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MINIREVIEW

Regulation of Myosin Expression in Developing and Regenerating Extrafusal and Intrafusal Muscle Fibers with Special Emphasis on the Role of Thyroid Hormones

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Summary

Expression of the muscle phenotype is the result of interaction between intrinsic and extrinsic factors, the latter including innervation, mechanical influences and hormonal signals. This minireview summarizes some of the current knowledge regarding the regulation of myosin heavy chain (MHC) isoform transitions during muscle development and regeneration. It describes the role of genetic factors, neural and mechanical influences and it focuses on the contribution of thyroid hormones to the differentiation of muscle fiber phenotypes as shown by the regulation of the expression of MHC isoforms and development of myofibrillar ATPase activity. Finally, it shortly summarizes results regarding the differentiation of MHC isoforms in regenerated muscle fibers of the graft after heterochronous isotransplantation in rats with different thyroid status.

Key words

Myosin heavy chains • Myofibrillar proteins • Muscle cell lineages • Muscle innervation • Thyroid hormones • Muscle fiber types • Muscle spindles

Diversity of mammalian skeletal muscles and myosin heavy chain (MHC) isoforms

Myogenesis and regeneration of skeletal muscles are characterized by fusion of replicating mononucleated myoblasts or satellite cells into syncytial myotubes and by their maturation into differentiated muscle fibers. This differentiation involves activation of cell-type specific genes which induces the synthesis of muscle-type specific proteins. The resulting ratio between phenotypically diverse fiber types differs from muscle to muscle reflecting their physiological function and usage. Furthermore, the fiber population of a given muscle is in a dynamic state, constantly adjusting to altered functional demands, hormonal signals and changes in neural input (for review see Miller and Stockdale 1987, Syrový 1987, Hausman and Watson 1994, Buonanno and Rosenthal

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1996, Schiaffino and Reggiani 1996, Haddad *et al.* 1997, Pette and Staron 1997).

Many muscle specific proteins, including myosin, exist in multiple, tissue-specific and developmentally regulated isoforms encoded by families of genes. While studying the expression of genes encoding myofibrillar proteins, it has been revealed that selection of the optimal phenotype is based on the activation of one gene – encoding the appropriate isoform – from a given gene family. These experiments have also confirmed that regulation of this expression can occur at the level of transcription, alternative splicing of primary transcripts, mRNA stability and/or translation (for review see Gunning and Hardeman 1991, Buonanno and Rosenthal 1996, Schiaffino and Reggiani 1996). The best known is the highly conserved multigene family encoding the MHC isoforms (Buckingham *et al.* 1986). Up to date, eight sarcomeric MHC genes, two cardiac (alfa and beta) and six skeletal (embryonic, perinatal, IIa, IIx/d, IIb and extraocular), have been identified in tightly linked clusters on human chromosomes 14 and 17, mouse 14 and 11, and rat 14 and 10 (Weiss *et al.* 1999). The individual MHC genes can be differentially expressed during development and respond to neural, hormonal and other physiological signals.

The structural and functional diversity of skeletal muscles reflects the content of distinct MHC isoforms, which are responsible for different myofibrillar ATPase (mATPase) activity and the maximum intrinsic velocity of shortening (V_{max}) of muscle fibers. In skeletal muscles of adult mammals such as mice, rats, rabbits and guinea pigs, four major extrafusal fiber types have been described: slow-twitch or type I and three fast-twitch fiber types, IIA, IIX/D and IIB. Each histochemical fiber type contains a corresponding MHC isoform: I, IIa, IIx/d and IIb (for review see Pette and Staron 1990, 1997, Schiaffino and Reggiani 1994). Besides these basic muscle fiber types, also transitional (hybrid) fibers can be identified, which contain various combinations of MHC isoforms. The best known are fibers designated as type IC and IIC that express both type I and type IIa MHC isoforms in various proportions (for review see Hämäläinen and Pette 1995). Coexpression of MHC RNA transcripts was demonstrated in both rat (DeNardi *et al.* 1993) and human (Smerdu *et al.* 1994) muscles. Embryonic and neonatal (perinatal) MHC isoforms are expressed in developing and regenerating muscle fibers or in specific adult mammalian muscles, such as extraocular (Wieczorek *et al.* 1985) or masseter muscles (Whalen *et al.* 1981, Butler-Browne and Whalen 1984, Butler-Browne *et al.* 1988). Other MHC isoforms, slow tonic (slow-developmental), extraocular, superfast and alpha cardiac-like are as a rule expressed either in highly specialized muscles of some species, e.g. in extraocular muscles and in muscles derived from the first branchial arch (e.g. in masticatory and the tensor tympani muscles) or in intrafusal fibers of muscle spindles in a great majority of skeletal muscles (Wieczorek *et al.* 1985, Pedrosa *et al.* 1990, Pedrosa-Domellöf *et al.* 1991, 1992, D'Albis *et al.* 1993, for review see Pette and Staron 1990, 1997, Soukup *et al.* 1995).

The expression of specific isoforms of a given muscle fiber in the adult mammal is the result of a combination of genetic programs intrinsic to the myoblast lineage from which the muscle fiber had developed, and extrinsic influences such as type of innervation, mechanical and/or hormonal factors; the latter, however, can change the muscle phenotype only within a genetically predetermined adaptation range (for review see Bandman 1985, Pette and Vrbová 1985, 1992, Miller and Stockdale 1986, Westgaard and Lømo 1988, Gunning and Hardeman 1991, Hoh 1991, Miller 1991, Stockdale 1992, D'Albis and Butler-Browne 1993, Rudnicki and Jaenisch 1995, Buonano and Rosenthal 1996, Schiaffino and Reggiani 1996, Pette and Staron 1997).

Diversity of muscle cell precursors

Muscle fibers are formed by the fusion of myoblasts to form myotubes that become innervated and mature into myofibers. In higher vertebrates they arise in at least two waves. The primary myotubes are the first to develop from primary myoblasts, followed by the secondary myotubes arising from secondary myoblasts. The primary myotubes are formed in the absence of nerves, whereas formation of secondary myotubes is impeded by denervation (e.g. Wilson and Harris 1993). Earlier, it was thought that all myoblasts belong to a fairly homogeneous population. There is now, however, considerable evidence that myoblasts are heterogeneous and that their lineage-derived genetically fixed differences interact with extramuscular factors to form a distinct muscle phenotype. In birds, three lineages of early myoblasts, each pre-programmed to express MHC isoforms in a special manner were revealed (for review see Miller and Stockdale 1986, 1987). Although studies in mammals did not report such clear results as in birds, it

is supposed that genetic differences exist between primary and secondary, fast and slow, axial and limb or between rostral and caudal myoblasts (for review see Hoh 1991, Miller 1992, Donoghue and Sanes 1994, Schiaffino and Reggiani 1996). Corresponding differences were also demonstrated among satellite cells. It was found that cultured satellite cells derived from slow rat soleus and fast tibialis anterior (TA) muscles differ in their MHC expression (Düsterhöft and Pette 1993). Under appropriate culture conditions, some myotubes derived from soleus satellite cells expressed slow MHC in addition to embryonic MHC isoform, whereas expression of the slow isoform was negligible in TA-derived satellite cells. These differences were further enhanced by slow-type pattern electrical stimulation of cultured cells (Wehrle *et al.* 1994). Soleus satellite cells are thus apparently predetermined (in contrast to those from fast muscle) to express the slow-twitch MHC isoform. Studies of mammalian muscles have also shown that the divergence of slow and fast fibers occurs early during fiber differentiation; the differences between myotubes emerge while they are still multiply innervated and the differences are not eliminated by denervation (for review see Gunning and Hardemann 1991, Hoh 1991). Another example of special muscle cell precursors could be related to intrafusal fibers of muscle spindles. The characteristic morphology and the specificity of the MHC isoforms in intrafusal fibers could reflect a unique pathway of muscle spindle differentiation from a special intrafusal cell lineage committed to differentiate after innervation by Ia afferent axons into intrafusal fibers (Pedrosa and Thornell 1990, for review see Soukup *et al.* 1995, Walro and Kucera 1999).

Neural control of myofiber phenotype

The pioneer studies of Buller *et al.* (1960) established that muscle properties were closely related to those of their innervating motor neurons. Subsequently, the role of motor innervation has been investigated by different methods among which denervation, cross-reinnervation and nerve or muscle electric stimulation have been frequently used (for review see Pette and Vrbová 1985). Many of the contractile and even fine-structural properties of muscle fibers were found to change according to the type of motor innervation ("slow" or "fast") received by ectopic muscle grafts not only in mammalian (Buller *et al.* 1960), but also in avian muscles (Hník *et al.* 1967, Zelená *et al.* 1967, Jirmanová and Zelená 1973). Cross-reinnervation and electrical stimulation at frequencies that resemble the normal slow-twitch muscle motor unit activities, specify the role of motor activity and result in the experimental transformation of muscle characteristics. These changes concern their metabolic properties (Buchegger *et al.* 1984, Gundersen *et al.* 1988, Leeuw and Pette 1993) and contractile characteristics (Eccles 1967, Salmons and Sréter 1976, Lømo *et al.* 1974, Westgaard and Lømo 1988). The latter are associated with a transformation of the spectrum of myosin isoforms and regulatory proteins of myofilaments (Weeds *et al.* 1974, Sréter *et al.* 1975, Srihari *et al.* 1981, Ausoni *et al.* 1990, Termin and Pette 1992; for review see Pette and Vrbová 1992). Although the role of nerve derived factors that travel down the axons of motoneurons has been acknowledged (for review see Gutmann 1976), it is the pattern of nerve impulses that could affect muscle properties, which is now preferentially accepted (Buller *et al.* 1960, Vrbová 1963).

The role of motor activity was especially stressed by Vrbová, who initially found that the contraction speed of the slow-twitch soleus muscle became higher after denervation and tenotomy (Vrbová 1963). Using implanted stimulators, Salmons and Vrbová (1969) demonstrated that the muscle contraction time could be altered by electrical stimulation of its nerves and that changes in the expression of contractile proteins are accompanied by a transition of muscle properties (for review see Pette and Vrbová 1985, 1992, Buonanno and Rosenthal 1996, Pette and Staron 1997). As regards extrafusal muscle fibers, many studies performed during the last two decades have demonstrated that after stimulation of a fast muscle at a chronic low frequency (CLFS) not only shifts in the expression of contractile proteins of thick and thin filaments were reported, but the vascularization and the oxidative capacity of the muscle was also enhanced (for review see Pette and Vrbová 1992, Buonanno and Rosenthal 1996, Schiaffino and Reggiani 1996). In the experiments mentioned above, the muscles were stimulated *via* their nerve so that the effects of nerve derived factors could not be completely excluded.

The experiments, in which denervated muscles were directly stimulated showed that such activity was sufficient to elicit changes in the contraction time of muscles (Lømo *et al.* 1974) and resulted in a change of MHC expression (Gorza *et al.* 1988). Stimulation of denervated muscles of rats with simulated intrinsic motoneuronal firing patterns (Hennig and Lømo 1985) was as effective as cross-innervation in causing transition

of muscle contractile properties (Eken and Gundersen 1988). During fast-to-slow transformation, fast-type myofibrillar protein isoforms are replaced by their slow counterparts and the direction of MHC isoform transition proceeds in the following order: MHC IIb → MHC IIx/d → MHC IIa → MHC I (Termin *et al.* 1989, for review see Pette and Vrbová 1985, Mira *et al.* 1992, Pette and Staron 1997). Conversely, a transformation of slow-twitch muscles into faster contracting muscles was provoked by phasic high-frequency stimulation (PHFS) (Lømo *et al.* 1974, Eken and Gundersen 1988). PHFS leads to an up-regulation of the fast type MHC isoforms in the rat slow soleus muscle (Gorza *et al.* 1988, Ausoni *et al.* 1990, Hämäläinen and Pette 1996). Hämäläinen and Pette (1996) were even able to demonstrate the appearance of MHC IIb – the fastest isoform present in rat limb muscles.

However, in the case of intrafusal muscle fibers, the induction and maintenance of fiber phenotypes are completely independent of neuronal activity. Special properties, including unusual MHC isoforms of intrafusal muscle fibers in muscle spindles, are induced and maintained by the trophic nonimpulse activity of Ia sensory neurons (for review see Zelená and Hník 1963, Zelená 1994, Soukup *et al.* 1995, Walro and Kucera 1999).

Contribution of mechanical factors to fiber type differentiation

Recent data (Goldspink *et al.* 1991, 1992) have indicated that mechanical signals, such as stretch, may be important for the expression of MHC I in the soleus muscle. It seems possible that, at the same time, stretch in antigravity muscles, such as the soleus, may also prevent expression of the MHC IIb in spite of the appropriate neural stimulation pattern experimentally imposed on these muscles by electrical stimulation or cross-reinnervation. Elimination of the effects of gravitational stretch following transposition of regenerating soleus muscle into the bed of extensor digitorum longus muscle apparently released the MHC IIb expression in the cross-transplanted and regenerated soleus muscle (Snoj-Cvetko *et al.* 1996b). Accordingly, unloading of hindlimbs triggered expression of MHC IIx/d and some MHC IIb in the unloaded soleus muscle (Fauteck and Kandarian 1995).

Thyroid hormone levels and MHC gene expression in developing and mature muscle fibers

It is well known that striated muscles are privileged targets for thyroid hormones (for review see e.g. D'Albis and Butler-Browne 1993, Hausman and Watson 1994, Haddad *et al.* 1997). In general, the action of thyroid hormones on gene transcription is mediated *via* their nuclear receptors. Nuclear hormone receptors regulate gene transcription by recognizing specific regulatory sequences, called hormone responsive elements, situated in gene promotors, enhancers or silencers. Heterodimers of thyroid hormone and retinoid X receptors have a constitutive DNA-binding activity (for review see Hatina and Reischig 2000). In the absence of the hormone, thyroid receptor is connected with a corepressor that decreases effectivity of transcription initiation. Activation after binding of the hormone leads to conformational changes represented by dissociating a corepressor and recruiting a coactivator; the receptor complex then switches into the transcription activator (Chin and Yen 1997).

Regulation of the MHC gene family is complex and the same MHC gene can be regulated by the thyroid hormone differently in various muscles (Izumo *et al.* 1986). In mammals, the perinatal development is associated with a change in the plasma concentration of the T_3 and T_4 thyroid hormones. The concentration of active hormone T_3 (3,3',5-triiodo-L-thyronine) is barely detectable in embryonic and newborn rats, it increases a few days after birth, reaches a peak at about 2 weeks, then slightly declines to reach a plateau in the adult rats (Dubois and Dussault 1977). Coincident with this increase in T_3 concentration, embryonic and perinatal MHC isoforms are progressively repressed and adult fast MHC isoforms are accumulated (for review see Bandman 1985). During development, hyperthyroid rats display an earlier switching from embryonic and perinatal MHC isoforms to adult-type fast MHC isoforms, while the contrary holds true for hypothyroid rats (Gambke *et al.* 1983, Butler-Browne *et al.* 1984, Izumo *et al.* 1986, D'Albis *et al.* 1990, for review see Bandman 1985). This scheme is generally true since it has been observed in limb muscles of all investigated mammals including humans (Hoh and Yeoh 1979, Whalen *et al.* 1981, Fitzsimons and Hoh 1983, Hoh *et al.* 1988, D'Albis *et al.* 1987, 1989, 1991, Butler-Browne *et al.* 1990, Diffee *et al.* 1991, Finkelstein *et al.* 1992). It therefore appears that

the T_3 thyroid hormone represses the expression of embryonic and perinatal myosin isoforms, while it activates that of adult fast isoforms. Brozanski *et al.* (1991) have shown that the disappearance of perinatal myosin isoforms during postnatal development is delayed in the diaphragm of undernourished rat pups compared to the controls; this undernutrition is also accompanied by a decrease in serum T_3 levels, which led the authors to suggest that the alterations in MHC isoform transitions are induced by hypothyroidism associated with the undernutrition. In humans, excessive levels of the thyroid hormones during fetal development have also been shown to produce a precocious accumulation of adult MHC isoforms as well as a precocious maturation of the muscle (Butler-Browne *et al.* 1990).

All muscles of the same animal respond to endogenous changes in thyroid hormone concentrations, but their response has been shown to vary depending on the muscle (Gustafson *et al.* 1986, Izumo *et al.* 1986, D'Albis *et al.* 1990, Petrof *et al.* 1992). While in the rat, the diaphragm displays a most precocious switch to adult fast myosin isoforms and the masseter is the last, in the rabbit, the tongue musculature displays the most precocious switching (D'Albis *et al.* 1991). It appears that different skeletal muscles do not contain the same number of thyroid hormone receptors, which results in a different sensitivity of individual muscles to this hormone. The same explanation has also been suggested for the MHC isoform switching in eight muscles in developing experimentally-treated hyperthyroid rats compared with euthyroid rats (D'Albis and Butler-Browne 1993). Hyperthyroidism barely modifies the MHC isoform switching in the diaphragm, which indicates that the endogenous physiological thyroid hormone concentration is almost optimal for inducing the expression of fast myosin. On the contrary, hyperthyroidism accelerates the MHC isoform switching in other investigated muscles, which become similar to the diaphragm. In the masseter of young adult rats, where the perinatal MHC isoform persists, the hyperthyroid treatment causes it to disappear, whereas hypothyroidism induces its reappearance (Mahdavi *et al.* 1987). The effect of hyperthyroidism, induced during early postnatal development, is not permanent and can be reversed by the interruption of treatment (D'Albis *et al.* 1990).

Thyroid hormone is also known to modulate MHC gene expression and the MHC isoform composition of adult skeletal muscles (Izumo *et al.* 1986, Kirschbaum *et al.* 1990, Caiozzo *et al.* 1991, 1992). In general, hypothyroidism increases the expression of slow MHC isoform in both skeletal and cardiac muscles, whereas hyperthyroidism inhibits slow MHC isoform expression in the soleus and diaphragm of the rat (Ianuzzo *et al.* 1977, 1991, Izumo *et al.* 1986, Caiozzo *et al.* 1992). In slow muscles such as the soleus, hyperthyroidism is associated with an upregulated expression of IIa MHC messenger RNA (mRNA), whereas hypothyroidism is associated with an upregulated I MHC mRNA expression. In fast muscles, hypothyroidism is associated with an increase in type IIa MHC mRNA (Izumo *et al.* 1986). These responses to an altered thyroid state have also been characterized at the protein level, since hyperthyroidism was found to increase relative amounts of fast MHC isoforms in slow muscles, whereas hypothyroidism resulted in a relative increase in the slow MHC isoform content (Fitzimons *et al.* 1990). In the soleus muscle, hyperthyroidism induced in rats by injections of T_3 every other day for 20 weeks, increased its contractile velocity and significantly decreased the proportion of slow myosin from 93 to 69% (Caiozzo *et al.* 1991). The expression of fast type IIa MHC gene was concomitantly increased (Izumo *et al.* 1986). In contrast, no significant changes, either in the native myosin or MHC isoform content or in contraction velocity, were observed in the plantaris muscle, which mostly contains fast type II MHC isoforms (Caiozzo *et al.* 1991). On the contrary, experimental hypothyroidism has an overall slowing effect on muscles, which is correlated with an increase in the amount of both native slow myosin and type I MHC isoform in fast-twitch and slow-twitch muscles (Leijendekker and van Hardeveld 1987, Caiozzo *et al.* 1992). Furthermore, slow muscles were found to be much more responsive to alterations in thyroid state than fast muscles (Fitzimons *et al.* 1990). Thyroidectomy also decreases the resting EMG activity of the soleus muscle (Hník *et al.* 1985).

Interesting results were obtained on a model of the mutant dwarf mouse, originally described in 1929 by Snell. The hypothyroid dwarf mouse mutant provided an excellent model system for investigating the direct influence of thyroid hormones on MHC isoform transitions because of the absence of a possible secondary effect due to stimulation of growth hormone production. In this mutant, the developmental isoform transitions are substantially delayed in skeletal muscles and completely blocked in the heart. A normal adult fiber phenotype could be restored by multiple injections of thyroxine (Whalen *et al.* 1985, Butler-Browne *et al.* 1987, Prulière *et al.* 1989). Studies using both the mutant dwarf mouse and hormone injections have shown that the expression of

an adult fast MHC gene is accelerated by the thyroid hormones (Whalen *et al.* 1985) and that the acceleration is independent of muscle innervation. These findings imply that the thyroid hormones exert direct effects on muscles (Russel *et al.* 1988).

When comparing the influence of different thyroid hormone levels with the effect of chronic low frequency stimulation (CLFS) on MHC isoform expression in fast-twitch muscles of hypothyroid, euthyroid and hyperthyroid rats, it was found that the thyroid hormone and CLFS had an antagonistic effect. Increased neuromuscular activity resulting from CLFS shifts myosin expression towards slower isoforms, whereas the thyroid hormone has an opposite effect (Ianuzzo *et al.* 1977, Kirschbaum *et al.* 1990). Under euthyroid conditions, CLFS mainly elicited a IIb → IIx/d → IIa MHC isoforms transition, but not the appearance of I MHC isoform. On the other hand, the slow or I MHC isoform was present in the hypothyroid state and its expression was further enhanced by CLFS, indicating that the "suppressive effect" of the thyroid hormones on this isoform is stronger than the "inductive influence" of CLFS. Hyperthyroidism alone suppressed the expression of IIa MHC and enhanced the transition of IIx/d MHC to IIb MHC. This shift to faster MHC isoforms was only partially counteracted by CLFS (Kirschbaum *et al.* 1990).

The hypothyroid state and hindlimb suspension are well characterized with regard to their impact on MHC isoform expression (Johnson *et al.* 1980, Asmussen and Soukup 1991). When studying the interaction of these competing influences, it was found that the hypothyroid state, and not mechanical unweighting factors, has a predominating controlling role on MHC expression in slow muscle. In the soleus and vastus intermedius muscles of both hypothyroid control and hypothyroid suspended groups, there was an increase in type I MHC isoform and a decrease in type IIa MHC. On the contrary, a decrease in I MHC and an increase in IIa MHC isoform were found in the normal euthyroid suspended rats (Asmussen and Soukup 1991, Diffee *et al.* 1991).

These results suggest that the thyroid hormones induce the expression of those MHC genes which are coding for isoforms with higher ATPase activity. In the soleus muscle, where the slow MHC isoform predominates, the expression of IIa MHC would increase together with muscle contraction velocity, while in the masseter, which contains IIa and IIb MHC, the latter fibers with the highest ATPase activity would be favored. It seems that changes in the concentration of the thyroid hormones support a preferential sequence for the transformation of MHC isoforms: I ↔ IIa ↔ IIx/d ↔ IIb. This suggests that the excess of thyroid hormones favors the appearance of fast MHC isoforms, whereas a lack of the thyroid hormones allows a preferential expression of slow MHC at the expense of fast type MHC isoforms.

MHC expression in intrafusal fibers of muscle spindles

Intrafusal muscle fiber types exhibit distinct morphological characteristics, as they contain typical nuclear accumulations and myofibrillar ultrastructure, possess complex sensory and motor innervation, exhibit specific histo- and immunocytochemical characteristics and are surrounded by a multilayered capsule (Zelená and Soukup 1973, 1974, Soukup 1976, Soukup *et al.* 1979, for review see Zelená 1994, Soukup *et al.* 1995). The three types of intrafusal fibers, nuclear bag_1, nuclear bag_2 and nuclear chain fibers are unique in co-expressing several MHC isoforms, including special spindle-specific ones, such as slow tonic (slow-developmental) and alpha cardiac-like MHC. Furthermore, isoforms typical for muscle development, such as embryonic and neonatal (perinatal) MHC, have also been found in intrafusal fibers of adults (for review see Soukup *et al.* 1995, Walro and Kucera 1999). In the rat, each intrafusal fiber type has a typical MHC pattern expressing several MHC isoforms (or expressing at least isoform(s) containing antigenic determinant(s) reacting with the specific mcAbs for each MHC isoform); it comprises at least 6 MHC isoforms in nuclear bag_2 fibers (embryonic, neonatal, slow twitch/beta cardiac, alpha cardiac-like, slow tonic/slow developmental and fast twitch), 4 MHC isoforms in nuclear bag_1 fibers (embryonic, slow twitch/beta cardiac, slow tonic/slow developmental and alpha cardiac-like) and 2 MHC isoforms in nuclear chain fibers (neonatal and fast twitch) (for review see Soukup *et al.* 1995). Although no gene analysis regarding intrafusal MHC isoforms is known, it was recently suggested that MHC composition in intrafusal fibers has some adaptive significance to proprioception (Walro and Kucera 1999). The distinct MHC pattern and expression of spindle-specific MHC isoforms which are not expressed in extrafusal fibers, distinguish intrafusal fiber types from each other and also from extrafusal fibers in all mammalian and human muscles (Soukup and Thornell 1999, for review see Soukup *et al.* 1995, Walro and

Kucera 1999). These distinct features make intrafusal fibers an attractive *in situ* model for investigating myogenesis, myofibrillogenesis and the mechanisms regulating MHC expression (for review see Soukup *et al.* 1995, Soukup and Novotová 1996, Walro and Kucera 1999).

The development and regeneration of intrafusal muscle fibers, similarly to those of the extrafusal fibers, start from embryonic and adult myoblasts (satellite cells), respectively. We have also suggested that the resulting phenotype of intrafusal fibers is a combination of genetically fixed properties of myoblast cell lineages and of extrinsic, especially neurogenic factors (Soukup *et al.* 1990, 1993, Pedrosa *et al.* 1990, Pedrosa-Domellöf *et al.* 1991, for review see Soukup *et al.* 1995). We have described differences in the reactivity using polyclonal antibodies against MHC isoforms of intrafusal fiber types in muscle spindles of newborn, de-efferented and adult rats (te Kronnie *et al.* 1981, 1982). These and other studies confirmed that the unique expression of slow tonic, alpha cardiac-like, embryonic and neonatal MHCs in limb muscles of adult mammals is restricted to intrafusal fibers (Pierobon-Bormioli *et al.* 1980, Maier *et al.* 1988, Pedrosa *et al.* 1989, 1990, Kucera *et al.* 1992, Kucera and Walro 1990, Pedrosa-Domellöff *et al.* 1991, 1992, 1993, Pedrosa-Domellöff and Thornell 1994, for review see Soukup *et al.* 1995, Walro and Kucera 1999). We have also reported that the reactions of different antibodies against MHC isoforms vary along the length of intrafusal fibers (Soukup *et al.* 1990, Pedrosa *et al.* 1990, Pedrosa-Domellöf *et al.* 1991). This finding explains the regional differences observed earlier in the mATPase reaction (Soukup 1976). These studies have shown that sensory innervation is required for the expression of „spindle-specific" MHC isoforms, whereas motor innervation contributes to the diversity in distribution of the different MHCs along the length of the nuclear bag fibers (Soukup *et al.* 1990, for review see Zelená 1994, Soukup *et al.* 1995, Walro and Kucera 1999). It is known that a number of proteins, including MHC isoforms, can remain localized in the vicinity of the nuclei responsible for their synthesis (Pavlath *et al.* 1989, for review see Hall and Ralston 1989). It remains an open question, whether the concept of nuclear domains can be applied to intrafusal fibers, as the typical regional variations in MHC expression along their length might reflect the existence of nuclear domains under the influence of either sensory or motor innervation (for review see Soukup *et al.* 1995).

The major question pertaining to spindle development is whether intrafusal fibers develop from the same pool of bipotential muscle precursor cells (myoblasts) as extrafusal fibers or from a separate population of myoblasts predestined to become intrafusal fibers. Primary myotubes, which give rise to the first generation of both extrafusal and intrafusal fibers, do not differ in their ultrastructure at the onset of spindle development. Hitherto, no difference in their immunocytochemical profiles has been detected until sensory axons had reached them. Although the expression of MHC genes in extrafusal fibers has been analyzed at both protein and mRNA levels, using biochemical separation techniques or the *in situ* hybridization technique with probes specific for MHC gene transcripts (for review see Pette and Staron 1990, Gunning and Hardeman 1991, Hoh 1991, Ontell *et al.* 1995), the biochemical or clonal analyses of intrafusal fibers are difficult due to their scarcity and corresponding difficulties in their isolation even in differentiated muscles of adult animals (cf. Pedrosa-Domellöf *et al.* 1993). Corresponding studies of tiny undifferentiated intrafusal fibers in developing muscle spindles are still virtually impossible, also due to the general problems in gaining access and manipulating mammalian fetuses. We have therefore tried to induce experimentally postnatal myogenesis and regeneration inside rat muscle spindles. These experiments enabled us to analyze the contribution of intrinsic myogenic (cell lineage) and extrinsic (mainly neurogenic) factors on the ultrastructural differentiation and expression of MHC isoforms in intrafusal fibers (Soukup *et al.* 1993, Zelená and Soukup 1993). The anticipated results partly filled the gap resulting from the lack of biochemical results in previous studies of differentiation of intrafusal muscle fiber types.

Our studies of the differentiation and MHC isoform expression of *de novo* formed supernumerary intrafusal fibers in neonatally de-efferented rat muscle spindles suggested the existence of at least two types of intrafusal satellite cells (Novotová and Soukup 1995, Soukup *et al.* 1990, 1993, 1999b, for review see Soukup *et al.* 1995, Soukup and Novotová 1996). One class of satellite cells is related to nuclear bag (bag_1 and bag_2) fibers; these satellite cells give rise to supernumerary fibers in which the sensory innervation can trigger the expression of slow tonic MHC isoform, thus inducing the differentiation of the nuclear bag phenotype. The other class of satellite cells is associated with nuclear chain fibers, but sensory innervation does not induce the expression of slow tonic MHC isoforms in these

supernumerary fibers. The latter satellite cells differentiate into fibers that exhibit the nuclear chain phenotype irrespective of the presence or absence of sensory innervation (Soukup *et al.* 1993). This property of satellite cells parallels the behavior of those myoblasts that give rise to nuclear chain fibers during normal development and which also do not express slow tonic or slow twitch MHC isoforms (Pedrosa and Thornell 1990), although they bear sensory contacts from the earliest stages of their development (Kucera *et al.* 1989).

Muscle grafting and differentiation of muscle fiber types

After muscle transplantation, the sequence of events proceeding during muscle regeneration is similar, although not identical, to normal muscle development (Gutmann and Carlson 1975, for review see Carlson 1976, Carlson *et al.* 1981, Schiaffino and Reggiani 1996). Muscle grafting thus represents an alternative model for examining postnatal differentiation of extrafusal and intrafusal muscle fiber types. Furthermore, it was found that regenerating fibers adapt more rapidly than surviving fibers (Donovan and Faulkner 1987) and that the adaptive range of MHC expression in regenerating rat soleus (SOL) or extensor digitorum longus (EDL) muscles is broader than in mature muscles (Snoj-Cvetko *et al.* 1996a,b, Eržen *et al.* 1999).

Because the muscle fiber phenotype is the result of interactions between genetic factors intrinsic to the myoblast lineage and extrinsic factors including innervation and the level of thyroid hormones, we have introduced a model of heterochronous isotransplantation (Jirmanová and Soukup 1995), when the slow SOL or fast EDL muscles from young rats are intramuscularly grafted into either EDL or SOL muscles of adult inbred recipients. This technique enables to compare directly – in one model – the influence of muscle cell lineage (depending on slow or fast donor muscle), innervation (given by nerve axons with a low or high nerve impulse frequency from the host muscle) and level of thyroid hormones (hypothyroid, euthyroid or hyperthyroid state of the host) on the muscle fiber phenotype. MHC isoform content and mATPase activity within fibers of regenerated muscle graft can then be analyzed using immunocytochemical, histochemical and stereological methods (Zachařová and Kubínová 1995, Zachařová *et al.* 1997, 1999).

When EDL and SOL muscles from 24- to 28-day-old rats were grafted intramuscularly into the EDL muscle and reinnervated by its nerve in adult inbred euthyroid, hypothyroid and hyperthyroid rats, both SOL and EDL isografts developed into fast muscle in euthyroid rats, since they contained about 90% of fast type II fibers. In hypothyroid rats, the slow to fast transformation of SOL graft was less pronounced, as only 60 % of extrafusal fibers were of the fast type. But it was accentuated in hyperthyroid rats, where the SOL graft contained 98 % of fast type II fibers (Fig. 1). In grafted EDL muscles, the proportion of fast or type II fibers reached almost 99% in euthyroid rats, significantly more than in hypothyroid rats. The hypothyroid state induced by methimazole treatment was effective only if applied immediately after the operation. If the treatment was started 12 weeks after transplantation and maintained for 20 weeks, the conversion of SOL muscle into a fast muscle was almost complete, as fast type II fibers represented 83.0 % of all muscle fibers (Fig. 1). We therefore concluded that after reinnervation by the fast EDL nerve, the thyroid hormone is necessary for the transformation of the regenerating slow soleus muscle into a fast muscle (Soukup *et al.* 1998a,b, 1999a, Zachařová *et al.* 1999).

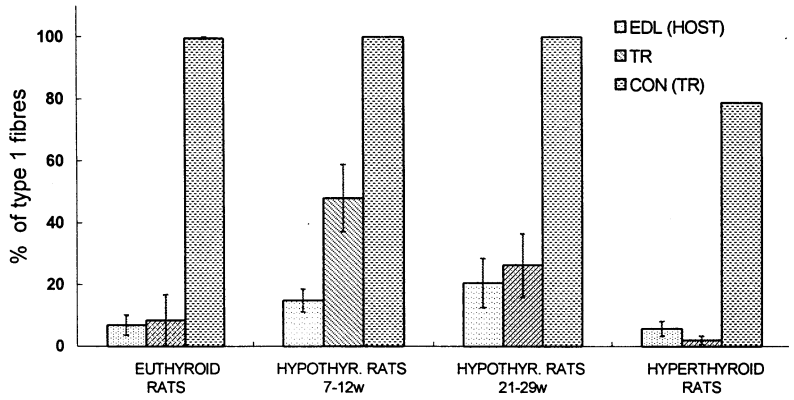

Fig. 1. *Percentage of slow type I fibers in the regenerated soleus muscle isotransplanted into fast EDL muscle of adult inbred host rat.*

The number and morphology of muscle spindles in regenerated EDL muscle grafts isotransplanted from young rats into EDL muscles of adult inbred recipients has been described previously (Jirmanová and Soukup 1995). We found that the regenerated „intrafusal" fibers did not express the typical spindle specific MHC isoforms, they did not exhibit the characteristic regional differences in MHC expression and in the mATPase reaction (Jirmanová and Soukup 1995, Soukup and Thornell 1997). These results have confirmed the previous findings in standard free grafts (for review see Carlson *et al.* 1981). On the other hand, the regenerated "intrafusal" fibers expressed either fast twitch or slow twitch MHC isoforms and exhibited an alkali- or acid-stable mATPase reaction along their whole length, similarly as extrafusal fast type II and slow type I muscle fibers. Since no sensory axons could be found in regenerated muscle spindles after heterochronous isotransplantation (Soukup and Novotová 2000), we concluded that intrafusal satellite cells, although derived from distinctly different nuclear bag_1, bag_2 and nuclear chain fibers, exhibited great plasticity as their MHC expression could be shifted towards the extrafusal muscle fiber phenotype by foreign alpha-motor innervation (Soukup and Thornell 1997).

The proportion of „intrafusal" fibers in regenerated EDL or SOL muscles after isotransplantation in animals with a different thyroid status has not yet been described. However, our pilot experiments of orthotopic (EDL/EDL, SOL/SOL) and heterotopic (EDL/SOL, SOL/EDL) transplantations in euthyroid, hypothyroid and hyperthyroid rats show that the regenerated „intrafusal" fibers (Table 1) attain a comparable proportion of fast (type II) and slow (type I) fibers in all muscle grafts as extrafusal fibers (Soukup and Novotová 1997, Soukup *et al.* 1999a, Zachařová *et al.* 1999, Mráčková *et al.* 1999). Regardless of the type of grafted muscle and thyroid status of the rats, the regenerated intrafusal fibers neither contained spindle specific MHC isoforms, nor did they exhibit a dual mATPase reaction. Furthermore, the characteristic regional differences in MHC isoform expression and mATPase reaction typical for the nuclear bag fibers have also not been found. On the contrary, normal and host muscle spindles always contained intrafusal fibers of three different types (nuclear bag_1, nuclear bag_2 and nuclear chain fibers) and expressed spindle specific (slow tonic, alpha cardiac-like and embryonic or neonatal) MHC isoforms. Hence, intrafusal fibers in regenerated SOL and EDL grafts isotransplanted into fast EDL host muscles and reinnervated solely by motor axons exhibited the same fiber type proportion as regenerated extrafusal fibers. The percentage of both extrafusal and "intrafusal" fast fibers then varied according to the animal's thyroid status; it was slightly increased in hyperthyroid rats and significantly decreased in hypothyroid rats as compared to euthyroid rats.

Table 1. Percentage of intrafusal fiber types in the rat slow soleus (SOL) muscle isotransplanted into fast extensor digitorum longus (EDL) muscle of adult inbred recipient.

	I+Ic/IIc	IIa	IIb	Number of muscles (number of IF analyzed)
Hypothyroid rats	18.0±13.3	47.6±6.2	34.3±13.7	5 (143)
Euthyroid rats	4.5±7.8	57.7±6.4	37.7±4.4	7 (207)
Hyperthyroid rats	5.3±0.8	72.8±11.5	21.8±10.7	2 (55)

Data are means ± S.E.M.

Conclusions and Aims of Future Research

Can we draw any conclusions from the results regarding the significance of thyroid hormones for the expression of MHC genes? We can suppose that thyroid hormones are necessary for "opening" the fast myosin genes to be responsive to fast nerve impulse frequency. In this sense they can to some extent counteract the effect of chronic low frequency nerve stimulation that would lead e.g. in the SOL muscle, to preferential expression of slow myosin. The establishment of a certain muscle fiber type is first of all determined by nerve and muscle activity. Under hyperthyroid conditions, when the organism becomes generally hyperactive, faster MHC isoforms

prevail, enabling "faster" contraction. If we hypothesize that the thyroid hormone is necessary for the transduction and decoding of "nerve or other signals" into the cell nucleus and/or to the MHC gene family, then a lack of this hormone in the hypothyroid state may prevent the transformation towards faster fiber types. The hypothyroid state, however, apparently does not repress the expression of the slow MHC isoform.

However, during phylogenetic development, vertebrates, especially mammals, have developed an elaborate system to maintain a steady-state of thyroid hormone concentrations. Therefore, we can presume physiological effect only after significant changes in thyroid hormone levels under experimental or pathological conditions. Indeed, both in animals and man, pathological situations with increased or decreased levels of thyroid hormones have been described and these lead to changes in muscle fiber type composition (Whalen *et al.* 1985, Butler-Browne *et al.* 1987, 1990, Prulière *et al.* 1989, Brozanski *et al.* 1991).

It can thus be concluded that thyroid hormones are necessary for expression of the fast muscle phenotype, but under normal conditions their significance is rather permissive than instructive, as the organism prefers to maintain their concentration, for many other reasons, within a narrow physiological range. Animals then use other extrinsic factors, especially neural input, for regulating the muscle phenotype to be in accord with the physiological demands of the organism. The expected results are thus primarily of cognitive value, as they provide a deeper insight into regulatory processes, controlling the expression of muscle genes. However, controlled regulation of muscle differentiation could be of considerable clinical importance, e.g. for muscle transplantation in humans.

Over the past decade, significant advances in techniques of molecular biology have substantially increased our understanding of *in vivo* myogenesis. Genes, encoding the MyoD family of myogenic regulatory factors and those genes encoding the isoforms of muscle proteins, as well as the role of multiple growth factors on myogenic cell proliferation and differentiation are now generally acknowledged (for review see Grounds *et al.* 1992, Miller 1990, Yablonka-Reuveni and Rivera 1994, Rudnicki and Jaenisch 1995). As has been shown in mice, the thyroid hormone (T_3) interacts with muscle regulatory gene MyoD in culture and thus affects myoblast proliferation and muscle regeneration (Anderson *et al.* 1998). A further approach to the triggering mechanisms of myoblast differentiation will be possible when fascinating techniques of molecular biology will be applied. Gene targeting experiments, for instance, have revealed the hierarchical relationship among different myogenic regulatory factors (MRFs): MyoD or MyF-5 are probably sufficient for the myoblast formation and their survival, whereas myogenin acts later during development and plays an essential role in the terminal differentiation of myotubes *in vivo* (for review see Miller 1992, Rudnicki and Jaenisch 1995). It was even found that MyoD and myogenin mRNAs accumulate selectively in fast and slow muscles and that this accumulation is controlled by innervation and hormones (Hughes *et al.* 1993). Whether MRFs are the genetic clues responsible for the formation of specific muscle cell lineages, or whether the basis of genetic predetermination occurs even earlier during myogenesis still remains to be elucidated. The molecular mechanisms triggering the differentiation of muscle spindles are only poorly understood. It is clear, however, that innervation of a subset of developing type I myotubes (whether forming a special intrafusal cell lineage or not) by peripheral sensory Ia afferents is that critical event for inducing differentiation of nascent intrafusal fibers (Zelená 1957, for review see Zelená 1994, Soukup *et al.* 1995, Walro and Kucera 1999). Mice lacking neurotrophin NT3 (or its tyrosine kinase receptor trkC) are devoid of muscle spindles because their Ia afferent axons fail to form and trigger spindle differentiation (Ernfors *et al.* 1994, Klein *et al.* 1994, Kucera *et al.* 1995). Another example of gene-targeted experiments is a mutant mice lacking a transcription regulating factor EGR3 (Tourtellotte and Milbrant 1998, O'Donovan *et al.* 1999). In this case, different mechanism(s) was involved in the spindle agenesis because central Ia afferents appeared to be normal (Tourtellotte and Milbrant 1998). Their data suggest that spindles were incapable of differentiating because the nascent intrafusal myotubes were unable to express autonomously the required EGR3 factor. Although specific genes regulated by EGR3 have not yet been identified, the increasing interest for muscle spindles suggests that also gene(s) encoding MHC isoform(s) in intrafusal muscle fibers will soon be recognized.

These results have shown that both extrafusal and intrafusal muscle fibers exhibit great variability of their phenotypic expression. Their variability can be ascribed to the plasticity of the muscle precursor cells, as the muscle diversification apparently depends on heritable lineage-derived properties interacting with environmental influences to give each muscle fiber its distinctive characteristics.

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