermuscular fat depot. Chemically, these lipids can be subdivided into phospholipids, triacylglycerols (TAG), mono- and diacylglycerols, cholesterol and cholesteryl esters, and free fatty acids. Phospholipids and TAG are the major constituents of IMF whereas the contribution of the other lipids is only marginal. However, the contribution of each of these constituents will vary with the method to determine IMF content: for example with the polarity of the extraction solution and the use of acid pretreatment (Reichardt *et al.*, 1998).

Phospholipids are the main constituents of cellular membranes and their contribution to IMF content in pigs (0.4–0.5%) is nearly constant within similar muscles (Lazo *et al.*, 1994) as was shown for cattle (Ender *et al.*, 1997), rabbits (Gondret *et al.*, 1998) and poultry (Marion, 1965). Between muscles, phospholipid content can differ; the proportion of phospholipids increases from white glycolytic to red oxidative muscle types (Leseigneur-Meynier and Gandemer, 1991). This observation is most likely due to smaller fibre diameters and a higher proportion of mitochondrial membranes in oxidative-type muscles.

Generally, an increase in IMF content is mainly due to an increase in TAG content as substantiated in different studies with pigs (Cameron and Enser, 1991; Essen-Gustavsson *et al.*, 1994; Fernandez *et al.*, 1999) and rabbits (Gondret *et al.*, 1998). Consequently, TAG metabolism in muscle tissue should be the subject for identification of genes involved in genetic variation in IMF deposition.

16.2.3 Triacylglycerol in muscle

Triacylglycerol contained within muscle is an important and readily mobilized source of energy during exercise. In muscle, TAG are stored in adipocytes and myocytes. Proton nuclear magnetic resonance analysis showed that two fat compartments exist in muscle tissue with similar fatty acid and triacylglycerol compositions (Schick *et al.*, 1993). One compartment corresponded to intramuscular adipocytes whereas the other was suggested to be fat stored in myocytes. Little information is available regarding the role of each distinct intramuscular fat depot in the variation of IMF content.

Within myocytes, TAG are stored in depots, mostly adjacent to mitochondria. The total TAG content in human muscles ranges from 0.56 to 3.33% depending on

fibre type, nutrition and physical exercise (reviewed by Jeukendrup *et al.*, 1998). Trained individuals have more intramuscular fat, which is in accordance with the higher utilization of TAG. In contrast, increased TAG accumulation within muscles of sedentary individuals is associated with insulin resistance and obesity (reviewed by Goodpaster and Kelley, 1998). However, it is still unclear whether elevated muscle TAG content causes insulin resistance or arises as a consequence.

Furthermore, it is well documented that oxidative (type I) muscle fibres contain considerably more intracellular TAG than glycolytic (type IIa and IIb) fibres (for more information about muscle fibre types see Reggiani and Mascarello, Chapter 2, this volume). However, the TAG content in muscles is only to a minor extent related to differences in muscle fibre type composition, but mainly due to variation in the accumulation of adipocytes between muscle fibres (Kaufmann and Safani, 1967; Gondret *et al.*, 1998). The fact that no correlation was found between TAG content and fibre type composition of muscles of different metabolic type in pigs agrees well with this conclusion (Leseigneur-Meynier and Gandemer, 1991; Essen-Gustavsson *et al.*, 1994). With respect to the IMF depot, age-related changes are due to adipocyte hyperplasia as well as hypertrophy in cattle, pigs and rabbits (Hood and Allen, 1973; Lee *et al.*, 1973; Gondret *et al.*, 1998). Thus both adipocyte hyperplasia and hypertrophy may be involved in genetic variation in IMF content in pigs. It should be noted that this has no consequences for consumer acceptability of meat due to visibility of fat because in the optimal range of 2.5–3% IMF, no fat is visually detectable in pig meat (Van der Wal *et al.*, 1992).

Thus, IMF content can be optimized in three ways: (i) optimized number of intramuscular adipocytes (hyperplasia); (ii) optimized TAG content in adipocytes (adipocyte hypertrophy); and (iii) optimized TAG content in myocytes. Obviously and most likely, combinations of those three might be responsible. In order to propose candidate genes that contribute to genetic variation in IMF content, each of these opportunities will be considered in the next sections.

16.2.4 Adipocyte hyperplasia

Adipocytes are highly specialized cells serving the crucial function of storage, metabolism and release of lipids. Unfortunately the pattern and regulation of normal adipocyte growth and differentiation are yet incompletely understood. Adipocytes are terminally differentiated cells that develop from multipotent stem cells of mesodermal origin that also give rise to the muscle (myocytes) and cartilage (chondrocytes) lineages. Studies in animals and humans have demonstrated that the potential to generate new fat cells continues throughout the lifespan (reviewed by Smas and Sul, 1995; Prins and O'Rahilly, 1997). Moreover, reduction of adipocyte number occurs by adipocyte apoptosis and possibly dedifferentiation (reviewed by Prins and O'Rahilly, 1997).

Adipocyte differentiation is influenced by a large number of mitogens and growth factors (reviewed by Hwang *et al.*, 1997). With regard to the transcriptional control of adipocyte differentiation, knowledge has been derived mainly from tissue and cell culture models, in particular the mouse 3T3-L1 preadipocyte cell line. In recent years some of the proteins regulating early differentiation of adipocytes have been identified (reviewed by: Smas and Sul, 1995; Hwang *et al.*, 1997; Castillo *et al.*,

1999; Lane *et al.*, 1999; Lazar, 1999). For example, during adipogenesis several transcription factors are involved, such as c-jun, c-fos, Sp1, CCAAT/enhancer binding protein (C/EBP), adipocyte determination and differentiation-dependent factor 1 (ADD1 or SREBP), C/EBP α undifferentiated protein (CUP or AP-2 α) and peroxisome proliferator-activated receptor (PPAR). The C/EBP and PPAR transcription factor families have been studied in most detail because certain members of these protein families are essential for adipocyte determination and differentiation. Namely, ectopic expression of C/EBP α and PPAR γ converts fibroblastic cells into adipocytes (reviewed by Hwang *et al.*, 1997). Interestingly, long-chain fatty acids as well as prostaglandins are potent activators of PPARs and hence induce adipogenesis in heterologous cell systems. The proposed mechanism is that PPAR δ can be activated by long-chain fatty acids and induces PPAR γ expression, which upon specific activation promotes adipocyte differentiation in fibroblasts (Bastie *et al.*, 1999). Interestingly, alleles of PPAR γ 2 have been associated with human obesity (Ristow *et al.*, 1998).

As well as in adipocytes, fatty acids themselves are also regulating fatty acid metabolism in cultured rat neonatal cardiac myocytes, but here PPAR α rather than PPAR γ is suggested to be involved (Van der Lee *et al.*, 2000). This observation is in agreement with the observation that the PPAR γ 2 isoform is expressed almost exclusively in adipose tissue whereas the PPAR γ 1 isoforms, PPAR α and PPAR δ , are expressed in a wider range of tissues and cell types (reviewed by Lazar, 1999). This tissue-dependent expression has also been demonstrated for porcine PPAR γ 1 and PPAR γ 2 isoforms (Grindflek *et al.*, 1998; Ding *et al.*, 1999; Kim *et al.*, 2000).

In conclusion, the search for the master regulatory factor in adipocyte differentiation still continues and identification may lead to understanding of those factors that switch on adipocyte determination and differentiation. This may also give insight into the key factors that ultimately regulate adipocyte hyperplasia.

16.2.5 Adipocyte hypertrophy

During adipocyte differentiation, TAG are continually deposited in lipid droplets that coalesce into larger droplets. This process progresses until most of the cell volume is occupied by a single large lipid droplet leading to hypertrophy. All tissues increase in mass due to a combination of hyperplasia and hypertrophy but the ratio between both processes varies between tissues and within the same tissue of different species. In cattle, sheep and pigs hypertrophy of adipose cells can be easily 1000-fold in volume as cells grow from 15 to more than 150 μm in diameter (Mersmann, 1991). During the first 4 weeks after birth, subcutaneous adipocyte volume increases almost ninefold in pigs (Mersmann *et al.*, 1997).

The synthesis of TAG in adipocytes and also myocytes proceeds from fatty acids synthesized *de novo* or supplied by the diet. The major sites for lipid synthesis are the liver and adipose tissue but differences occur between species with respect to the relative importance of each tissue. In humans the liver is the main site of *de novo* fatty acid synthesis, in pigs adipose tissue is the most important site, whereas in rat and mouse both tissues are contributing (reviewed by Mersmann, 1986). In pigs, *de novo* fatty acid synthesis plays a major part in the origin of fatty acids because diets consist predominantly of carbohydrates (Mersmann, 1986). Fat metabolism of intramuscu-

lar adipocytes is in part identical to myocytes and therefore these will be described jointly in the next section.

16.2.6 Muscle fatty acid metabolism

Recently, Hocquette *et al.* (1998) reviewed the role of TAG in energy metabolism, its control and the consequences of manipulating muscle energy metabolism in meat-producing animals. Here we focus on the fatty acid metabolism of the muscle tissue, in particular within myocytes and adipocytes.

The mechanism of uptake of fatty acids by intramuscular adipocytes and myocytes is facilitated by the enzyme lipoprotein lipase (LPL) located on the capillary endothelial cells, which binds and hydrolyses lipoprotein TAG (see Fig. 16.2). Overexpression of human LPL in mouse myocytes and cardiomyocytes is associated with a small decrease in body fat suggesting that LPL may be a rate-limiting step in fatty acid uptake (Levak-Frank *et al.*, 1995). However, manipulation of LPL expression proved to be hazardous. Overexpression of LPL was associated with extensive proliferation of mitochondria and peroxisomes and development of lethal myopathy. Furthermore, amino acid substitutions identified in human LPL are associated with coronary artery disease, atherosclerosis and/or obesity (reviewed by Murthy *et al.*, 1996).

Fatty acids are translocated across the endomysial and sarcolemmal membranes and taken up by intramuscular adipocytes or myocytes. Fatty acids can be translo-

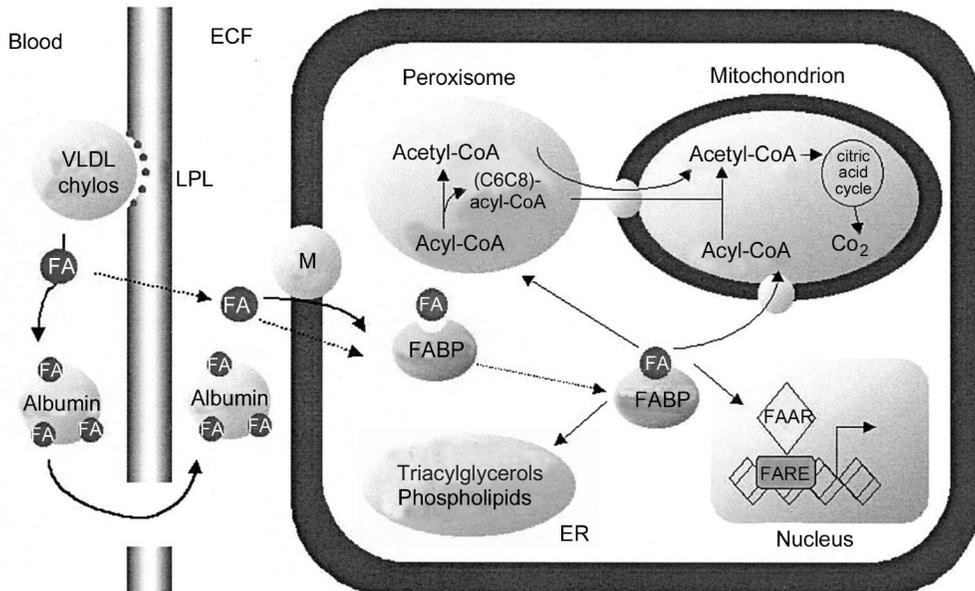


Fig. 16.2. Schematical representation of the fatty acid trafficking from plasma to intracellular compartments. Abbreviations: FA, fatty acids; VLDL, very low density lipoproteins; Chylos, chylomicrons; LPL, lipoprotein lipase; ECF, extracellular fluid; M, membrane-associated fatty acid transport proteins; ER, endoplasmic reticulum; FAAR, fatty acid-activated receptor; FARE, fatty acid-responsive element (from Veerkamp and Maatman, 1995).

cated across these membranes by a simple diffusion mechanism or facilitated by membrane-associated proteins such as plasma membrane fatty acid-binding protein (FABP_{pm}), fatty acid translocase (FAT or CD36) and fatty acid transporter protein (FATP) (reviewed by Van Nieuwenhoven *et al.*, 1996; Abumrad *et al.*, 1999). The process of fatty acid uptake is not yet fully understood but various parts of this process have been reviewed recently (Luiken *et al.*, 1999; Glatz and Storch, 2001). Although simple diffusion of fatty acids may occur, the major fraction of cellular fatty acid uptake is protein facilitated under physiological conditions (Abumrad *et al.*, 1999). This is supported by FAT (CD36) deficiency in rats and man, and over-expression of FAT, which results in reduced peripheral triacylglycerols and non-esterified fatty acids (Aitman *et al.*, 1999, and references therein).

Intracellularly, fatty acids are bound by fatty acid-binding proteins (FABPs), which are considered to be the important carriers for intracellular fatty acids. FABPs facilitate the transport of fatty acids from the plasma membrane to the sites of fatty acid oxidation or to the sites of fatty acid esterification into TAG or phospholipids. Moreover, FABPs may also be involved in bidirectional transport of fatty acids in adipocytes. In this respect the interaction between the hormone-sensitive lipase (HSL) and adipocyte-specific FABP (A-FABP) may be of significance (Shen *et al.*, 1999). Moreover, HSL is also present in skeletal muscle-derived myofibres (Langfort *et al.*, 1999).

Other functions of FABPs are thought to be the protection of the cell from the deleterious effect of high concentrations of intracellular free fatty acids, and modulation of the action of (long-chain) fatty acids and other ligands, hence influencing enzymes, membranes, receptors, ion channels or genes (Veerkamp *et al.*, 1993; Graber *et al.*, 1994; Veerkamp and Maatman, 1995; Glatz and Van der Vusse, 1996, and references therein). The role of FABPs in regulating fatty acid-dependent cell signalling has been reviewed by Glatz *et al.* (1995).

16.3 Intramuscular Fat and Meat Quality

Due to the increased consumer awareness with respect to eating quality and nutritional aspects of meat, qualitative aspects of meat are attracting increasingly more attention. Several studies involving consumer and trained panels revealed that IMF content is one of the important traits that influences eating quality characteristics such as meat tenderness, juiciness and taste (reviewed by Hovenier *et al.*, 1993; Verbeke *et al.*, 1999). Although some studies were unable to detect this relationship, it is now generally agreed that variation in IMF content explains an important part of the genetic variation in eating quality of porcine meat (Wood and Cameron, 1994). Other recent studies have confirmed this relationship (Kirchheim *et al.*, 1997; Fernandez *et al.*, 1999).

The IMF content of porcine meat is an optimum trait with respect to the influence of meat tenderness (reviewed by Hovenier *et al.*, 1993). An IMF content below the recommended optimum range of 2.5–3% diminishes eating quality whereas a higher IMF content will not further improve this parameter and will have adverse effects on consumer acceptability due to increased visibility of fat in the meat (i.e. marbling).

In the early 1990s, IMF content was already well below the desired range of 2.5

to 3% in pigs produced in Europe (De Vries *et al.*, 1994; Casteels *et al.*, 1995; Cameron *et al.*, 1999; Von Rohr *et al.*, 1999, and references therein) but not in pigs produced in the USA (NPPC, 1995). In addition, IMF content in pigs has been decreasing ever since as a result of unfavourable genetic correlations with the selection criteria of higher lean meat content and reduced backfat thickness. For example, each percentage improvement in carcass lean percentage would be accompanied by a reduction in IMF content of 0.07% (De Vries *et al.*, 1994). Therefore, strategies to improve IMF content need to be considered.

Ultimately, the implementation of strategies to improve meat quality depends on the willingness of consumers to purchase and pay more for meat of better quality. To market meat with better eating quality, consumers need to be able to discriminate between different categories of eating quality, thus favouring meat grading, branding and labelling. It was concluded from a survey among Dutch consumers that the willingness to pay extra for meat with improved eating quality depends on the kind of meat (e.g. chops or steaks), the attitude towards quality and the age of consumers (Steenkamp and Van Trijp, 1988). Recently, a survey among meat quality experts from the Swiss slaughter and retail industry reported a willingness to pay different prices for carcasses from different quality classes for several meat quality traits including IMF content (Von Rohr *et al.*, 1999). This indicates that improvement of meat quality by optimizing IMF content will not only be appreciated by the consumer but also be profitable to the meat industry.

16.4 Strategies to Improve Intramuscular Fat Accretion

In order to improve IMF content of pig meat, different strategies can be exploited. The most obvious strategy would be to take advantage of the positive genetic correlation between IMF content and body weight; that is, a higher weight at slaughter would result in increased IMF content and hence improved meat quality. However, this strategy would result in a lower lean percentage of the carcass and would need a new conformation of the slaughter industry to higher carcass weights.

The most promising strategy is based on the observation that IMF content can be improved by selection. Heritability estimates (h^2) for IMF content indicate substantial genetic variation in this trait but differ considerably among studies from 0.26 to as high as 0.86 with an average of 0.5 (reviewed by Hovenier *et al.*, 1993; De Vries *et al.*, 1994; Sellier, 1998; Verbeke *et al.*, 1999). Such a high heritability estimate indicates a considerable selection response in direct selection programmes. However, genetic correlations of IMF content with production and other meat quality traits and the accuracy of current methods to assess IMF content should also be taken into account.

16.4.1 Genetic correlations with production traits

As stated above, each of the four major fat depots is genetically associated with the others. Genetic correlation estimates between IMF content and carcass fatness traits range from 0.04 to 0.60 with an average of 0.30 (reviewed by Sellier, 1998). Similarly, genetic correlation estimates between IMF content and carcass leanness

traits range from -0.55 to -0.07 with an average of -0.34 (reviewed by Sellier, 1998). These genetic correlations indicate that selection for increased IMF content will have adverse effects on the efficiency of selection on production traits. However, the size of the genetic correlations indicates that substantial genetic variation in IMF content is independent of these main production traits. In other words, selection for increased IMF content is feasible especially in situations where improvement in meat quality rather than production traits would be economically more beneficial.

16.4.2 Genetic correlations with other meat quality traits

Intramuscular fat content has been reported to be genetically correlated to various other meat quality traits such as drip loss, water-holding capacity, cooking loss, reflectance, tenderness, juiciness, overall acceptability, marbling, flavour and firmness of the meat (reviewed by Hovenier *et al.*, 1992; Sellier, 1998; Huff-Loneragan *et al.*, 2002; see also Hopkins and Taylor, Chapter 17, this volume, and Freking *et al.*, Chapter 15, this volume). Some of these correlations are considerable, such as that between IMF and firmness of the meat (0.31; Huff-Loneragan *et al.*, 2002) and between IMF content and overall acceptability (0.61; Cameron, 1990). Although for other meat quality traits these genetic correlations are only marginal, these correlations do indicate that selection for increased IMF content will affect other meat quality traits as well.

16.4.3 Methods to assess intramuscular fat content

As well as unfavourable genetic correlations with production traits, conventional selection for IMF content is not very effective for two additional reasons. First, IMF content must be determined in slaughtered animals excluding these animals from further selection. Therefore, selection is based on data from slaughtered siblings and hence genetic improvement is less optimal and accurate. Furthermore, the currently applied fat extraction methods to determine IMF content are laborious and expensive. These limitations can be circumvented by developing and introducing accurate, non-invasive, cost-effective techniques to assess IMF content in live pigs. One such technique is real-time ultrasound scanning, which has recently been shown to allow assessment of IMF content in live pigs (Newcom *et al.*, 2002), making direct selection on IMF content in pigs more feasible in the near future.

16.5 Marker-assisted Selection of Intramuscular Fat Content

Another technique is the direct exploitation of the genes or genomic loci that control IMF content. The size of the genetic correlations between IMF content and production traits, as mentioned above, indicate that part of the genetic variation is independently inherited. In other words, these traits are partially controlled by different genes. Hence, traits may be treated independently when the respective genes that contribute to the genetic variation of IMF content but not of production traits are identified. Evidence for the existence of genes with a substantial effect on IMF

content has been provided by segregation analysis in pigs (Janss *et al.*, 1997). Upon identification, these polymorphic genes or genomic regions may be applied in breeding by eliminating detrimental allele(s) from the population, by marker-assisted selection (MAS; see Meuwissen and Goddard, 1996; Spelman and Bovenhuis, 1998) or by introgression of beneficial allele(s) in populations or breeds lacking these alleles (Visscher *et al.*, 1996). MAS can considerably increase the selection response, in particular for traits with a low heritability and/or for carcass and meat quality traits, such as IMF content (Meuwissen and Goddard, 1996). To identify the genes and genomic loci that are responsible for genetic variation in IMF content two approaches can be employed: the genome scan and candidate gene analysis approach.

16.5.1 Quantitative trait loci

The genome scan approach uses large numbers of available genetic markers and linkage maps to identify loci affecting quantitative traits (QTL). This approach relies on the premise that trait-affecting alleles will be in linkage disequilibrium with flanking genetic markers over a sufficiently large distance that association can be detected. However, the relatively large marker intervals used (typically 10–20 cM) allow only linkage disequilibrium mapping within families, as opposed to across families in a population, with inherently low accuracy to locate genes.

The advantage of the genome scan approach is that all genomic regions that have a substantial contribution to the genetic variation of the trait of interest will be identified. However, the reliability of identifying real QTL is of some concern because of the large number of tests performed in the analysis that are not independent, as markers or marker brackets are situated on the same chromosome. Moreover, the size of QTL regions is of major concern, because smaller QTL regions increase the genetic response from MAS (Spelman and Bovenhuis, 1998) and reduce the number of positional candidate genes substantially.

Recent technological advances such as microarray-based genotyping of single nucleotide polymorphisms (SNPs) will eventually lead to high-density QTL maps and more accurate determination of the responsible loci.

With respect to IMF content several total or partial genome scans have been performed in different pig populations (Table 16.1). In a Meishan × Large White and Landrace F2 crossbred pig population initial autosomal analysis revealed suggestive QTL affecting IMF content on chromosomes 2, 4 and 6 (line cross model) and 4 and 7 (half-sib model) (De Koning *et al.*, 1999). Adding a single marker to chromosomes 4 (FABP4) and 6 (FABP3) in the line cross analysis revealed only a suggestive QTL affecting IMF content for chromosome 6 but not for chromosome 4 (Gerbens *et al.*, 2000). Moreover, another seven additional markers to chromosome 4 removed the ‘half-sib’ QTL whereas the suggestive QTL affecting IMF content under line cross assumptions was regained (Rattink *et al.*, 2000). Analysis of the X chromosome revealed a highly significant QTL affecting IMF content in the same population (Harlizius *et al.*, 2000). Another autosomal genome scan based on line cross assumptions and accounting for imprinting identified two significant QTL affecting IMF content on chromosome 6, one maternally and one paternally expressed (De Koning *et al.*, 2000).

Table 16.1. Reported main QTL for intramuscular fat content or its related traits, muscle total lipid percentage and marbling, in various experimental crosses of pigs.

Pig population	Chromosome(s) scanned	Quantitative trait locus		Reference
		IMF	BFT	
Meishan × Large White or Landrace	1–18, X	4	No	Rattink <i>et al.</i> (2000)
		6p	No	De Koning <i>et al.</i> (2000)
		6q	Yes	De Koning <i>et al.</i> (2000)
		X	Yes	Harlitzius <i>et al.</i> (2000)
Meishan × Large White	1–18, X	7	Yes	Bidanel <i>et al.</i> (1998)
Meishan × Large White	1–18	4p	No	Paszek <i>et al.</i> (2001)
		4q	Yes	Paszek <i>et al.</i> (2001)
		6p	Yes	Paszek <i>et al.</i> (2001)
		6q	Yes	Paszek <i>et al.</i> (2001)
		12	No	Paszek <i>et al.</i> (2001)
Duroc × Norwegian Landrace × Large White	4, 6, 7	6	No	Grindflek <i>et al.</i> (2001)
Iberian × Landrace	1–18	6	Yes	Ovilo <i>et al.</i> (2002a)
	X	X	ND	Perez-Enciso <i>et al.</i> (2002)
Berkshire × Large White	1–18, X	1	Yes	Malek <i>et al.</i> (2001)
		2	No	Malek <i>et al.</i> (2001)
		8	No	Malek <i>et al.</i> (2001)
		10	No	Malek <i>et al.</i> (2001)
Meishan × Duroc or Hampshire or Landrace	13	13	Yes	Yu <i>et al.</i> (1999)

ND, not determined; IMF, intramuscular fat; BFT, back fat thickness.

In another Meishan × Large White crossbred pig population, a significant QTL affecting IMF content was reported on chromosome 7 (Bidanel *et al.*, 1998). On the other hand, Malek and co-workers (2001) identified significant QTL affecting total lipid content of muscle and marbling close to each other on chromosome 1 in a Berkshire × Large White crossbred population. Moreover, a significant QTL affecting IMF content was identified on chromosome 6 in a Duroc × Norwegian Landrace × Large White population (Grindflek *et al.*, 2001) as well as in an Iberian × Landrace population (Ovilo *et al.*, 2000, 2002a). In this latter pig population a QTL affecting IMF content was also reported for chromosome X (Perez-Enciso *et al.*, 2002).

These results should be interpreted cautiously since several studies did not scan the complete (autosomal) genome (see Table 16.1). In these studies, other chromosomes may also contain QTL for IMF in the respective pig populations. Moreover, results from QTL studies may vary according to the prior assumptions in the analyses (De Koning *et al.*, 1999) as well as due to the genetic background of the pigs under investigation.

Despite these reservations, it is worth mentioning that several studies report chromosome 6 to contain one or two QTL that affect genetic variation of IMF content. These QTL are located in a similar region on the long arm of chromosome 6 (De Koning *et al.*, 1999, 2000; Gerbens *et al.*, 2000; Ovilo *et al.*, 2000, 2002a;

Grindflek *et al.*, 2001). However, the QTL region in each study is still too large to identify positional candidate genes. However, a joint analysis of all available material for IMF content in a similar approach to that taken for backfat thickness (Walling *et al.*, 2000) may prove these QTL to be genuine and may significantly reduce the QTL region.

Actual application of QTL data in breeding schemes depends on the absence of genes that adversely affect other important breeding traits such as average daily gain and backfat thickness in the QTL region affecting IMF content. It should be noted that a lot of the aforementioned QTL affecting intramuscular fat content are in the proximity of QTL affecting backfat thickness and eventually may prove to be the same genes.

16.5.2 Candidate genes

In addition to the positional candidate genes from the QTL regions affecting IMF content mentioned above, genes may also be candidates based on existing knowledge of physiological and biochemical processes, also known as the candidate gene approach. From a statistical genetic point of view, the candidate gene approach is a linkage disequilibrium mapping approach using linkage disequilibrium across families in a population. Benefits of the candidate gene approach are the relative straightforwardness and low costs. However, success largely depends on the amount and quality of prior information to identify candidate genes and the pig population under investigation.

Various candidate gene approaches in livestock have been successful in identifying linkage association (*ESR*, *A-FABP*, *H-FABP*, *FUT1*, *MC4R*, *MYOG*, *PRLR*) or even causative relationships (*GDF-8*, *Ryr-1*, *KIT*, *MSHR*) with various traits like (re)production, disease resistance, meat quality and coat colour traits (reviewed by Rothschild, 1998). With respect to IMF content there are only three candidate genes reported to have associations, *FABP3*, *FABP4* and *LEPR*. These genes will be described here in more detail.

The *FABP3* gene encoding muscle-specific fatty acid-binding protein (H-FABP) was significantly associated with genetic variation in IMF content, backfat thickness and growth in purebred Duroc pigs (Gerbens *et al.*, 1999). The *FABP3* gene resides on porcine chromosome 6 within or close to the QTL region affecting IMF content identified in several crossbred pig populations (De Koning *et al.*, 1999; Ovilo *et al.*, 2000; Grindflek *et al.*, 2001). Subsequent analysis showed that H-FABP genotypes have a significant effect on IMF content in the Duroc × Norwegian Landrace × Large White crossbred population (Grindflek *et al.*, 2000), in a Iberian × Landrace crossbred population (Ovilo *et al.*, 2002b) and a considerable but non-significant effect in a Meishan × Large White and Landrace population (Gerbens *et al.*, 2000). On the other hand, others found no significant influence of the *FABP3* gene on IMF content in Austrian Piétrain, Large White and Landrace breeding populations (Nechtelberger *et al.*, 2001) or Australian commercial pigs (Chen *et al.*, 2000).

Obviously, a number of genes have been shown to be closely linked to the *FABP3* gene in human and mice. One particular gene should be highlighted, the leptin receptor gene (*LEPR*), which mediates the effects of leptin, the major hormonal controller of long-term energy balance (Friedman and Halaas, 1998). The

FABP3 and *LEPR* genes are located about 20 cM apart on porcine chromosome 6 with *LEPR* telomeric to the *FABP3* gene (Stratil *et al.*, 1998; Ovilo *et al.*, 2002b). Candidate gene analysis showed that the *LEPR* gene is also significantly associated with IMF content (Ovilo *et al.*, 2002b).

Of course, correlated traits may result in similar effects in candidate gene analyses. The *FABP3* gene also affects backfat thickness in the purebred Duroc pigs but these effects were shown to be independent (Gerbens *et al.*, 1999). Similar results were found by Ovilo *et al.* (2002b) for the *FABP3* gene but not for the *LEPR* gene. Grindflek and co-workers (2001) found no QTL for backfat thickness on chromosome 6 indicating also an independent association with IMF content.

Taking all data into account, the existence of significant associations with IMF content for *FABP3* and *LEPR* in independent and distinct pig populations indicates that these or closely linked genes may be responsible for part of the genetic variation in IMF content in particular pig breeds or populations. Data suggest that two QTL may reside in a similar region, one affecting IMF content and the other backfat thickness. So far, a causal relationship with variation in IMF content has not been reported for either *FABP3* or *LEPR*. However, H-FABP variants may still exist in pigs. Interestingly, naturally occurring protein variants were identified for human and cattle H-FABP (Bartetzko *et al.*, 1993; Phelan *et al.*, 1996) and human intestinal-specific FABP (I-FABP) (Baier *et al.*, 1995). In general, variation in the I-FABP-encoding gene has been shown to affect energy metabolism in several ethnic human populations (reviewed by Hegele, 1998).

The *FABP4* gene encodes adipocyte-specific FABP (A-FABP) and was shown to be significantly associated with IMF content independently from backfat thickness in purebred Duroc pigs (Gerbens *et al.*, 1998). The *FABP4* gene resides within a QTL region affecting IMF content on chromosome 4 in a Meishan × Large White and Landrace pig population (De Koning *et al.*, 1999) and other fatness traits in other pig populations (Andersson *et al.*, 1994; Knott *et al.*, 1998; Walling *et al.*, 1998; Perez-Enciso *et al.*, 2000). However, the effect of *FABP4* on fatness traits could not be substantiated in these populations (Gerbens *et al.*, 2000; Andersson, personal communication) or in other Austrian pig breeds (Nechtelberger *et al.*, 2001). Neither do the results of QTL studies suggest a general QTL affecting IMF content on chromosome 4 (Grindflek *et al.*, 2001; Malek *et al.*, 2001; Ovilo *et al.*, 2002a). In conclusion, in specific pig breeds, genes closely linked to the *FABP4* gene on chromosome 4 of the pig are most likely to be responsible for the effect on IMF content.

16.6 Prospects for Breeding on Intramuscular Fat

With several genome scans being performed a number of genomic regions have been identified as affecting IMF content in various pig breeds. The challenge for the near future is to identify positional candidate genes that will be responsible for the observed genetic variation in IMF content. Meta-analysis of several genome scan studies involving IMF content and fine mapping of these QTL regions may help to more accurately pinpoint the interesting genomic regions. This, together with the ongoing elucidation of genes involved in fatty acid metabolism and site-specific fat deposition, should eventually lead to the genes controlling the amount of fat deposited in muscle tissue.

In this respect a considerable number of proteins that are physically involved in fatty acid uptake and trafficking have already been elucidated and in some cases shown to be rate-limiting (reviewed by Abumrad *et al.*, 1999; Luiken *et al.*, 1999). On the other hand, information on the factors that regulate these genes, such as the PPAR family and long-chain fatty acids, is just emerging (Bastie *et al.*, 1999). Interestingly, the *PPAR γ* gene was shown to be associated with several meat quality traits but not IMF content in pigs (Emnett *et al.*, 2000). Others have described a considerable number of genes involved in human adipocyte differentiation and obesity (Bray and Bouchard, 1997; Hwang *et al.*, 1997). Furthermore, the lamin A/C gene (*LMNA*), which causes partial lipodystrophy syndrome in humans (Shackleton *et al.*, 2000), may also give insights into the factors regulating site-specific fat deposition.

Insulin resistance may also be important because this is associated with increased TAG content in skeletal muscle. Some proteins have been shown to be involved in the mechanisms that lead to insulin resistance such as TNF- α and both its receptors, A-FABP and possibly free fatty acids and leptin (reviewed by Hotamisligil, 1999; Scheja *et al.*, 1999). Indeed, a TNF- α promoter polymorphism was associated with obesity in women but not in men (Hoffstedt *et al.*, 2000) and the region containing the *TNF- α* gene on SSC7 is implicated in the variation of back fat thickness in various pig populations (Wang *et al.*, 1998, and references therein). However, there is evidence that insulin resistance is a consequence of elevated TAG content and not the cause (reviewed by Goodpaster and Kelley, 1998).

Finally, information on validated responsible genes can be used in marker-assisted selection of pigs within, of course, the context of standard breeding purposes. Therefore, these gene effects on IMF content should not be paralleled by adverse effects on other fatness or performance traits such as backfat thickness, lean growth or average daily gain. Moreover, since these genes are most likely to be involved in intricate pathways in fat metabolism, effects on fat quality, such as fatty acid composition, may also be foreseen. Therefore these considerations should be examined carefully before implementation in practice.

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17 Post-mortem Muscle Proteolysis and Meat Tenderness

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17.1 General Introduction

The final toughness of meat depends on the degree of alteration of the structural components of muscle and associated proteins during and subsequent to rigor. Proteolysis contributes to this alteration through a degradation of muscle proteins as observed in muscle held at chiller temperatures. However, physical factors like the degree of muscle shortening also contribute to final toughness.

17.2 Introduction to Mechanisms of Tenderization

Even though there is good evidence that specific myofibrillar muscle proteins are degraded during the immediate post-mortem period (Bandman and Zdanis, 1988), the total proportion of protein degraded is considered to be only small (Davey and Gilbert, 1966), whilst there are substantial reductions in toughness. There is also debate over whether the cysteine proteases, in particular the calpains (Dransfield, 1993; Uytterhaegen *et al.*, 1994; Koohmaraie, 1996), are largely responsible for this degradation, or whether the cathepsins and other enzyme systems (Goll *et al.*, 1992; Ouali, 1992; Zeece *et al.*, 1992; Roncales *et al.*, 1995; O'Halloran *et al.*, 1997) have a role. These issues will be discussed in this chapter in relation to general principles drawn from bovine and ovine muscle.

Quantification of the contribution of individual proteins to tenderization remains to be resolved and, although the rate of disappearance of individual proteins may not always match changes in toughness (Dransfield, 1997), this does not diminish the strategic and cumulative contribution such degradation may have.

The mechanism that drives tenderization post-rigor has demanded significant attention and several explanations for this process have been proposed. There are a number of reviews summarizing the biochemical changes that occur in meat during the post-mortem period (Ouali, 1992; Koohmaraie, 1996; Geesink *et al.*, 2000; Hopkins and Thompson, 2002b; Sentandreu *et al.*, 2002) and in terms of proteolysis we will draw heavily on the overview provided by Hopkins and Thompson (2002b).

Most of the reduction in toughness is attributed to changes in the myofibrillar proteins as discussed below. In general connective tissue is stable post mortem (as reviewed by McCormick, 1994; Purslow, 1994) although after extended periods of storage intramuscular connective tissue (IMCT) does show signs of structural changes (Nishimura *et al.*, 1995). In fact Nishimura *et al.* (1998) described a new method to examine the mechanical strength of IMCT and showed that after 10 days of ageing there was a decrease in strength of this tissue in beef meat. A curve illustrating the reduction during storage of toughness post-rigor (i.e. tenderization) is shown in Fig. 17.1. There are two important features of this curve: the post-rigor slope and the maximum toughness (measured as shear force) obtained at rigor. It is accepted that individual curves for the same muscle from different animals will show considerable variation (Koohmaraie *et al.*, 2002), but the generalized curve serves as a useful model for discussing mechanisms. The absolute values obtained at maximum contraction (Fig. 17.1) are of great importance, for these will influence the rate of ageing post-rigor (Herring *et al.*, 1967). Temperature during pre-rigor storage has been found to have a large impact on the extent of shortening (Locker and Hagyard, 1963), and this varies considerably between different muscles in the carcass (Cena *et al.*, 1992). As such, there is opportunity to alter the maximum toughness achieved (measured as shear force) by managing chilling conditions pre-rigor, within the restraints imposed by food safety regulations.

The degradation of myofibrillar muscle proteins during the post-mortem period has been studied using a number of techniques including determination of free amino acids, fragmentation of myofibrils, measurement of protein solubility, measurement of non-protein nitrogen and gel electrophoresis. It is relevant to start with a brief overview of the results of published work in which the degradation of specific myofibrillar proteins has been reported, as such consideration helps to clarify which

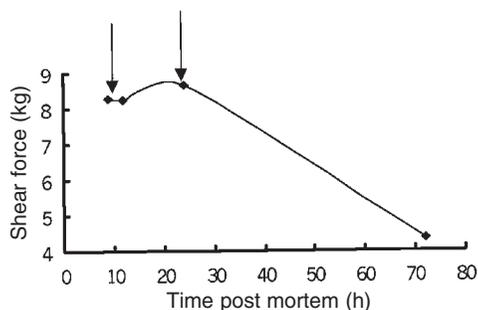


Fig. 17.1. Proposed time course changes in shear force of ovine longissimus during post-mortem storage. Maximum contraction occurs somewhere between the arrows as the muscle enters rigor. (Adapted from Wheeler and Koochmarai (1994) *Journal of Animal Science* 72, 1232–1238, with permission of the American Society of Animal Science.)

enzymes are responsible for this degradation. The recent review by Hopkins and Thompson (2002a) covering myofibrillar degradation of ovine and bovine muscle assessed by electrophoresis (denaturing) provides additional information on this topic.

17.3 Degradation of Myofibrillar Proteins

17.3.1 Titin and nebulin

Titin or connectin is a large protein (up to 3700 kDa; Labeit and Kolmerer, 1995) that is crucial to the orderly organization of the sarcomere (sarcomere and cytoskeletal structure has been reviewed by Greaser, 1991; Small *et al.*, 1992). This protein provides along its length binding sites for other sarcomere proteins and the carboxyl-terminal region of titin is reported to bind to myosin (Maruyama, 1997). The PEVK region of the protein in the I-band provides the elasticity to the sarcomere (Labeit and Kolmerer, 1995). Another large protein, nebulin, is associated with actin and it has been reported that this protein is involved in the regulation of actin–myosin interactions. Taylor *et al.* (1995a) provides a schematic diagram of how titin interacts with actin and myosin and illustrates where other proteins such as desmin and vinculin are located in the sarcomere; Hopkins and Thompson (2002a) provide a schematic diagram of the structure of the Z-disk. A summary of studies in which these two proteins have been examined shows that titin is degraded post mortem into at least two fragments (Table 17.1). The fragment T_2 , which appears as titin is degraded, does not contain the portion of the titin molecule attached to the Z-disk (Robson *et al.*, 1991) and has a weight of 2100–2400 kDa (Takahashi *et al.*, 1992). A further degradation product with a weight of 1200 kDa has also been found, which Tanabe *et al.* (1994) concluded anchored titin to the Z-disk by binding to α -actinin. For this conclusion to be consistent with that of Robson *et al.* (1991) the 1200 kDa fragment must have come from the Z-disk end of T_1 , but not from the portion within the Z-disk. Data presented by Boyer-Berri and Greaser (1998) suggested that titin was cleaved in the region close to the Z-disk during post-mortem storage of muscle,

Table 17.1. Important observations about the degradation of selected muscle proteins in beef and sheep muscles.

Protein	Observations
Titin	Degraded into at least two fragments (T_2 and 1200 kDa) and the latter fragment will also disappear Rate of degradation varies between different muscles and between the same muscle in different carcasses T_2 has appeared at day 1 in both lamb and beef muscle In 'tender' muscle the complete protein (T_1) disappears earlier than in 'tough' muscle and in the latter muscle has still been detected at day 14 in beef Rate of degradation is faster at higher pH and temperature and has been reported to occur faster in stimulated carcasses
Nebulin	Rate of degradation varies between muscles and between the same muscle in different carcasses Disappears faster than titin Nebulin present in day 1 'tender' muscle, but in lesser amounts at days 3 and 7 in 'tough' muscle Rate of degradation is faster at higher temperature and has been reported to occur faster in stimulated carcasses
Vinculin	Degradation is apparent by day 1, but the intact protein has still been detected at day 21 in lamb muscle A 90 kDa fragment appears when the protein is degraded
Desmin	Degradation observed within 1 day Both 45 kDa and 38 kDa fragments have been detected Rate of degradation varies between muscles and between the same muscle in different carcasses, with faster degradation in 'tender' muscle compared with 'tough' muscle Rate of degradation is faster at higher temperature
Filamin	240 kDa fragments detected at 3 days in 'tender' muscle and at 14 days in 'tough' muscle

Derived from the paper of Hopkins and Thompson (2002a) *Journal of Muscle Foods* 13, 81–102 and reproduced with permission of Food and Nutrition Press.

with some variation according to muscle type. More recently a model was proposed for the degradation of titin in a conference review paper by Greaser *et al.* (2000). The proposed model developed using titin antibodies suggested that titin can be degraded at both the region near the Z-line and in the PEVK region resulting in retraction of the protein and emergence of multiple bands. However, it should be stressed that this proposed model has yet to be confirmed in less stretched muscle (i.e. with sarcomere lengths < 2.7 μm). According to the reports of Fürst *et al.* (1988) and Taylor *et al.* (1995a), nebulin is also degraded in the region close to the Z-disk, which is the C-terminal end of the polypeptide, whereas degradation is at the N-terminal end of the titin polypeptide.

Degradation of both titin and nebulin was the suggested reason for the increased fragility of myofibrils in the I-band region (Taylor *et al.*, 1995a) and was linked to the major decrease in toughness that occurs between 24 and 72 h post mortem

(Dransfield *et al.*, 1992a; Wheeler and Koohmaraie, 1994). Despite the anomalies between some of the studies it is apparent that the degradation rates of titin and nebulin are accelerated at high post-mortem temperatures (Lusby *et al.*, 1983; Rhee *et al.*, 2000) and that, overall, the disappearance of these proteins contributes to tenderization. The difficulty has been to explain tenderization rate quantitatively in terms of the degradation of these two large proteins with only limited attempts reported (Steen *et al.*, 1997). Also, as stressed by Greaser *et al.* (2000), experiments based on muscle from large sets of animals with the same genetic background and age and examined over the same post-mortem period have been limited.

17.3.2 Myosin, actin and α -actinin

Proteolytic cleavage of myosin has been reported in muscle held at an elevated temperature (Yates *et al.*, 1983), but not in muscle held at 4°C or in samples held at pH 7 (Bandman and Zdanis, 1988). Such post-mortem conditions (high temperature for sustained periods at low pH) are atypical for most chilled meat, although heat-shortened meat maybe an exception. Both actin and α -actinin have been found even more resistant to degradation under 'normal' chiller conditions and purified calpain does not degrade actin or α -actinin (Goll *et al.*, 1991). It is evident that degradation of myosin, actin or α -actinin does not contribute to tenderization (based on electrophoresis) and this is one piece of evidence used to discount the role of cathepsins in tenderization (Roncales *et al.*, 1995; Koohmaraie, 1996).

17.3.3 Degradation of troponin-T

Degradation of troponin-T gives rise to a 30-kDa protein fragment (Ho *et al.*, 1994), which has been associated with a decrease in toughness (Huff-Lonergan *et al.*, 1995, 1996a). Penny and Dransfield (1979) showed that the disappearance of troponin-T was accelerated as the temperature of storage increased, whereas in cold-shortened muscle (which only showed a slight improvement in shear force) troponin-T disappeared at a rate similar to that in muscle held at 5 and 10°C. When all their data were pooled, Penny and Dransfield (1979) found that variations in the amount of troponin-T accounted for 60% of the variation in shear values. Steen *et al.* (1997) found a negative correlation between shear force at 8 days post mortem and the concentration of the 30-kDa fragment. A more recent report by Wheeler and Koohmaraie (1999) showed that the rate of degradation of troponin-T in the longissimus muscle was independent of sarcomere length so that shortened muscle, which had double the shear force of control muscle, exhibited similar disappearance of this protein. Interestingly, for the psoas muscle the response was not the same, such that even though shortened muscle had nearly double the shear force there was a trend for less degradation of troponin-T than control muscle.

Whether the degradation of this protein can be considered responsible for reductions in toughness, or is simply an indicator of proteolysis, still remains to be resolved (Robson *et al.*, 1997), but given that it does not have a structural role in myofibrils the former conclusion seems unlikely. This conclusion was supported by recent work (Hopkins and Thompson, 2001c), which showed that the rate constant

for the appearance of what was considered the 30 kDa fragment did not explain variation in shear force of ovine longissimus muscle.

It should be stressed, however, that it is probably unrealistic to expect that the disappearance of a single myofibrillar protein could be used to explain tenderization. The evidence indicates that a number of proteins associated with the thin and thick filaments and others found at the periphery of the Z-disk are degraded under normal post-mortem chiller conditions. Contrary to earlier suggestions that Z-disk degradation was significant during post-mortem storage of muscle (Davey and Gilbert, 1968) the evidence from the work of Taylor *et al.* (1995a) shows that degradation of proteins near the Z-disk is what actually occurs. This conclusion can now extend at least for titin to other regions of the sarcomere (Greaser *et al.*, 2000). In fact several ultrastructural studies from the 1970s clearly show that the myofibre breaks in the I-band region and that the Z line is stable (Davey and Graafhuis, 1976; Abbot *et al.*, 1977; Gann and Merkel, 1978).

17.3.4 Degradation of vinculin, desmin and filamin

Other proteins that are important in maintaining the functional capability of muscle are those of the costameres and the cytoskeleton. Costameres connect Z-disks to the sarcolemma and are made up of proteins such as vinculin, talin, desmin, dystrophin and β -spectrin. A summary of the characteristics of these proteins was given by Robson *et al.* (1997), and Taylor *et al.* (1995a) provided a schematic illustration of how they interact within the sarcomere.

Vinculin, one of the costamere proteins, was shown by Taylor *et al.* (1995a) to be readily degraded under normal post-mortem chiller conditions and a decrease in shear force and an increase in myofibrillar fragmentation index (MFI) in ovine muscle paralleled its disappearance. This degradation in part explains the detachment of the sarcolemma from the myofibrils, which was observed in electron micrographs of aged versus fresh muscle (Taylor *et al.*, 1995a). Related to this, the degradation of vinculin and filamin has been implicated in the detachment of adjacent myofibrils (Taylor *et al.*, 1995a; Taylor and Koohmaraie, 1998).

Work with human skeletal muscle using confocal microscopy and immunofluorescence (Mondello *et al.*, 1996) suggests that vinculin, desmin and talin are located at both the Z-disk and the M line. If this is also true for bovine/ovine muscle, then their degradation will be of more significance for detachment of myofibrils from the sarcolemma and thus tenderization. Robson *et al.* (1998) indicated that talin directly interacts with the protein actin further illustrating the importance to myofibril integrity of proteins associated with intermediate filaments.

Wheeler and Koohmaraie (1999) have shown that desmin, an intermediate filament protein, was degraded at the same rate in shortened as in control muscle over 10 days of ageing and that the reduction in shear force was similar between shortened and control muscle. They also reported that disappearance rate of desmin was highly correlated with the disappearance of troponin-T. Huff-Lonergan *et al.* (1996a) found that the disappearance of desmin and filamin was more rapid in meat that had a faster rate of tenderization.

From the numerous studies of protein degradation in meat the important observations have been summarized in Table 17.1; further information on the specifics of

these studies is available in Hopkins and Thompson (2002a). Collectively the results show that desmin and titin are important substrates whose degradation contributes significantly to tenderization. There has been extensive work to discover which enzyme groups are responsible for this degradation in post-mortem muscle and the main contenders will be discussed in subsequent sections.

17.4 Cathepsins

The calpains and a number of the cathepsins contain a cysteine residue that binds covalently to a substrate (Ertbjerg, 1996). Goll *et al.* (1989) provided a classification of the cathepsins with indicative pH levels for optimal activity, which are all in the acidic range, and Zeece *et al.* (1992) have reviewed their potential role in meat ageing. Much of the focus on cathepsins and their role in meat tenderization has been directed towards B (EC 3.4.22.1), L (EC.3.4.22.15) and D (EC.3.4.23.5), which are endopeptidases that cleave peptide bonds internally in peptides. Cathepsins B and L (cysteine proteases; EC 3.4.22) have also been shown to exhibit exopeptidase activity. The endogenous family of cystatins located in the sarcoplasm inhibits their activity and a review of these inhibitors was provided by Turk and Bode (1991).

Cathepsins are located in the lysosomes (Goll *et al.*, 1983) and thus to play a part in myofibril degradation they must be released from the lysosomes, which is feasible given the failure of ion pumps in membranes during the development of rigor. Pommier *et al.* (1987) showed that the amount of free cathepsin D (an aspartic protease) increased during ageing, which they attributed to a fall in pH and lysosomal rupture, but concluded that this did not have an impact on tenderization, whereas others (Whipple *et al.*, 1990) have suggested that there is no lysosomal rupture during ageing. Attempts to study the structural integrity of lysosomes during ageing have used the electron microscope (Chambers *et al.*, 1994) and more recently in a preliminary report histochemistry was used (Mobark *et al.*, 1999). Both studies suggested that leakage from the lysosomes does occur during ageing, although the activity of cathepsins D and B was minimal in the latter study. The low activity was attributed to either a lack of sensitivity of the technique or the action of cystatins. In addition lysosomal enzyme activity is often associated with lipid droplets in muscle and is also detected in endoplasmic reticulum after 14 days of post-mortem storage (Taylor *et al.*, 1995b). It is perhaps the reticulum staining that was reported by Dutson and Lawrie (1974) as diffuse staining near the Z line within 5 days of post-mortem storage. It is noteworthy that Chambers *et al.* (1994), Dutson and Lawrie (1974) and Taylor *et al.* (1995b) all found intact lysosomes during post-mortem storage for up to 14 days, indicating that lysosomes are stable structures. In addition Jung *et al.* (2000) have shown that beef muscle lysosomes are stable in beef aged for 2 days and subjected to pressures up to 600 MPa.

Many of the studies on cathepsins provide no support for the conclusion that these enzymes have a role in post-mortem proteolysis and thus tenderization, as invariably they were conducted under conditions atypical for the myofibrillar environment during the post-mortem period. Indeed, as reported by Zeece *et al.* (1986a), at 15°C and a pH of 5.5 there was minimal myofibre degradation by cathepsin D, but when the incubation was at 37°C and pH 5.5 myosin and titin degradation were observed.

Matsukura *et al.* (1981) and Mikami *et al.* (1987) demonstrated that of the cathepsins, type L degraded the largest number of different myofibrillar proteins. In both studies, degradation was studied under high temperature, low pH conditions and was evidenced throughout the sarcomere. Robbins and Cohen (1976) also reported that incubation of bovine muscle with cathepsins causes loss of Z lines, a structural change that is not normally observed post mortem. In some cases Z-disk structure was lost in addition to changes in the I-band. The fact that actin and myosin are not degraded during normal chilled storage of meat is the second major hurdle for those that propose a role for cathepsins in tenderization, because, as evidenced in a number of studies (Schwartz and Bird, 1977; Matsukura *et al.*, 1981; Okitani *et al.*, 1981), these proteins are commonly degraded when myofibrils are incubated in the presence of cathepsins.

Ouali (1992), in his review of the mechanisms controlling tenderization, suggested that the cathepsins do contribute to degradation, citing results from experiments in which both rabbit and beef muscles were studied. However Koochmarai *et al.* (1991) showed that, although lamb and beef had similar cathepsin B and B + L activity measured at death, the rate of tenderization was very different. Whipple *et al.* (1990), working in the same laboratory, found that the activity of cathepsins B and B + L did not explain the differences in tenderness of muscle from *Bos indicus* and *Bos taurus* cattle. Erbjerg *et al.* (1999) made very similar conclusions: that cathepsin B and L activity in beef is closely related to actin and myosin degradation but not to the appearance of degradation products normally found in meat such as the 30-kDa protein. O'Halloran *et al.* (1997) suggested that the higher activity of cathepsins B and B + L in the soluble fraction from fast-glycolysing muscle was in part responsible for a lower shear force measured at 2 and 6 days post mortem, compared with slow-glycolysing muscle. Such conflicting conclusions may well reflect variations in extraction techniques, methods of assaying enzyme activity and in general a lack of rigorous statistical techniques to quantitatively link variation in cathepsin activity with measures of toughness such as shear force.

The accumulated evidence makes it difficult to conclude that cathepsins are a major contributor to tenderization particularly in the early post-mortem period, given the following points: (i) the requirement for these enzymes to leak from lysosomes and into the cytosol; (ii) the acidic conditions which these enzymes require for optimum activity; (iii) the fact that under normal chilling conditions proteins such as myosin and actin are not degraded, yet these are readily degraded when myofibrils are incubated with cathepsins; and (iv) the lack of significant studies that have demonstrated a link between tenderization and the activity of these enzymes. This conclusion is reinforced by the results of studies in which inhibitors to these enzymes have been used as discussed in a subsequent section.

17.5 Calpains

Busch *et al.* (1972) showed that an endogenous protease activated by calcium could degrade myofibrillar proteins, resulting in a significant decrease in isometric tension. Optimal activity of the unidentified enzyme was at pH 7.0 and required levels above 0.1 mM Ca^{2+} *in vitro*. Subsequent data showed that the action of the Ca^{2+} ions was towards the enzyme itself and not on the myofibril substrates (Dayton *et al.*, 1976).

Additionally, it was suggested that cysteine side chains on the enzyme were important for its proteolytic activity, but that at 37°C the enzyme underwent autolysis in the presence of Ca^{2+} ions. The first report of calpain activity related to meat was by Olson *et al.* (1977) and is discussed further below.

Murachi *et al.* (1981) described the enzyme (EC 3.4.22.17) as having two forms that were given the names calpain I and calpain II, the latter form requiring much higher levels of Ca^{2+} ions for activation. These authors indicated that the enzymes had been found in a range of tissues, with the earliest report in brain tissue (Guroff, 1964). An endogenous inhibitor of the enzymes was named calpastatin (Murachi *et al.*, 1981). It was reported that less of this inhibitor was required to reduce calpain II activity and that inhibition occurred by the binding of Ca^{2+} ions to the calpains (I and II), resulting in a conformational change, thereby allowing interaction with calpastatin and inactivation.

More recently another member of the calpain family has been identified, usually referred to as p94 or calpain 3. Unlike calpains I and II, p94 is reported to be specific to muscle tissue (Sorimachi *et al.*, 1989) and has been reported to bind to two distinct sites on titin (Labeit *et al.*, 1997). Other ubiquitous forms in skeletal muscle have also been characterized. These include isoforms such as calpain 10, which has been reported to be associated with the sarcolemma and does not possess domain IV, which binds calcium (Ma *et al.*, 2001).

17.5.1 The calcium requirement for calpain activity

Etherington (1984) suggested that calpain II required a Ca^{2+} ion concentration of 1–2 mM and calpain I, 50–100 μM Ca^{2+} for maximal activity. Subsequent studies suggested that calpain II (now commonly called m-calpain) required somewhere between 300 and 800 μM Ca^{2+} for half maximal activity (Boehm *et al.*, 1998), a level well above that found in living muscle cells (Etherington, 1984).

In terms of post-mortem tenderization, the calcium requirement of the calpains presents some important constraints. Jeacocke (1993) stated that the intracellular concentration of free Ca^{2+} must rise to more than 100 μM at rigor, although the maximum level was not specified and the use of the dye arsenazo may have inflated the estimate as this dye can cleave Ca^{2+} bound to proteins and anions. Protein degradation reported in some initial studies was at very high Ca^{2+} ion concentrations (Busch *et al.*, 1972; Dayton *et al.*, 1976), beyond the levels expected under intracellular conditions, even those after rigor. Further to this, Ducastaing *et al.* (1985) showed that the activity of calpain I (μ -calpain) decreased significantly during the onset of rigor mortis. By comparison the activity of m-calpain was largely unaltered for up to 3 days post mortem and in some cases this has extended to 56 days post mortem (Geesink and Koohmaraie, 1999a). Insufficient free calcium to activate the enzyme is the suggested reason for m-calpain stability (Veiseth *et al.*, 2001). Beltrán *et al.* (1997) presented an exception for bovine muscle with a pH above 6.3, which showed a higher activity of m-calpain after 7 days of ageing than meat with a pH less than 6.3. Veiseth and Koohmaraie (2001) suggested that this effect was due to the use of a buffer with insufficient buffering capacity.

Recent data presented by Hopkins and Thompson (2001c) showed that the free

calcium concentration in ovine muscle reached a plateau post-rigor of approximately 110 μM when measured with a Ca^{2+} sensitive electrode, a level too low to activate m-calpain in support of the conclusion of Geesink and Koohmaraie (1999a). Similar results were reported by Geesink *et al.* (2001) who found a free calcium level of 106 μM in ovine longissimus muscle at 24 h post mortem when determined by atomic absorption (AA) and 60 μM when determined using the dye arsenazo III.

It is notable that several studies have considered the activity of extractable μ -calpain, m-calpain and calpastatin maximal 'at death' with a subsequent decline post mortem under *in vitro* conditions (at very different rates) (Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1987; Boehm *et al.*, 1998). By contrast, Dransfield (1993), using modelling, predicted minimal activity of these enzymes 'at death' under *in situ* conditions. According to his model μ -calpain reached a maximum activity approximately 10 h after death (Fig. 17.2) and he proposed that this enzyme was not active at death, probably because the Ca^{2+} ion concentration is too low (Dransfield, 1992). There is reason to question this conclusion given new knowledge about 'free' Ca^{2+} ion concentrations after death (Geesink *et al.*, 2001; Hopkins and Thompson, 2001c) and the fact that calpains have been implicated in protein turnover in living muscle under presumably much lower 'free' Ca^{2+} ion concentrations.

The autolysis (self proteolysis) of the calpains has been shown to lower the Ca^{2+} ion concentration required for activation of both μ and m autolysed types (Edmunds *et al.*, 1991), but the requirement is still beyond the level that is reported to exist in living skeletal muscle cells (Etherington, 1984). However, in kidney tissue taken from rats, Yoshimura *et al.* (1983) reported that μ -calpain only required a Ca^{2+} ion concentration of 10 μM for full activity and it is apparent that stimulation of living cells will cause an elevation in Ca^{2+} ion levels intracellularly. Kerr *et al.* (2000), using optical imaging techniques and proteins that fluoresce in the presence of calcium,

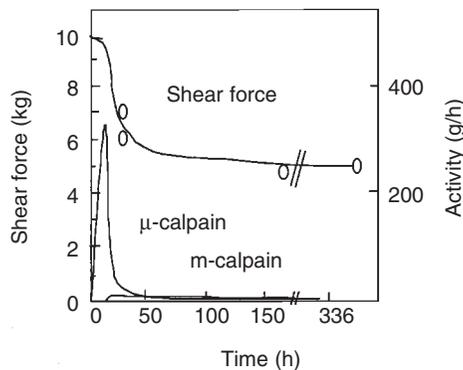


Fig. 17.2. Change in calpain activity in relation to change in shear force post mortem adapted from Dransfield (1993) and based on the data of Koohmaraie *et al.* (1991) and Whipple *et al.* (1990). Reproduced from *Australian Journal of Agricultural Research* 53, No. 2 (Hopkins and Thompson, 2002) with permission of CSIRO Publishing.

have recently verified this in 'real time'. An electrostatic switch mechanism whereby m-calpain may be activated at physiological calcium concentrations *in vivo* was proposed by Strobl *et al.* (2000) and it may be that under post-mortem conditions, this mechanism is ineffective, explaining the significantly increased calcium requirement. Related to this, Lawson and Schäfer (2002) in a preliminary paper reported that m-calpain co-localizes with the protein integrin on muscle cell membranes and exhibits activity, but the mechanism of activation was not discussed. Despite our incomplete understanding of the control of *in vivo* calpain activity, the current evidence does not indicate a role for m-calpain in tenderization.

17.5.2 The temperature, pH and ionic strength conditions required for calpain activity

Early research showed that the optimal conditions for *in vitro* calpain activity were pH 7.5 at 25°C and this has been confirmed repeatedly. However, this is a combination that does not generally occur in post-mortem muscle, but which indicates a temperature/pH dependency for the activity of the calpains *in vitro*. Minimal activity has been reported at 5°C with a pH of 5.5, conditions that prevail approximately 24 h post mortem and Boehm *et al.* (1998) suggested that the level of activity after 24 h would be < 10%. In contrast, Koohmaraie *et al.* (1986) suggested that μ -calpain retained 24–28% of its maximum activity at a pH of 5.5–5.8 and 5°C (as measured against pH 7.5 and 25°C) when myofibrils were used as the substrate, but this was challenged by Kanawa *et al.* (2002) who reported no activity under these conditions when using myofibrils. Activity was reduced to < 15% when casein was used as the substrate (Koohmaraie *et al.*, 1986), which the authors suggested could have been due to a reduced solubility of casein at the low pH and temperature. It should be stressed that the type of assay used will determine the ability to detect μ -calpain activity and when ¹⁴C-labelled casein was used by Geesink and Koohmaraie (1999a) activity could still be detected at 56 days post mortem in ovine muscle. Another aspect that may have impacted on previous estimates of calpain activity was the pH of the extraction buffer used during homogenization as already mentioned (Veiseth and Koohmaraie, 2001; Section 17.5.1). It was shown that unless the homogenate pH was kept above 6.5, there was a significant reduction of calpain activity due, it was suggested, to precipitation of these enzymes. These estimates of activity are invariably made without the presence of calpastatin and they highlight the impact that different experimental methodologies can have on results.

Olson *et al.* (1977) demonstrated that the inherently tender psoas muscle had a significantly lower 'calpain' activity than either semitendinosus or longissimus muscle and that by 24 h post mortem the level of activity was close to zero. This lower 'calpain' activity for the psoas muscle was later verified by Koohmaraie *et al.* (1988) who reported muscle-specific levels, but it should be remembered that this muscle is stretched in the carcass and has a low connective tissue content, which also contributes to the low shear force of the muscle. Indeed, the relative contribution of these three variables (enzyme activity, sarcomere length and connective tissue content) varies between muscles and thus impacts on the extent of tenderization (Wheeler *et al.*, 2000).

Published results indicate large variability in the loss of calpastatin activity post

mortem (Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1987; Boehm *et al.*, 1998), but in all cases the rate of loss of μ -calpain activity was the greatest. There are differing opinions about the influence of declining pH on the ability of calpastatin to inhibit the calpains. The model proposed by Dransfield (1993) relies to some degree on the claim that the ability of calpastatin to inhibit calpain activity is reduced below a pH of 6.5. Goll *et al.* (1997) in a conference review paper questioned this conclusion and suggested there is evidence, albeit from cardiac muscle, that there was no inhibition until the pH drops below 6.2 (Otsuka and Goll, 1987). More recently Geesink and Koohmaraie (1999b) have suggested that pH has little effect on the inhibition of μ -calpain by calpastatin under *in vitro* conditions of ideal temperature and Ca^{2+} levels.

A further consideration with respect to the activity of the calpains is the effect of ionic strength. As muscle enters rigor there is a rapid rise in the ionic strength of the sarcoplasmic fluid, with a peak and subsequent plateau (Ouali, 1992). It was demonstrated by Geesink and Koohmaraie (1999b) that the activity of partly autolysed μ -calpain in the absence of calcium was reduced by 50% when incubated in a buffer containing 0.3 M NaCl, after 30 min of incubation. This NaCl concentration is similar to that found in post-rigor muscle (Ouali, 1992). Geesink and Koohmaraie (1999b) indicated that the loss of μ -calpain activity occurred because the autolysed calpain was unstable under high ionic conditions. This conclusion was used to explain why minimal proteolysis was observed in myofibrils after 7 days of incubation (under optimal temperature and pH conditions) in a μ -calpain/calpastatin solution. However, given that ionic strength increases significantly at rigor (well before 7 days post mortem) and then plateaus it would therefore seem reasonable to expect to see a large reduction in proteolysis before 7 days in post-mortem muscle under conditions where the temperature will be less than optimal for activity. In a recent preliminary report Goll *et al.* (2000) also concluded that the activity of autolysed μ - and m-calpain was significantly reduced when the ionic strength was increased above 0.3 M and this effect was attributed to a rise in ionic strength and not NaCl (Geesink and Koohmaraie, 2000). Related to this it has been reported by Goll *et al.* (2000) that the autolysed μ -calpain bound irreversibly to the separation columns used during elution, which would explain the reported decrease in activity, due to low recovery. It was apparent from the results of these studies that methods of determining calpain activity *in vitro* had real limitations and there was conflict about whether the calpains can be separated from calpastatin using elution with differing concentrations of NaCl (e.g. McDonagh, 1998; Geesink and Koohmaraie, 1999c). An alternative approach based on determination of mRNA levels may prove useful (Ilian *et al.*, 1998), but this approach also has some limitations. Other alternatives that have been used include ^{14}C -labelled casein and casein zymography. There is a complication in extrapolating from different assay conditions where variables such as pH, temperature or calcium concentration are often held constant compared with the dynamic post-mortem muscle environment where pH and temperature both decline as muscle passes through rigor and the 'free' calcium concentration rises.

17.5.3 Degradation products due to calpain activity

Early studies found that calpains led to the release of α -actinin from the Z-disk and degradation of troponins T and I, and C-protein (Etherington, 1984). Whilst a

number of the structural and cytoskeletal proteins are degraded by the calpains (Goll *et al.*, 1989), actin and myosin are not (Dayton *et al.*, 1976) and this mirrors the resilience of these proteins to post-mortem degradation as previously discussed. This adds further support to the argument for the role of the calpains in post-mortem tenderization.

After incubating myofibrils with m-calpain for up to 30 min at temperatures ranging from 25°C to 5°C and at pH 6.5–7.5, Zeece *et al.* (1986b) found that titin and troponin-T were degraded, but this was reduced as the temperature and pH decreased. Degradation of titin was still detected at 5°C and pH 7.5, but at 5°C and pH < 6.5 there was no significant decrease in the number of sarcomeres per myofibril (a measure of myofibril fragmentation) after a 30 min incubation. In general the degradation of troponin-T to the 30-kDa fragment by calpain extracts mimics the pattern observed for meat during post-mortem ageing. Huff-Lonergan *et al.* (1996b) followed the degradation of specific muscle proteins, using Western blotting techniques, after myofibrils were incubated with μ -calpain for varying times at 5°C and pH 5.6 with 100 μ M Ca^{2+} . This approach revealed rapid degradation of titin and nebulin and slower degradation of filamin, desmin and troponin-T, with the production of the same proteolytic fragments found in studies of post-mortem meat degradation (Huff-Lonergan *et al.*, 1995; Taylor *et al.*, 1995a). It has been argued that the degradation of myofibrillar proteins such as titin and desmin is minimal during the first 24 h after death (Boehm *et al.*, 1998) and hence a role for μ -calpain in tenderization was questioned. However, Koohmaraie *et al.* (1988) state that ‘about 50% of the ageing response is completed by 24 hrs of postmortem aging’ in beef, and Wheeler and Koohmaraie (1994) have also demonstrated significant proteolysis in lamb within the first 24 h. Related to this, Hopkins and Thompson (2001b,c) challenged the claim of minimal proteolysis in the 24 h period after death and presented evidence using a range of indicators that proteolysis was very rapid during this period, an outcome consistent with activation of μ -calpain and the significant tenderization of muscle post-rigor. Indeed autolysis of μ -calpain as early as 3 h post mortem in ovine muscle has recently been shown by Veiseth *et al.* (2001), indicative of proteolytic activity before the onset of rigor.

It has been shown using autoradiography that Ca^{2+} ions bind to titin (Takahashi *et al.*, 1992) and, more specifically using fluorescence detection, to the major sub-fragment of this degraded protein where the 1200-kDa fragment is cleaved (Tatsumi *et al.*, 1999). Since calpains bind Ca^{2+} ions for activation and titin is a good substrate for calpain (Robson *et al.*, 1997) these results are not inconsistent with the proposed role of the calpains and the suggested degradation sites of titin (Greaser *et al.*, 2000). Additionally the calpain p94 has been reported to bind to titin (Sorimachi *et al.*, 1995; Robson *et al.*, 1997). Despite the emphasis on Ca^{2+} ions there have been few studies which have measured the levels of these ions in meat throughout the rigor process as outlined earlier. The Ca^{2+} ion has a central role in tenderization. Thus studies which quantify the rate of accumulation of ‘free’ ions during the post-mortem period with a focus on the activation of the calpains are considered important.

17.5.4 The role of calpain-mediated proteolysis in tenderization

Considering the circumstantial evidence linking the activity of calpains to proteolysis there have been few attempts to model enzyme activity with the rate of tender-

ization, or rate of proteolysis. Koohmaraie *et al.* (1987) demonstrated that MFI values increased for 6 days post mortem when measured initially at death (plateaued after 6 days) and Taylor *et al.* (1995a) also showed an increase from death through the post-mortem period in MFI values, although the rate varied between different muscles. As MFI increased, the activity of extractable μ -calpain decreased in the work of Koohmaraie *et al.* (1987) to almost a plateau by 1 day post mortem. Adopting the Ouali (1992) approach of basing comparisons on an index of the enzyme to its inhibitor, the relationship of this index to MFI for the data of Koohmaraie *et al.* (1987) appeared highly correlated up to 6 days post mortem. It was debatable, however, whether the at-death measures of MFI are meaningful, as pre-rigor muscle will shorten when excised (Parrish *et al.*, 1973) and this probably explained the low MFI values reported at death.

Taking a slightly different approach Dransfield *et al.* (1992b) predicted that tenderization (defined as a decrease in shear force) commenced at pH 6.1, which even in fast-glycolysing muscle would be at least 3 h after death according to their data. Subsequently Dransfield suggested that μ -calpain was not fully activated until this pH (6.1) was reached (Dransfield, 1993) and that this coincided with the start of tenderization. However, this does not preclude proteolysis occurring prior to this pH, as the prediction by Dransfield (1993) sees an increase in μ -calpain activity up to a maximum at 10 h (Fig. 17.2). Thus, proteolysis could proceed before tenderization commences. It could be speculated that since single fibres do not enter rigor simultaneously (Jeacocke, 1984) then neither would the activation of μ -calpain occur uniformly throughout fibre bundles.

The interaction between enzyme activity, pH and temperature cannot be ignored when modelling tenderization. Dransfield *et al.* (1992a) indicated that μ -calpain activity was unaffected by temperature until the pH dropped to 6.2 and was reduced thereafter at a faster rate as the temperature increased. However, it should be noted that this estimate was based on limited data and without consideration to the change in activity of calpastatin. This latter point is very important. Dransfield (1992) stated that the *in vivo* activity of μ -calpain below a pH of 6.1 was likely to be similar to the *in vitro* (extractable by chromatography) activity because the calpastatin activity was largely removed. However, this conclusion was questioned by Goll *et al.* (1997). Clearly, this must have an impact on the result of Dransfield (1992) who indicated that 68% of the variation in shear values could be accounted for by μ -calpain activity. Hwang and Thompson (2001), using an experimental design aimed at unravelling the impact of pH and temperature on calpain activity, reported an interaction between temperature and pH at 1.5 h post mortem. As a result, when the chilling was slow the activity of the μ -calpain and calpastatin decreased when pH decline was rapid, whereas when chilling was rapid the activity of the μ -calpain was largely unaffected by pH decline. In the former case this led to a reduction in ageing potential due to an exhaustion of μ -calpain.

Another consideration for this modelling was the well-documented reduction in ageing rate for meat with an intermediate ultimate pH (Watanabe *et al.*, 1996). Further to this, although MFI values were lower for high ultimate pH meat (> 6.3) in the data of Watanabe *et al.* (1996) (as would be predicted from the modelling of Dransfield, 1992), indicative of reduced proteolysis, shear force values were not the highest as would be expected. The effect on shear force was contrary to the prediction from the modelling, but was consistent with other reports for high pH meat

(Silva *et al.*, 1999). Steen *et al.* (1997) discussed some of the contrasting results reported between ultimate pH and shear force, and it should not be forgotten that changes to the water-holding capacity of meat are also likely to impact on shear force values. Thus, meat with a high pH will have an increased water-holding capacity (Purchas, 1990). Also worthy of consideration from the data of McDonagh *et al.* (1999) is the fact that indices of calpain/calpastatin activity are likely to be more strongly related to MFI than to shear force.

Regulation of calpain activity post mortem will not only be influenced by the pH decline and temperature interaction, but also by the activity of calpastatin. A correlation between ultimate toughness and calpastatin activity at 24 h post mortem has been shown (Steen *et al.*, 1997). O'Halloran *et al.* (1997) reported a lower activity of calpastatin at 3 h post mortem in fast-glycolysing muscle, than in slow-glycolysing muscle, but not at subsequent times post mortem. Interestingly, Jones *et al.* (1999) has reported fibre type-specific differences in p94 calpain expression, but until this enzyme is purified and its activity characterized we will not know its importance in meat ageing. In a recent conference review paper, Geesink *et al.* (2000) discussed the apparent paradox of the activity of μ -calpain in the presence of excess calpastatin. They suggested that it is likely that calpastatin's ability to bind and therefore inhibit calpain is modified as they interact, or that the degradation of calpastatin by the calpains enables the enzyme group to maintain activity. Another explanation is that calpastatin distribution in cells is altered by rises and falls in intracellular 'free' calcium levels and this regulates activity of the calpains (Averna *et al.*, 2001).

Zamora *et al.* (1996), using a measure of the resistance (measured by compression) of raw meat, showed a positive correlation ($r = 0.65$) between calpastatin activity measured at 1 h post mortem and resistance measured at 14 days. Further to this, the rate of ageing was strongly correlated ($r = 0.90$) with the rate of decline of calpastatin activity. The work of Zamora *et al.* (1996) was more informative than most reports because of the repeated measurements of calpain and calpastatin activity throughout the post-mortem period. Most other studies have relied on one or two measurements and correlations have been used to examine relationships.

Given the slow decrease in m-calpain activity (Ducastaing *et al.*, 1985; Koohmaraie *et al.*, 1987; Boehm *et al.*, 1998) most attention has focused on the activity of μ -calpain. This activity can only be measured for the μ -calpain that can be extracted. Geesink and Goll (1995) in a preliminary report suggested that μ -calpain becomes associated with an unextractable fraction in post-mortem muscle during storage (is myofibrillar bound) and that as a consequence the proteolytic activity of the enzyme was underestimated. This would have important ramifications for attempts to model enzyme activity and changes in toughness, but in follow-up work, Boehm *et al.* (1998) presented evidence that suggested that the bound μ -calpain was virtually inactive under *in vitro* conditions. However, based on the results of Delgado *et al.* (2001) it could be postulated that under *in vivo* conditions the bound μ -calpain is protected from the action of calpastatin. The limitation of the studies to date has been the inability to measure the *in situ* activity of both the calpains and their inhibitor calpastatin, although recent evidence from work using rat satellite cells has implicated these enzymes in degradation and remodelling of muscle cytoskeletal proteins during cell fusion (Temm-Grove *et al.*, 1999). In this work rat satellite cells were microinjected with calpastatin, which stopped fusion of myoblasts, whereas injection with m-calpain increased the rate of fusion.

The discovery of calpain 3 or p94 has raised new challenges and possibilities for the development of a tenderization model. This calpain is responsible for limb-girdle muscular dystrophy type 2A due to a mutation in the gene for calpain 3 and was observed to undergo very rapid autolysis in muscle cells when expressed under *in vitro* conditions (Branca *et al.*, 1999) even though it is expressed abundantly at the mRNA level (Ono *et al.*, 1998). There have been limited studies undertaken to examine whether the amount of calpain 3 is related to variation in toughness. This is because the unstable nature of the enzyme prevents the proteolytic activity from being defined and hinders elucidation of its structure. However, by raising antibodies to a protein sequence based on a specific region of calpain 3, Parr *et al.* (1999) presented data for porcine muscle that showed no association between the level of this enzyme and toughness assessed by shear force after 8 days of ageing. Probably of more importance was the finding that the stability of the enzyme during the first 24 h post mortem did not vary between samples with very different rates of tenderization. Koohmaraie *et al.* (2002) stressed, however, that the use of antibodies for detecting the activity of calpain 3 could give misleading results due to a lack of specificity, but this is likely to be resolved with further research. By comparison, however, Ilian *et al.* (2001) recently claimed using ovine and bovine muscle, that there was a significant correlation between the abundance of mRNA for calpain 3 (determined by use of a DNA probe) and toughness. However, determination of mRNA abundance does not necessarily reflect the activity of an enzyme and unfortunately, the analysis conducted by Ilian *et al.* (2001) was restricted to correlation so it was impossible to draw conclusions about causation. The role of calpain 3 in tenderization remains to be validated, but with the continuing development of antibodies this role is likely to be clarified. It is worth remembering that calpain 3 was identified 25 years after calpains I and II and so research focusing on the isolation and identification of muscle-specific enzymes will continue to fill knowledge gaps in meat science and provide a more complete understanding of the processes that drive tenderization. Research on other calpain isoforms like calpains 3 and 10 falls into this category.

17.6 Inhibition of Cathepsins and Calpains

There are a few studies in which inhibitors of the cysteine proteases have been used *in situ* to quantify their contribution to post-mortem tenderization. Sugita *et al.* (1980) showed that in an *in vitro* system E-64 (*trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane) inactivated the calpains; Barrett *et al.* (1982) reported that this compound also inhibited the cathepsins B, H and L. In contrast to this Tatsumi *et al.* (1998) questioned the action of protease inhibitors, suggesting that a number of these inhibitors bound to proteins such as titin and nebulin causing a conformation change, and as a result suppressing their ability to bind calcium. This result was advanced as evidence in support of the calcium theory of tenderization. In this theory a role for enzymes in tenderization was dismissed (Takahashi, 1996) and it was claimed that tenderization was due to the binding of calcium to specific sites on proteins. However, the claim of the binding of protease inhibitors to sarcomere proteins appears in conflict with the reported action of inhibitors such as E-64 (Fujishima *et al.*, 1997). In the literature several workers attempted to use infusion techniques to introduce enzyme inhibitors such as E-64 into a carcass immediately

post mortem, but the use of low concentrations and problems with the permeability and diffusion of the inhibitors to muscle cells resulted in disparate outcomes.

To study the process of tenderization Uytterhaegen *et al.* (1994) utilized a range of enzyme inhibitors injected into muscle at 24 h post mortem. It should be stressed that Uytterhaegen *et al.* (1994) used small cores of muscle to ensure the various inhibitors were effectively dispersed in the treated muscle. This *in situ* approach was an attempt to overcome the limitations of *in vitro* studies, where often the environment is atypical of that within muscle during ageing. The results of these workers clearly showed that catheptic enzymes (B, L, D and H) could not be implicated in protein degradation in post-mortem muscle when held at chiller temperatures at least up to 8 days post mortem. By contrast, however, the data of Alarcon-Rojo and Dransfield (1995) suggested that cathepsin D and cathepsins B + L may contribute to tenderization late in the ageing period. At 6 days post mortem muscle strips soaked at 10°C from day 1 in a number of cathepsin inhibitors were tougher than control samples. However, enzymes such as cathepsin D have a reduced activity at chiller temperatures (Zeece *et al.*, 1986a). In addition, when Uytterhaegen *et al.* (1994) injected muscle with either pepstatin (60 µM) or a peptidyl-diazomethane (0.2 mM) at 1 day post mortem these inhibitors of cathepsins D and B + L, respectively, did not inhibit ageing.

Uytterhaegen *et al.* (1994) also found that a range of calpain inhibitors, including leupeptin and E-64 injected at concentrations of 400 µM and 1.4 mM, respectively, at 24 h post mortem, effectively prevented tenderization. This approach provided strong evidence for the role of the calpains in meat tenderization. Use of other inhibitors selective for the cathepsins B, L, D and H ruled out their contribution to post-mortem tenderization as measured by Uytterhaegen *et al.* (1994) and this was supported by the work of Hopkins and Thompson (2001b,c). When muscle was injected with E-64, indices of proteolysis such as protein solubility and myofibrillar fragmentation were reduced, and there was no reduction of shear force with ageing. By comparison, an inhibitor of cathepsins B and L (peptidyl-diazomethane) had no such effect and injected muscle behaved like control muscle exhibiting extensive proteolysis and tenderization. Like Uytterhaegen *et al.* (1994), Hopkins and Thompson (2001b,c) used a method of injection which they had previously demonstrated was able to deliver an inhibitor throughout muscle (Hopkins and Thompson, 2001a). It was also verified that the peptidyl-diazomethane inhibitor was soluble at the temperature and pH characteristic of muscle soon after death (Hopkins, 2000). The one cautionary note is the possibility that the inhibitor was in some way blocked from accessing the active site of the target enzymes. Despite this possibility the evidence adds further weight to the conclusion that cathepsins play little or no role in early post-mortem ageing and the evidence pointed to the calpains as being responsible for tenderization.

Based on the results of studies published by Uytterhaegen *et al.* (1994) and Hopkins and Thompson (2001b,c), the conclusion by Dransfield (1999), that studies based on the use of inhibitors have not clarified the role of enzyme groups in tenderization, must be reviewed. An interesting observation in the results of Uytterhaegen *et al.* (1994) and Hopkins and Thompson (2001c) was the partial degradation of the myofibrillar protein troponin-T in the presence of the inhibitor E-64, despite no tenderization. This may indicate that the degradation of particular proteins does not relate to the toughness of the cooked meat, which could be associ-

ated with the labile nature of some proteins after heating. Alternatively such degradation in the presence of effective synthetic calpain inhibitors suggests that other enzymes may also have a role in tenderization.

In spite of the limitations of extracting cathepsins and calpains from muscle, the evidence does suggest that the latter group of enzymes have a pivotal role in proteolysis and post-mortem tenderization. The contrast in degradation patterns for myofibrils incubated with the respective enzymes shows that the calpains can mimic the pattern observed when meat is chilled. It is considered that research focusing on developing new approaches to the isolation and characterization of muscle enzymes will provide significant benefits to meat science.

17.7 Serine Proteases and Proteasomes

The evidence suggests that the calpains can be implicated in the tenderization of meat through proteolytic action, and that although their exact activity pattern is yet to be decisively modelled, the changes they elicit are likely to be significant. Thus, they may only be required to be active for relatively short periods of time since initial proteolysis has been suggested as potentially more important than subsequent proteolysis (Dransfield, 1998).

Despite these conclusions it is interesting to consider what drives tenderization after long storage periods such as 60 days (Simmons *et al.*, 2000). Such findings suggest that, as meat science gleans new insight from medical and biochemical research about the activity of different enzyme groups, other candidate groups may well emerge that can explain apparent anomalies. The serine proteases such as thrombin fall into this category as this enzyme has been found in skeletal muscle (Citron *et al.*, 1997), and recently a serine protease (M) was identified in mice that could bind to myofibrils and cause degradation (Sangorrín *et al.*, 2002). The evidence for the involvement of these enzymes in myofibrillar degradation is, however, not compelling at the moment. For example when Uytterhaegen *et al.* (1994) injected meat with the serine protease inhibitor phenylmethane sulphonylfluoride (PMSF) the meat aged and exhibited proteolysis suggesting no involvement of this enzyme group in myofibrillar degradation.

This also applies to the multicatalytic protease complex (MCP) named by some as proteasome. According to the summary provided by Koochmaraie (1992) it is latent upon extraction from muscle, but a range of conditions can activate the enzyme once extracted from muscle, such as heating or incubation with sodium dodecyl sulfate. Given the fact that MCP was incubated under conditions atypical for post-mortem muscle before it became active, there appeared little evidence to implicate this protease in the process of tenderization. However, very recently, using crude extracts, the activity of proteasome was measured under conditions which mimicked those in post-mortem muscle with related changes in pH (Lamare *et al.*, 2002). This showed that proteasomes maintained activity during the development of rigor and post-rigor and it has been suggested that under high pH conditions these enzymes may play a role in myofibrillar degradation. When myofibrils are incubated with purified proteasome, structural changes are induced after 24 h at 37°C (Taylor *et al.*, 1995c), whereas under similar conditions calpain will cause extensive structural changes within 10 min. These results indicate, again, that calpains are the major system causing structural change post mortem.

17.8 Conclusions

Connective tissue provides the support at a number of levels via the endomysium, perimysium or epimysium and maintains the integrity of the contractile apparatus made up of myofibrillar proteins such as actin and myosin and important associated proteins such as titin and nebulin. Proteins such as desmin, talin and α -actinin also help to maintain the framework within which the contractile proteins function. Degradation of proteins such as titin, nebulin and desmin will lead to myofibril disruption and contribute to tenderization.

The available evidence does not support a role for the cathepsins B and L in early post-mortem proteolysis and points to the calpain system as the major candidate, despite the fact that the mode of action of the calpains is not yet fully defined. This is consistent with protein turnover in living muscle and with studies that have shown that factors leading to a reduction in protein degradation (i.e. β -agonists) also lead to lower calpain activity. The rise in ionic strength as muscle enters rigor must be considered complementary to biochemical processes, which lead to tenderization post-rigor. The development of rigor and the shortening of fibres counter early proteolysis, but post-rigor the cumulative effect of proteolysis (largely driven by the calpains) reverses the rise in toughness and tenderization will occur. This proposed model is far from complete and in part this reflects our incomplete knowledge of the action of endogenous muscle enzymes.

There are several issues that remain to be resolved with respect to the calpains: (i) elucidation of the controlling mechanism for activation in the *in vivo* environment; (ii) the role of m-calpain and whether the activation of this enzyme can be manipulated post mortem; (iii) the contribution of p94 to tenderization; and (iv) the contribution of myofibril-bound μ -calpain to proteolysis and tenderization. Additionally the observation that the degradation of myofibrillar proteins occurs in the presence of effective synthetic and natural calpain inhibitors suggests that other enzymes may also have a role in tenderization. Inevitably the accumulated evidence points to a complex system likely to involve interacting proteases and ions and only through open-minded investigation with reliance on developments in the medical and biochemical fields will a more complete model of tenderization be developed.

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18 Water-holding Capacity of Meat

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18.1 Introduction

Lean muscles contain about 75% water. Proteins are the second main constituent part at about 22%; thus a water:protein ratio of about 3.5:1 exists in meat. A considerable proportion of the water interacts with the proteins and their highly ordered structures, the myofibrils and substructures. The water that interacts is inhibited in its molecular movement and is called immobilized water. A further limitation of water movement in muscles and also in meat for a considerable period of time post

mortem is caused by the lipid bilayers of membrane structures, which allow only a slow movement of water across the bilayers. In the extracellular space the water is immobilized by capillary forces. Therefore, water of unlimited movement is rarely found in meat. In contrast, however, tightly bound water in the sense of chemical binding (like crystal water in salt) is only a very small proportion, about 0.1% of tissue water (Fennema, 1977). Another 5–15% of the water shows a rather restricted mobility (Hamm, 1975). This water may be called interfacial water, and is located at the surface of proteins; water–solute and water–water interactions are involved. Their binding energies are larger than those between normal water molecules (Hamm, 1986).

Chemical and physical changes alter the labile state of immobilization, and these changes happen during the formation of meat and its processing (Honikel, 2002). We call meat the final state of a sequence of biochemical and physical events which take place in cross-striated muscles of animals after slaughter. The processes may take 1 day or last 2 weeks. During the post-mortem processes, changes of the water structure and water movements into the extracellular space and between cellular substructures are of paramount importance for the quality of meat especially with regard to tenderness, juiciness and water retention (Hamm, 1960, 1985).

The attractive forces, however, with which the water is bound and immobilized by muscle proteins do not change only during the post-mortem processes, but also during processing of meat, manufacturing of meat products and preparation of meat for eating. Processing involves chilling, storage, freezing, thawing, drying and mincing; manufacturing furthermore includes salting (curing) and comminuting. Almost all the procedures change the water retention of meat, the water-holding capacity (WHC). Thus knowledge of factors of influence on the WHC of meat handled under various conditions is important for the quality of meat and also of considerable economic interest. Moreover, investigations into the causes of changes in WHC of meat teach us about alterations in muscle proteins, especially the myofibrillar ones, which play the most important role not only in the function of the muscle but also in its WHC. Changes in WHC are very sensitive indicators of changes in the structure of myofibrillar proteins (Hamm, 1960; Honikel *et al.*, 1986).

Only selected aspects of water in meat, and its importance in terms of quality, will be discussed in this chapter. Several important animal-specific factors related to WHC that will not be considered include species, sex, age, muscle type and treatment of animals before slaughter.

18.2 Methods for the Measurement of Water-holding Capacity

18.2.1 General remarks

WHC is the ability of meat – or more generally of meat systems – to hold all or part of its own and/or added water. This ability depends on the method of handling and the state of the system. As the state of meat and its treatment differ considerably the meaning of WHC varies to a large extent. Therefore, the methods applied and the state of meat at the time of measurement must be exactly defined in order to obtain comparable results. In spite of all the variations in methods used there are three main ways of treatment, which can be divided into three different basic methods of measuring WHC.

18.2.2 Methods of measurement

18.2.2.1 Applying no external force

This group comprises the measurement of evaporation and weight loss, free drip, bag drip (Penny, 1977; Honikel *et al.*, 1986), cube drip and related methods (Howard and Lawrie, 1956), whereby the meat is left to itself under different environmental conditions. These methods are very sensitive but time consuming (one to several days) and are often sped up by the following methods.

18.2.2.2 Applying external mechanical force

By using positive or negative pressure the WHC of meat can be detected within a few minutes or an hour. This group comprises centrifugation methods (Wierbicki and Deatherage, 1958; Honikel and Hamm, 1987), filter paper press methods (Grau and Hamm, 1957) and suction loss methods (Fischer *et al.*, 1976). With these methods the amount of water released is far higher than with methods without external force as the pressure applied enforces the release of water from the intra- and extracellular space of the muscle structure. In drip loss measurements only extracellular water exudes from the meat (Offer, 1984). Therefore, a factor must be known to evaluate the actual drip loss of the meat. The matter becomes even more complicated as the state of meat changes during the period of conditioning and ageing, which also influences the WHC. Therefore methods applying mechanical force reveal only the tendency of meat behaviour in subsequent days but the absolute values are not directly comparable with the drip loss measurements.

18.2.2.3 Applying thermal force

As meat is usually consumed after heating, the WHC of meat on cooking is of interest. The cooking loss is measured in a wide variety of methods (Bendall and Restall, 1983). During heating, the meat proteins denature and the cellular structures are disrupted, which have a strong influence on the WHC of meat. Extra- and intracellular water will be released by the meat sample on cooking. The influence of the method of heating and the final temperature are important for the resulting WHC. The influence of these parameters is, however, not fully recognized. Also the relationship between the above-mentioned methods and cooking loss is not known in detail.

These different methods of measurement of WHC in research and practice arise from the different interests of people who handle meat. It becomes evident that WHC also means different things to different people. The WHC of all methods depends on the pH of the meat, which changes after death due to the formation of lactic acid. Furthermore the WHC depends on the muscle type and species of animal owing to their varying composition and structure. A standardized procedure for cooking concerning shape, size and environment is possible and has been published (Honikel, 1998).

18.3 Muscle Structure and Water Distribution

The constituent parts of lean meat besides water and proteins are lipids and lipoids of cellular and subcellular membranes (see also Gerbens, Chapter 16, this volume),

amounting to 1–2%, inorganic salts (1%), small amounts of low molecular weight substances such as amino acids and high molecular weight compounds such as DNA and RNA. In the muscle of a living animal, glycogen (0.7–1%) is also present.

The muscle is composed of fibre bundles which are surrounded by a collagen network, the perimysium. Bundles are an aggregation of fibres (see also Rehfeldt *et al.*, Chapter 1, and Stickland *et al.*, Chapter 3, this volume) representing the muscle cells. The cells are surrounded by a connective tissue sheath, the endomysium, and the cell membrane, the sarcolemma, which separates the intracellular water from the extracellular fluid. The transport of water through this cellular membrane is rather slow (Honikel *et al.*, 1986).

The filaments, as well as other subcellular structures such as sarcoplasmic reticulum, mitochondria and lysosomes, are embedded in the fluid of the sarcoplasm. This plasma contains dissolved proteins, salts and other low molecular weight compounds. It is supposed that about 20% of the water of the cell is in the sarcoplasm; the main part, however, is located in the myofibrillar space between and within the filaments (Bendall and Restall, 1983).

On contraction the basic units of the fibrils, the sarcomeres, shorten by sliding of the thick and thin filaments into each other. As this occurs the myofibrillar space decreases and a part of the water in the fibrils must become translocated in the sarcoplasmic space. As a contraction usually lasts only a short time in a live animal, the exchange of water from one substructure to another is rather short and reversible. But after a continuous shortening has occurred as may happen post mortem, the displacement of water from the myofilaments permanently increases the amount of sarcoplasmic water. This water is then no longer immobilized in and between the filaments. The effect of displacement is further enhanced after death as the pH falls from 7.0 to a value of around 5.5, causing a shrinkage of myofilaments.

18.4 Changes Post Mortem and Water-holding Capacity

18.4.1 Biochemical changes post mortem

With the death of an animal the bloodstream stops and with it the supply of energy-rich compounds and oxygen to the muscle; the removal of metabolites also stops. But the cell, which contains glycogen in a concentration of 0.7–1.0% of its weight, can still convert energy anaerobically and produce ATP. The metabolism ends now with lactic acid. This acid amounts finally to concentrations of about 0.1 M equal to about 9 g/kg. Due to lactic acid production the pH falls normally from 7.0 to a value between 5.3 and 5.8, depending on the type of muscle and animal. This process may take only 1 h in extreme cases. In pigs with a normal rate of glycogenolysis the ultimate pH values are reached within 6–12 h post mortem; in beef this process lasts 18–40 h (Honikel and Kim, 1985).

As stated above the post-mortem pH fall to 5.5 causes a shrinkage of myofilaments. This is due to the fact that, with falling pH, the myofibrillar proteins approach their isoelectric point (IP), which is at pH 5.3 (Grau *et al.*, 1953). At the IP the protein–protein interaction is high, as the number of negative- and positive-charged amino acid side chains are equal and attraction forces are at a maximum. The attraction decreases the space within and between the myofilaments (Kristensen

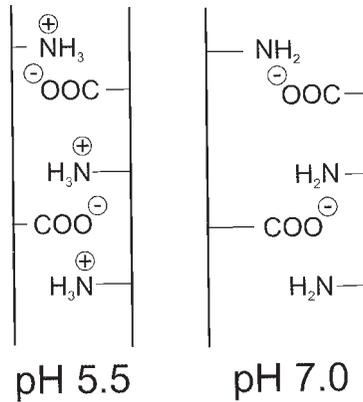


Fig. 18.1. Scheme of influence on pH value of swelling or unswelling of proteins due to changes in charges of amino side chains of proteins.

and Purslow, 2001). Less water can be immobilized in the reduced interfilamental space (Fig. 18.1).

Expressed in colloid chemical terms the swollen gel of myofilaments in a muscle of a live animal turns into a less swollen gel post mortem, leading to a reduced immobilization of water.

18.4.2 Abnormal biochemical changes post mortem

In a number of countries of the world, due to the handling of the animal ante, intra- and post mortem, the breeds of slaughter animals show abnormal changes in muscles.

In pigs, which are stress-susceptible, the killing of the animal causes such stress that hormones are excreted that initiate an extreme stimulation of glycogenolysis in the muscle. Within 45–60 min after death the final pH is reached at prevailing temperatures of 35°C and higher (up to 42°C). This combination of low pH and high temperature causes protein denaturation and additionally membrane disorders, leading to a rapid leakage of intracellular water into the extracellular space. This meat is exudative and, due to protein denaturation, also pale and is called PSE (pale, soft, exudative) meat, occurring mainly in pork. With regard to the drip loss of chilled meat the WHC of PSE pork is extremely poor.

18.4.3 Structural changes post mortem

As mentioned above, muscles in the pre-rigor state can contract if, in the presence of ATP sufficient for contraction, the temperature is lowered too rapidly or too slowly (Fig. 18.2). Contraction enhances drip loss (compare Figs 18.2 and 18.3). Therefore biochemical changes and chilling conditions must fit each other (Honikel, 1987). In order to keep shortening to a minimum the temperature at pH values above 6.0 must be between 18°C and body temperature, below pH 6.0, but before

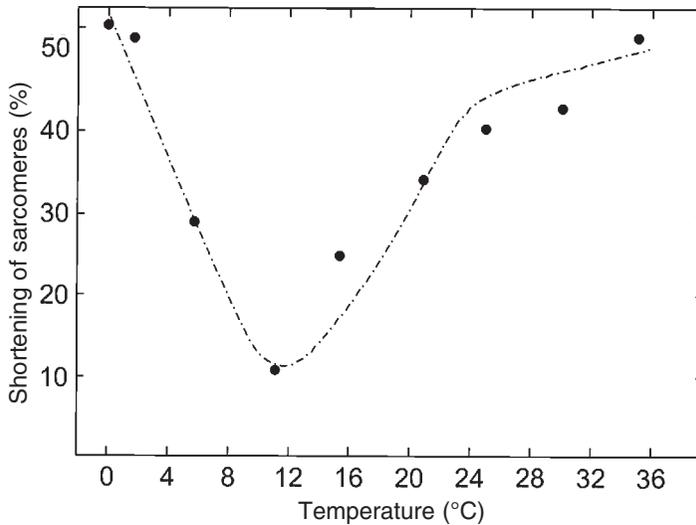


Fig. 18.2. Influence of temperature on sarcomere shortening in the pre-rigor state; original sarcomere length (0% shortening was 1.9 μm) (longissimus dorsi of pork) (Honikel *et al.*, 1986).

the onset of rigor mortis (pH 5.7–5.9) the temperature must be around 12–18°C; after the onset of rigor, the temperature can drop below 10°C. Observing these values, contraction or shortening post mortem can be avoided (Honikel *et al.*, 1983). The onset of rigor mortis with or without shortening takes place in any case.

After the onset of rigor mortis the ageing process by proteases as stated above must take place in order to tenderize meat (see also Hopkins and Taylor, Chapter 17, this volume). During the period of ageing the cellular membranes become leaky and water from the intracellular space moves into the extracellular fluid. Whereas the proteolytic action of proteases does not change the WHC of meat, the disintegration of membranes does affect it.

18.5 First Conclusion

Water is immobilized in the muscle in the myofibres and is restricted in movement by subcellular and cellular membranes. In the muscles post mortem the pH falls under normal conditions from pH 7 to about 5.5. Approaching the IP (pH 5.3) of the muscle the proteins shrink and the interfilamental space is lowered, translocating water into the sarcoplasm. This ‘free’ sarcoplasmic water penetrates slowly into the extracellular space and finally appears at the muscle surface.

A rapid fall in pH occurring in PSE-prone muscles at prevailing temperatures above 35°C causes a partial protein denaturation and membrane leakage. Fluid moves easily into the extracellular space. Fast or very slow chilling causes shortening of myofibrils decreasing the interfilamental space further, above the action of pH fall.

Ageing itself does not change the immobilization of water in the myofibres, but

with progressing time membrane structures disintegrate and water leaves the muscle cell more easily, enhancing the drip loss.

18.6 Processing of Meat and Water-holding Capacity

18.6.1 Chilling

Due to the danger of spoilage meat must be chilled soon after slaughter. From a hygienic as well as an economic point of view rapid chilling is preferable as with a more rapid fall in temperature fewer microorganisms grow and less water evaporates from the meat. For the consumer who wants a tender and juicy meat, ageing at elevated temperatures seems preferable. These differing opinions have introduced a variety of chilling procedures.

We are looking at meat from the point of view of people who are interested in WHC. As mentioned above (Fig. 18.2), on rapid or very slow chilling, contraction takes place, leading to an increase in 'free' water in the muscle cell. This 'free' water appears after a delay as water at the meat surface evaporating or dripping to the floor. Figure 18.3 shows how the chilling temperature influences the drip loss. One day post mortem there is no big difference between the drip loss of meat kept at 0°C or at 15°C (difference about 1%). The difference increases day by day. At 7 days post mortem meat kept all the time at 0°C exhibited 8.3% drip loss, meat kept for the first day at 15°C and on days 2–7 at 0°C showed 3.3% drip loss (difference 5%). At 35°C within the first 24 h post mortem the drip increased to 7% at day 7; that is a difference between 15 and 35°C of 3.7% in drip loss. If one compares Figs 18.2 and 18.3 the similarity between the curves becomes obvious. There is a linear relationship between sarcomere length as an indicator of shortening and the drip loss at several

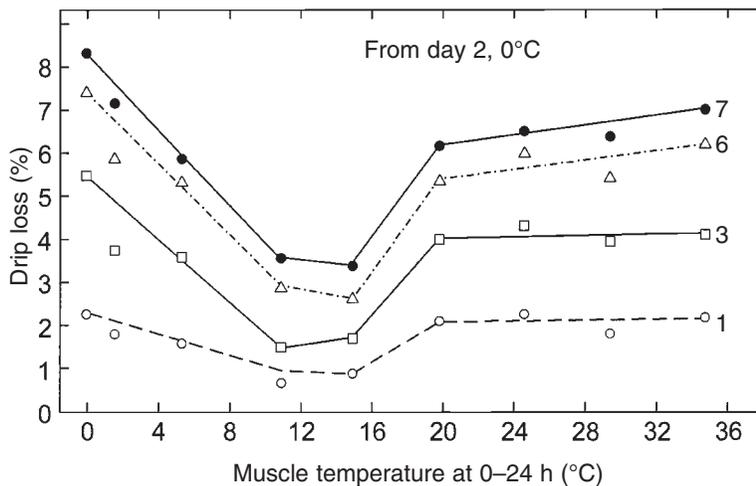


Fig. 18.3. Influence of temperature post mortem (0–24 h) on drip loss of pork longissimus dorsi 1–7 days post mortem (indicated on the right of the graph) (Honikel *et al.*, 1986).

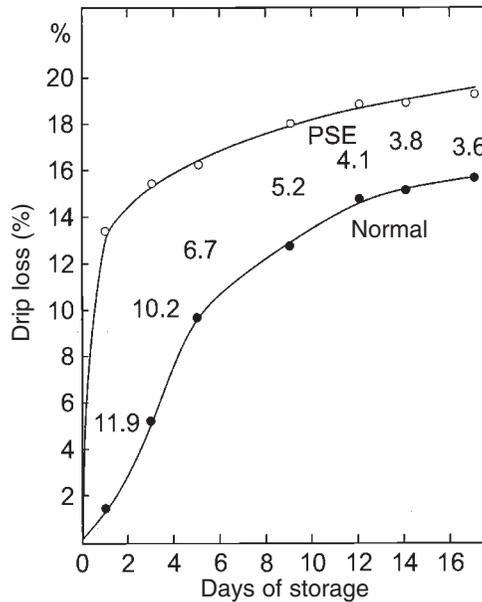


Fig. 18.4. Drip loss during storage of slices of 'normal' and 'PSE' longissimus dorsi with a pH_1 value of 5.8. Slices of longissimus dorsi were obtained within 45 min post mortem with pH of 5.8. Two slices were stored at 38°C between 45 min and 2 h, at 35°C between 2 and 3 h, and 33°C between 3 and 4 h post mortem. From 4 h up to 17 days post mortem the temperature of storage was 0°C (PSE). Two further slices of the same muscle were stored at 20°C between 45 min and 4 h post mortem. Then the temperature was 0°C up to 17 days post mortem (normal). Drip loss was measured at the days indicated. The figures between the curves are the differences in percentage drip loss at the days of measurement. Data for drip loss are the mean of the two slices used (Honikel and Kim, 1985).

days post mortem. In order to keep the drip loss of meat to a minimum, meat must be chilled in such a way that none or a minimum shortening occurs. The chilling conditions depend on the velocity of pH fall as mentioned above.

With PSE-prone muscles, which show an accelerated pH fall post mortem, the poor WHC of this meat expressed as drip loss is due to the high temperature at low pH . In this case, however, no high temperature shortening occurs as shown by Honikel (1987). However, in these muscles, in addition to protein denaturation, there is an early disintegration of cellular membranes, which allows the cellular water to appear rapidly at the meat surface. Figure 18.4 shows the increase in drip loss of PSE and normal pork. At 1 day post mortem a slice of a PSE muscle exhibits a drip loss of 13.5%, 11.9% higher than the normal muscles with 1.6%. This difference becomes lower with time. At 17 days post mortem PSE meat has a drip loss only 3.6% higher than in normal meat at 15.8%. This difference is due to protein denaturation of the PSE muscle, whereas the early rapid appearance of drip is due to an early membrane leakage in PSE meat. As mentioned above, in normal meat membrane disintegration occurs slowly showing the highest increase in drip loss at 2–6 days post mortem (Fig. 18.4).

The rapid pH fall in PSE-prone muscles cannot be stopped, but the chilling

Table 18.1. Influence of chilling rate post mortem on drip loss in slices (c. 75 g) of PSE-prone ($\text{pH}_1 = 5.45$) longissimus dorsi of pork (Honikel, unpublished).

Min post mortem to 34°C	% drip after days post mortem				
	0.3	1	2	5	8
45	1.1	2.7	5.0	9.1	10.8
51	4.8	6.9	8.3	10.3	11.7
78	7.1	10.0	11.8	13.1	14.5
138	9.9	10.8	12.2	13.6	14.4

rates can be enhanced. The increase in chilling rate has a very pronounced effect on drip loss, as Table 18.1 shows. The time to reach 34°C in the muscles expresses the velocity of chilling. At 0.3 days post mortem the drip loss varies from 1.1% with the fastest chilling rate to 9.9% in those samples where 34°C is reached 138 min post mortem. At day 1 differences from 2.7 to 10.8% are observed. With progressing time the difference becomes smaller, being 3.6% at 8 days post mortem.

In PSE pork the pale meat loses water very early and rapidly (Fig. 18.4, Table 18.1). It is extremely exudative in the first 5–6 days post mortem, in the period of time when pork is sold and consumed. Rapid chilling (34°C must be reached within 50–55 min post mortem) improves the WHC of PSE-prone muscles; that is, it reduces drip loss.

In conclusion, muscles with a normal rate of glycolysis must not be chilled too rapidly or below 10–15°C before the onset of rigor mortis. PSE-prone muscles must be chilled rapidly and very early in order to reduce the high drip loss. Unfortunately in a carcass both types of muscles occur and the chilling conditions must be adjusted to the needs of different muscle types.

18.6.2 Heating

During heating the most dramatic changes occur in meat, such as shrinkage and hardening of tissue and release of cooking juice. These changes are caused by structural changes of myofibrillar proteins and of membrane structures.

The heat-induced structural changes apparently lead to reduced immobilization of water; with progressing heating time and with increasing temperature of heating the cooking loss increases as shown in Fig. 18.5. The cooking loss can be as high as 45% of the raw muscle weight on heating to 95°C. As observed in all aspects of WHC the cooking loss also depends on the pH of the meat. The higher the pH, the lower the cooking loss (Fig. 18.5). It is interesting to note that pre-rigor shortening induced by storage at various temperatures has no effect on cooking loss (Table 18.2). This fact indicates that cooking causes more significant changes in meat than do shortening or fast glycolysis in raw meat (Allison *et al.*, 2002). Drip loss never exceeds 20%; cooking may cause a loss of 45% of the original weight.

Table 18.2. WHC (percentage cooking loss) of sternomandibularis of beef during post-mortem pH fall, incubated at various temperatures that cause various degrees of shortening (cooking to 95°C and keeping it for 10 min) (Honikel *et al.*, 1981).

Temp. (°C)	pH 6.8	pH 6.1	pH 5.9	pH 5.5
0.5	34	42	44	—
4	34	40	41	44
5	36	41	42	45
7.5	37	40	42	45
10	27	38	40	45
14	33	39	41	44
17	32	40	43	44
20	37	42	43	44
20	35	39	41	43
23	33	40	42	44
24	37	44	46	53
27	37	39	40	42
30	35	41	42	45
\bar{X}	34.4	40.4	42.1	44.8
SD	± 2.8	± 1.6	± 1.7	± 2.7

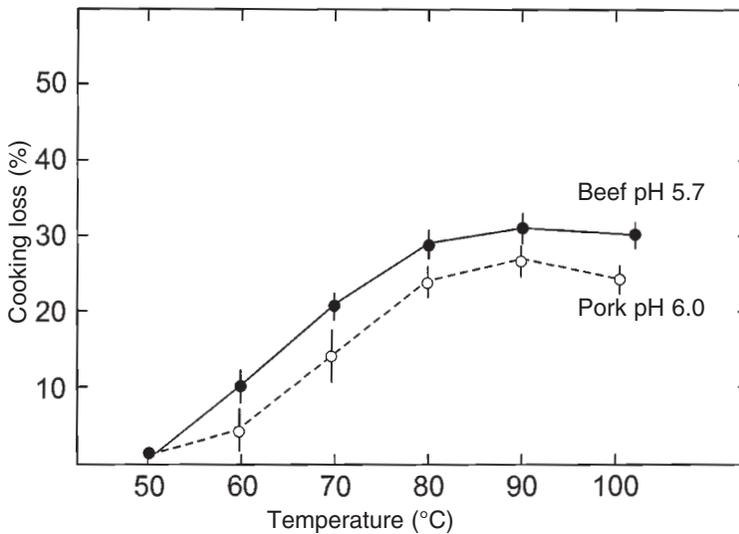


Fig. 18.5. Temperature of heating and cooking loss at various final temperatures and the influence of the pH of the meat (heating velocity was constant 2.5°C/min) (Honikel, unpublished results).

18.7 Conclusion on the Effects of Processing on Water-holding Capacity

Processing of meat is necessary in order to store and prepare meat. If chilling is done in the proper way, the influence on WHC remains small; pre-rigor shortening must

be avoided. Meat is usually consumed after heating. The meat we eat should be tender but also juicy. Juiciness is related to water retention. Thus the demand for tenderness of heated meat must be balanced with its WHC. We have found in our own investigations that this can be done (Seuß and Honikel, 1987).

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Perspectives

In this book we have presented many aspects of general mammalian and livestock muscle biology, from prenatal development to post-mortem meat quality. Although meat quality data can be used directly in society much of this knowledge is still in an abstract, fundamental scientific phase. In these Perspectives the editors try to look forward into the future and express their view on how this knowledge can be exploited to increase welfare for animals producing high skeletal muscle masses for meat, and to improve meat quality for consumers for healthy nutrition. How can skeletal muscle physiology (from prenatal to post mortem) and genetics (genomics) contribute to these goals?

It has been suggested many times that knowledge of the functioning of the genome will solve many problems. However, although the number of genomes with known sequences is rapidly increasing we are still far from implementing this knowledge. Knowing the sequence of the genome is not synonymous to understanding its function. Knowledge of the map position of the genes may help us to understand the way the genome is built. Knowledge of the transcriptomes and proteomes may help us to understand which genes are expressed and how proteins may interact to show their functioning. But basically all this needs the hard, sometimes called 'old fashioned', work of physiologists to understand the mechanisms by which the genes/proteins interact in the set of networks of complex reactions called life. Also the interactions between proteins and non-coding parts of the genome may be an important part of the interactions that needs to be studied. Only this integrated approach will enable us to build the knowledge that can be used for future exploration of the possibilities for improving both human and animal welfare. This book describes the functioning of several independent genes and proteins. Combining the results of many such studies will shed light on the physiological possibilities and borders of the genomes of livestock. This will enable us to rear high-producing, balanced livestock well within its physiological capabilities without extremes. Also, improving the quality of human nutrition is less a matter of product mass, but more of balancing the mineral and micronutrient contents of food products. Thus, the new but still little-explored capacities of the metabolomics may be equally important to knowing

the constitution of a tissue, important for both healthy animals with high quality of life and healthy consumers with high quality food.

An exciting scientific post-genomic period lies ahead with many promises for both livestock welfare and human nutrition. The editors hope this book will provide readers with up-to-date knowledge and new ideas to develop strategies to tackle the relevant questions in these areas in the coming years.

Dr Marinus F.W. te Pas
Professor Maria E. Everts
Professor Henk Haagsman

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