CONTROL OF MYOSIN HEAVY CHAIN EXPRESSION IN REGENERATING RAT SOLEUS

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ABSTRACT

The expression of different myosin heavy chain (MHC) isoforms is under the control of several factors, including the state of innervation and thyroid hormone levels. Muscle regeneration following injection of venom from the snake *Notechis scutatus scutatus* into rat soleus was used to study the role of innervation and excess thyroid hormone in influencing MHC gene expression. MHC protein and mRNA content was determined in muscles regenerating in the presence/absence of innervation or in the hyperthyroid state.

In summary, MHC production appears to be primarily controlled at the level of transcription or mRNA stability; however, translational or post-translational control may play a role for IIA MHC protein production. The results also suggest that satellite cells are not preprogrammed to express a particular MHC isoform, but rather are capable of expressing either fast or slow MHC. Subsequent expression of MHC isoforms is modified by factors such as innervation and thyroid hormone.

RESUME

L'expression des diverses isoformes des chaînes lourdes de la myosine (myosin heavy chain ou MHC), est sous le contrôle de plusiers facteurs dont l'innervation et le niveau circulant d'hormone thyroïdienne. La régénération musculaire a été étudiée à la suite d'une injection de venin du serpent *Notechis scutatus scutatus* dans le muscle soléaire de rat. Lors de cette régénération, les rôles de l'innervation et d'un excés d'hormone thyroïdienne, sur l'expression des gènes codant pour les diverses MHC; ainsi qu'en ARNs messagers correspondants, ont été déterminés dans les muscles en régénération en présence ou en absence de l'innervation, ou en présence d'un excés d'hormone thyroïdienne.

En résumé, l'expression des MHC semble être tout d'abord contrôlée soit à l'échelle transcriptionelle soit par la stabilité de l'ARN messager; cependent un contrôle au niveau de la traduction ou postérieur à la traduction pourrait jouer un rôle dans la synthèse de la protéine MHC IIA. Les résultats suggèrent également que les cellules satellites ne sont pas déterminées pour l'expression d'une isoforme particulière, mais sont la chaîne lourde lente. L'expression ultérieure des isoformes des chaînes lourdes de la myosine est modifiée par des facteurs tel que l'innervation et l'homone thyroïdienne.

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LIST OF ABREVIATIONS

1. MHC= myosin heavy chain
2. MLC= myosin light chain
3. Tm= tropomyosin
4. Tn= troponin
5. mRNA= messenger ribonucleic acid
6. cDNA= complementary deoxyribonucleic acid
7. PCR= polymerase chain reaction
8. LM= light microscopy
9. EM= electron microscopy
10. SR= sarcoplasmic reticulum
11. ATP= adenosine triphosphate
12. ACh= acetylcholine
13. AChR= acetylcholine receptor
14. AChE= acetylcholinesterase
15. SO= slow oxidative (muscle fibres)
16. FG= fast glycolytic (muscle fibres)
17. FOG= fast oxidative-glycolytic (muscle fibres)
18. MCK= muscle creatine kinase
19. CAM= cell adhesion molecule
20. VLA-4= very late-activation antigen
21. VCAM= vascular cell adhesion molecule
22. MEF-2= myocyte enhancer-binding factor
23. TRE= thyroid hormone response element

24. TSH= thyroid stimulating hormone
25. T3= tri-iodothyronine
26. T4= thyroxin
27. iRNA= inhibitory ribonucleic acid
28. tcRNA= translational control ribonucleic acid
29. mRNP= messenger ribonucleic protein
30. LMM= light meromyosin
31. HMM= heavy meromyosin
32. DTNB= 5, 5'-dithiobis-2-nitrobenzoic acid
33. CAT= choline acetyltransferase
34. PBS= phosphate buffered saline
35. MOPS= morpholinopropanesulfonic acid sodium salt
36. ACT= actin
37. CA= cardiac actin
38. NTX= innervated regenerates
39. NDEN= denervated regenerates
40. NTH= hyperthyroid regenerates
41. NTD= hyperthyroid/denervated regenerates
42. CLFS= chronic low frequency stimulation
43. UTR= untranslated region
44. EPP= end-plate potential
45. SOL= soleus
46. EDL= extensor digitorum longus
47. TA= tibialis anterior

INTRODUCTION

The myosin contractile protein plays a well known structural and enzymatic role in muscle function. In addition, its various isoforms are characteristic of developmental stages and of the individual adult muscle fibre types. Expression of these isoforms is subject to modulation by hormonal influences and the state of innervation of the muscle fibres. To date nine isoforms of the myosin heavy chain (MHC) gene are specific to striated muscle. The embryonic and neonatal isoforms are typical of developing muscle, and β (slow), slow tonic, fast IIA, IIB, IIX/D, extraocular and superfast are typical of adult muscle (Whalen *et al.*, 1979 and 1981; Mahdavi *et al.*, 1982; Wieczorek *et al.*, 1985; Bar and Pette, 1988; Schiaffino *et al.*, 1989).

Skeletal muscle development is characterized by the sequential expression of MHCs. The embryonic MHC is the earliest myosin isoform expressed followed by the neonatal MHC in fast-twitch muscle fibres of a newborn animal. These early MHC isoforms are replaced by one of the adult isoforms β, IIA, IIB, or IIX (also called IID). A population of muscle fibres, which predominates in slow-twitch muscle, initially expresses embryonic MHC followed by the adult β MHC (Whalen *et al.*, 1981; Butler-Browne and Whalen, 1984; Narasuwa *et al.*, 1987; Bar and Pette, 1988; Schiaffino *et al.*, 1989).

The expression of the various MHCs is under the influence of external factors such as innervation and thyroid hormone. The role that these factors play has not been fully elucidated. Butler-Browne *et al.* (1982) determined that denervation of the newborn rat had no effect on fast MHC accumulation in the gastrocnemius. Denervation has been shown to abolish the accumulation of β/slow MHC in newborn denervated rat soleus muscle, which indicates that innervation is required to maintain its production (Narasuwa *et al.*, 1987). Analysis of aneural avian muscle indicates that β MHC is detected in some myotubes, thus the initial production of β MHC is innervation-independent.

Manipulating the firing frequency of the nerve can also alter MHC accumulation. Low frequency stimulation of rabbit fast-twitch muscle results in a complete transition in MHC isoforms from fast to slow (Brown *et al.*, 1983; Staron *et al.*, 1987). In addition, this transition is reversible once the normal stimulation pattern is re-introduced.

Thyroid hormone is required for the normal growth and maturation of many tissues, including muscle. All of the MHCs respond to altered thyroid hormone levels; however, this response is highly variable depending on the tissue examined (Izumo *et al.*, 1986). In general, under hyperthyroid conditions there is an increased content of fast IIA and IIB MHC proteins and mRNAs. Conversely, under hypothyroid conditions the β MHC predominates (Nwoye *et al.*, 1982; Whalen *et al.*, 1985; Izumo *et al.*, 1986; Butler-Browne *et al.*, 1987; Russell *et al.*, 1988).

Skeletal muscle regeneration is a useful model with which to study the role of innervation and thyroid hormone on MHC expression because it is possible to create a population of regenerating myoblasts in an environment with a set but altered neuronal and/or hormonal input. Muscle regeneration mimics some aspects of muscle development, including the cellular maturational steps leading to muscle fibre formation. Also, there is an expression of some of the developmental forms of the contractile proteins, including myosin.

To gain further insight into the role played by innervation and thyroid hormone on MHC expression we examined MHC content at the protein and mRNA levels in regenerating rat soleus. The use of regenerating muscle allowed us to look at the effects of these two factors on muscle fibre type throughout muscle maturation. Injection of snake venom from *Notechis scutatus scutatus* caused a complete and homogenous degeneration of the muscle fibres followed by their regeneration. The effect of this toxin has been well documented (Harris *et al.*, 1975; Harris and Johnson, 1978; Harris, 1980); although blocking neuromuscular transmission at the neuromuscular junction, it causes muscle degeneration by its phospholipase A activity. In addition, the phenomenon of muscle regeneration is important clinically because of its occurrence in diseases like muscular dystrophy.

Earlier studies by Whalen *et al.* (1990) and Sesodia *et al.* (1993), using *Notechis scutatus scutatus* venom or the more purified version notexin, indicated that innervation is required

for the accumulation of β MHC. In the absence of innervation the neonatal MHC persisted longer and the fast MHC isoforms accumulated.

We have undertaken a more thorough analysis of MHC mRNA content in regenerating muscle subjected to denervation and/or excess thyroid hormone to further understand a) how these factors affect MHC expression in regenerating muscle and b) at what level e.g. transcription or translation. The mRNA analysis involved a qualitative (PCR) and a quantitative (slot blot hybridization) assessment of various MHC isoforms. Protein analysis, using immunohistochemistry, was conducted on the same samples. This allowed for the identification of different MHC isoforms at different stages during regeneration.

Our results indicate that the initial expression of the β MHC gene is nerve independent; however, innervation is required for β MHC mRNA and protein accumulation. Innervation also acts to suppress the accumulation of the immature and fast isoforms of MHC. The denervated regenerates contain IIB and/or IIX MHC protein and large amounts of IIA MHC mRNA, but little of its protein accumulates. This suggests that (i) innervation suppresses IIA MHC mRNA accumulation and (ii) a regulatory mechanism exists which modulates IIA MHC protein accumulation. Thyroid hormone causes a transition to the fast isoforms of MHC, from β to IIA MHC in hyperthyroid regenerates and from IIX to IIB MHC in hyperthyroid/denervated regenerates. The results suggest that innervation plays a greater role than thyroid

hormone in determining MHC expression in regenerating rat soleus.

Generally, the control of MHC expression by innervation and thyroid hormone is mostly at the level of transcription and/or mRNA stability. However, accumulation of IIA MHC protein is regulated additionally by translational or post-translational mechanisms.

LITERATURE REVIEW

I. MUSCLE

A. General Anatomy

Skeletal muscle has a highly ordered and distinctive structure. With light microscopy (LM), skeletal muscle cut in cross-section is seen to be composed of fascicles or bundles of muscle fibres, between which is found connective tissue.

The connective tissue surrounding individual muscle fibres is called the endomysium; that surrounding a fascicle of muscle fibres is the perimysium; and that surrounding the entire muscle is the epimysium. Within the perimysium and epimysium are located blood vessels and muscle spindles. At each end of the muscle, all three layers of connective tissue become continuous with each other and the tendon which attaches the muscle to bone, or sometimes to another tendon.

Muscle fibres, also known as myofibres, are the basic cellular units of muscle tissue. They are long, cylindrical, polynucleated cells, which in cross section, appear polygonal with multiple peripherally placed nuclei, and in longitudinal section, appear rod-shaped with nuclei situated along their sides.

The muscle spindles are sensory organs, which are situated between the fascicles of myofibres. They appear as a bundle of small-diameter muscle fibres encapsulated by layers of flat cells and collagenous fibrils. They function as mechanoreceptors, which provide sensory information regarding position and

stretch, since they are sensitive to muscle length and changes in muscle length (Barker and Banks, 1986).

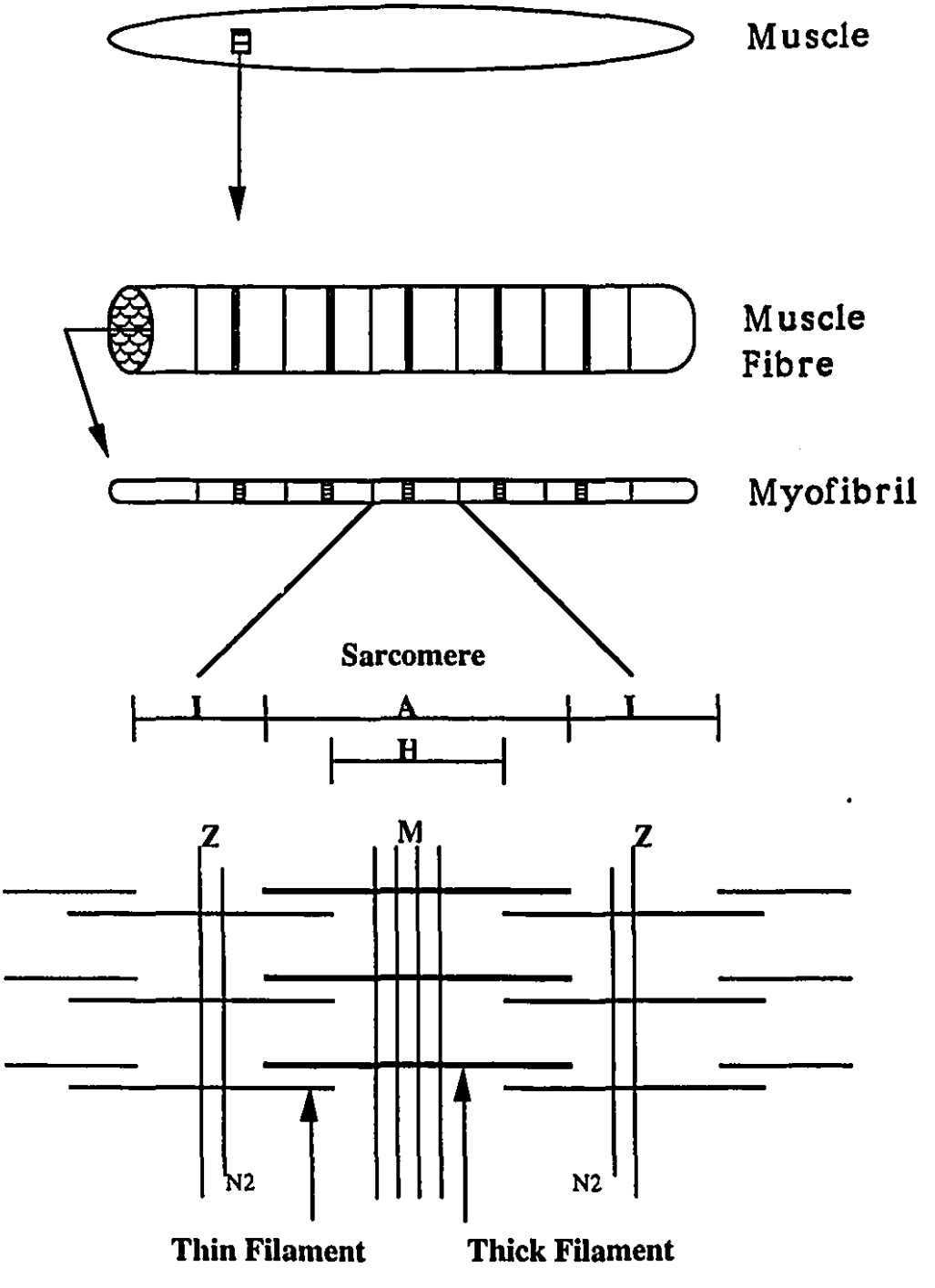
Using electron microscopy (EM), each muscle fibre, which is enclosed in a basement membrane, is found to be composed of many rod-shaped myofibrils, which have a diameter of 1-2 μm. In turn, each myofibril is composed of many myofilaments.

The myofilaments appear as two sizes: thick and thin (Fig. 1). The thick filaments are composed primarily of the protein myosin (Hanson and Huxley, 1953). The thin filaments are composed primarily of actin, but there are two regulatory proteins, tropomyosin (Tm) and troponin (Tn), associated with the actin (Hanson and Lowy, 1963; Pepe, 1966; Ohtsuki, Masaki, Nonomura and Ebashi, 1967; Caspar, Cohen and Longley, 1969).

The particular organization of the thick and thin filaments is responsible for the striated appearance of skeletal muscle. They are arranged in a regular and repeating pattern along the length of the fibre. A single unit of this pattern is known as a sarcomere, and is the basic contractile unit of the muscle fibre. A sarcomere is delineated at either end by a Z-line, the distance between the two Z-lines being the length of the sarcomere. The thick filaments are located in the central region of the sarcomere and do not reach the Z-line. The thin filaments are attached to the Z-lines, and extend towards, but do not reach the centre of the sarcomere. The presence of overlapping and non-overlapping portions of the thick and thin filaments make several bands which are recognized under light microscopy (Fig. 1). A cross section through the sarcomere where thick and thin

Figure1. Levels of myofibrillar organization. Muscle is composed of bundles of muscle fibres which are long cylindrical, multinucleated cells. The muscle fibres are composed of multiple myofibrils. The muscle fibres and myofibrils have a striated appearance which is due to the organization of the contractile proteins into the basic contractile unit of muscle, the sarcomere. A sarcomere is delineated by Z-lines. The A band is the region where thick and thin filaments overlap. The I band contains thin filaments and the H band contains thick filaments. The M line marks the centre of the sarcomere and the N_2 line, which consists of the protein nebulin, is located near the Z line.

Figure 1. Levels of Myofibrillar Organization



myofilaments overlap (A band) reveals a hexagonal arrangement of the thick and thin filaments. Each thick filament is surrounded by three thin filaments (Huxley, 1968). The I band of the sarcomere is the region containing thin filaments only, and it extends from the end of the A bands to the Z-line. The I bands of two adjacent sarcomeres are contiguous. The central part of the sarcomere, where only thick filaments are present, is called the H zone. The M line, which is found in the centre of the H zone, is produced by processes which link the thick filaments together to maintain their parallel arrangement (Huxley, 1953).

Under electron microscopy, projections can be seen along the sides of the thick filaments. Each projection is the "head" of a myosin molecule, and is the site on the myosin which binds to actin and which generates the force required to bring about the sliding of the filaments past each other during muscular contraction (Huxley and Hanson, 1954).

A population of cells, known as satellite cells, are located between the basement membrane and the sarcolemma of the muscle fibre. These spindle-shaped cells have a very high nuclear to cytoplasmic ratio and were first identified as being separate from myonuclei by Mauro (1961) using electron microscopy. Trauma to a muscle activates satellite cells to divide and differentiate into myoblasts, which eventually fuse with each other and mature into new muscle fibres (Hall-Craggs, 1974). This series of events occurs only during the process of muscle regeneration.

The electron microscope permits the highly organized sub-cellular structure to be seen. The highly specialized endoplasmic reticulum, which is known as the sarcoplasmic reticulum (SR), forms a sleeve-like cover surrounding each of the myofibrils. It is divided into two distinct regions based on anatomical features. The "junctional SR" is a foot-like projection which lies alongside a t-tubule (Peachey, 1965; Franzini-Armstrong, 1980). From it extends the "free SR", or lateral sacs, which contain the Ca^{++} transport ATPase that is responsible for pumping free Ca^{++} from the sarcoplasm into the SR, where it is stored (Deamer and Baskin, 1969). The stored Ca^{++} is subsequently released upon membrane excitation and functions to initiate muscle contraction (Franzini-Armstrong, 1980).

The t-tubule is an invagination of the sarcolemma (plasma membrane), which runs transversely around the myofibril at the level of the A-I junction. The lumens of the t-tubules communicate with each other and with the extracellular space of the muscle. A t-tubule and the lateral sacs of SR found on either side of it are called a triad (Peachey, 1965; Franzini-Armstrong, 1980).

When one looks at whole fresh muscles with the naked eye, they can be divided into two broad categories based on color: red and white. The red coloring is due to a high content of myoglobin, which is an oxygen-binding protein, and good vascularization. Together, these lead to an increased rate of oxygen diffusion into the muscle cell. White muscles contain less myoglobin and are more poorly vascularized than red ones. The

supply of oxygen to a muscle is important because of its use in aerobic energy generation in the form of ATP. Muscles using aerobic mechanisms to generate ATP are more fatigue resistant than those using anaerobic mechanisms. Therefore it is necessary that tonically active antigravity muscles such as soleus (SOL), use aerobic energy production.

Red and white muscles also differ in their internal structure, which influences their contractile behavior. The fully mature white fast-twitch fibres have a well developed SR network, which in turn forms many triads with t-tubules. The abundance of these triads permits the rapid release and resequestering of Ca^{++} for short contraction and relaxation times. Conversely, the red, or slow-twitch muscle fibres contain a less well developed SR and fewer triads (Vrbova, Gordon and Jones, 1978).

B. The Motor Unit and Neuromuscular Junction

The nerve trunk entering a muscle carries both motor and sensory fibres. The sensory fibres synapse with the intrafusal fibres of the muscle spindle or with the Golgi tendon organs. The motor component of the nerve contains myelinated large diameter axons, which branch and lose their myelin sheaths upon reaching the muscle. Each branch further divides into many fine terminal arborizations, each of which synapses with a single muscle fibre. One motoneuron and all the muscle fibres it

innervates make up a motor unit (Brodal, 1969; Vander, Sherman and Luciano, 1975).

The neuromuscular junction can be divided into three basic areas: the presynaptic terminal, the postsynaptic terminal and the synaptic cleft which separates the two. The presynaptic terminal consists of the nerve ending, covered by a Schwann cell. It contains vesicles of acetylcholine (ACh), which is the neurotransmitter at the neuromuscular junction (Hall, 1972). The postsynaptic terminal, or motor end-plate, is characterized by extensive folding of the sarcolemma. Located on the sarcolemma in this region are receptors specific for ACh (AChR) (Fertuck and Salpeter, 1974). The presence of the junctional folds greatly increases the surface area of the postsynaptic membrane and therefore, allows for a large number of AChRs to be accessible to the ACh.

Within the cleft is found the synaptic basal lamina, which has special lateral projections extending into the pre- and post-synaptic membranes. The synaptic basal lamina is similar to that in extrasynaptic regions in that it contains laminin, collagen IV, and fibronectin; however, it also contains binding sites for acetylcholinesterase (AChE) (McMahan, Sanes and Marshall, 1978). AChE is an enzyme that catalyzes the hydrolysis of ACh and as such is responsible for stopping the transmission of the nerve impulse. AChE is also associated with the postsynaptic membrane (Salpeter, 1967; Hall and Kelly, 1971).

C. Contractile Proteins

1. Actin

Actin, the primary component of the thin myofilaments, is a globular-shaped molecule, 1000 nm long and 5.5 nm in diameter. Two actin chains combine to form a double helix known as filamentous actin (F-actin) (Caspar *et al.*, 1969). Along the surface of the actin molecule are "active sites" which can interact with myosin. Actin is closely associated with two contractile regulatory proteins: tropomyosin (Tm) and troponin (Tn).

Several different isoforms of actin exist, and these are expressed in a tissue-specific fashion (Garrels and Gibson, 1976). The isoforms β and γ actin are found in many different types of non-muscle cells. The α-skeletal and α-cardiac isoforms are present in skeletal and cardiac muscle, respectively (Minty, Alonso, Caravatti and Buckingham, 1982).

2. Myosin

The myosin molecule is a hexamer composed of two heavy chains (MHC) and four light chains (MLC) (Lowey and Risby, 1971) (Fig. 2). The C-terminal portions of the two heavy chains are individual α-helixes, which together form a double α-helical coiled-coil tail, 155 nm long. The N-terminal portion of each heavy chain folds to form a globular head. The MLCs are associated with the head portion of the MHC molecule, one pair being associated with each of the two heads. The MHCs and

MLCs exist in different isoforms, which are expressed in a tissue- and stage-specific manner (Lowey and Risby, 1971; Whalen, Sell, Butler-Browne, Schwartz, Bouveret and Pinset-Harstrom, 1981; Pette and Staron, 1990) (Table 1).

The myosin head, which makes up the cross-bridge, is specialized for interacting with actin. It contains an actin-binding region and a myosin ATPase site, both of which are essential for muscle contraction and force production (Engelhardt and Ljubinowa, 1939; Ebashi, 1963). The sliding filament theory for muscle contraction says that the changes in the pattern of cross-striations during stretch and contraction are due to the thin and thick filaments sliding past one another (Huxley and Hanson, 1954). This accounts for the decreased widths of the I and H bands but constant width of the A bands seen during muscle contraction. The myosin filaments are arranged such that the heads of the myosin molecule, or the cross-bridges, are located regularly along the thick filament except at the centre of the A band. The heads of the myosin molecule project laterally and when activated, bind to actin and swivel so that the actin slides along the myosin filament (Huxley, 1957; Huxley, 1963; Reedy and Holmes, 1965; Huxley, 1969). After each stroke, actin and myosin dissociate and the myosin head is free to undergo another cycle of binding and movement. This model has since been refined with different states of actin-myosin binding (weakly and strongly binding states) (see Simmons, 1992).

Energy for the movement of cross-bridges is provided by myosin ATPase, which acts on ATP derived from three possible

sources: (i) creatine phosphate, (ii) substrate phosphorylation in glycolysis and (iii) oxidative phosphorylation (Vander *et al.*, 1975). Different muscles vary in their ability to utilize aerobic oxidative phosphorylation or glycolysis in producing ATP and these different metabolic properties are reflected in the contractile properties.

It is possible to identify three broad groups of muscle fibres based on their myosin ATPase and ATP production characteristics. The first group utilize anaerobic glycolysis and although they have a low oxidative capacity and thus fatigue quickly, they are able to produce energy quickly because of a high myosin ATPase activity and therefore, can contract rapidly. These are termed "fast-glycolytic" muscle fibres (FG). The second group of muscle fibres, which are called "slow oxidative" (SO), use predominately oxidative phosphorylation for energy production, which results in a large but slow production of ATP. This, coupled with a low myosin ATPase activity, results in slowly contracting muscle fibres which are fatigue resistant. The third group, the "fast-oxidative glycolytic" (FOG) fibres, have contractile properties intermediate to those of SO and FG muscle fibres in that they have high oxidative phosphorylation capabilities, yet they are fast contracting due to a highly active myosin ATPase (Banker and Engel, 1986).

3. Tropomyosin

Tropomyosin is a two stranded α-helical coiled-coil molecule that is 41 nm long and 2 nm in diameter (Caspar *et al.*,

1969). It is positioned along the groove of the F-actin α-helix (Hanson and Lowey, 1963). There are four tropomyosin genes, α, β, δ and γ, which are expressed in a tissue-specific manner. The α and β tropomyosin genes are expressed in skeletal muscle and they each code for a number of different isoforms (Wieczorek, Smith and Nadal-Ginard, 1988).

4. Troponin

Troponin (Tn) is a complex consisting of three subunits, which are designated as Tn-I (inhibitory), Tn-C (Ca^{++}-binding), and Tn-T (tropomyosin-binding unit) (Matsuda, Obinata and Shimada, 1981). In association with tropomyosin, troponin responds to the regulatory effects of Ca^{++} during muscle contraction.

5. Accessory Proteins

Several additional proteins have been identified as being associated with the sarcomere. These include the C-protein, M-line protein, muscle creatine kinase (MCK), and two components of the gap filament system, titin and nebulin.

The C-protein is a polypeptide located within the A band of the sarcomere in association with the myosin filaments (Offer, Moos and Starr, 1973). It is able to bind both myosin (Pepe and Drucker, 1975) and F-actin (Moos, Mason, Besterman, Feng and Dubin, 1978). Due to its close association with myosin it has been suggested that it functions to stabilize the organization of the myosin molecule. However, it also can modify actomyosin

ATPase, indicating a possible role in cross bridge activity (Moos *et al.*, 1978). Recently, C-protein has also been shown to interact with titin, a myofibrillar component extending from the Z line to the A band (Furst, Vinkemeier and Weber, 1992). It is postulated that C-protein may serve to link titin to myosin in the A band region.

The proteins MCK and M-protein are two components of the M-band of the sarcomere (Masaki and Takaito, 1974; Wallimann, Pelloni, Turner and Eppenberger, 1978). MCK is the major protein component of the filamentous bridges joining neighboring thick filaments (Cain, Infante and Davies, 1962). The M-proteins make up the M filaments, which link adjacent MCK molecules (Knappeis and Carlsen, 1968; Moos *et al.*, 1978). These two proteins likely act to stabilize the transverse and longitudinal order of the thick filament lattice (Luther and Squire, 1978).

The presence of an elastic component within the sarcomeres was suggested when it was observed that a sarcomere could be stretched beyond the point of overlap between thick and thin filaments and it would still return to its original length. Analysis of the "gap" region between the A and I bands of the over-stretched sarcomere revealed the presence of gap filament proteins, including titin and nebulin. Titin stretches from the Z line to the M line (Furst, Osborn, Nave and Weber, 1988). Due to titin's elastic nature and its ability to form complexes with myosin it has been proposed to be a key component of an elastic lattice that serves as an organizing

scaffold or lattice for the thick and thin filaments (Wang, McClure and Tu, 1979; Wang, Ramirez-Mitchell and Palter, 1984). Nebulin, which is located along a line in the I band (the N_2 line) (Wang and Williamson, 1980), is also elastic in nature and may be associated with titin in joining successive Z lines (Trinick, Knight and Whiting, 1984).

D. Excitation Contraction Coupling

The transmission of an electrical impulse from a nerve to the muscle fibres it innervates, initiates a sequence of events leading to muscle contraction. Electrical impulses arriving at the axon terminus trigger the fusion of ACh-containing vesicles with the presynaptic membrane, resulting in the release of ACh into the synaptic cleft. The ACh then traverses the cleft and binds to ACh receptors (AChR) on the postsynaptic membrane. This event alters the permeability of the postsynaptic membrane to K^+ and Na^+ so that there is a net influx of positive charge, which depolarizes the sarcolemma and produces an end-plate potential (EPP) (Vander *et al.*, 1975). The amplitude of the end-plate potential is proportional to the amount of ACh binding to the AChRs. If the membrane is depolarized to a certain threshold level, it generates an all-or-none action potential, which is propagated along the sarcolemma and into the t-tubular membrane system of the myofibre.

The depolarization of the t-tubule membrane causes, by an unknown mechanism, the adjacent junctional SR to release Ca^{++}

into the cytoplasm (Catterall, 1991). The released Ca^{++} then associates with the Tn-C subfragment, or Ca^{++}-binding component, of the Tn complex, resulting in a conformational alteration of the F-actin, such that the myosin-binding site on actin becomes free to interact with the myosin cross-bridges (Vander *et al.*, 1975). After contraction is complete, relaxation occurs via the re-polarization of the sarcolemma and t-tubules. As a result, the Ca^{++}-releasing channels of the SR close. Simultaneously, Ca^{++} is pumped back into the SR via an ATP-dependent Ca^{++}-ATPase pump, lowering the intracellular Ca^{++} concentration (Ebashi, 1960). This reduces Ca^{++} binding by Tn-C, which causes the myofilaments to resume an inhibited (relaxed) state (Franzini-Armstrong, 1980).

The internal structure of the muscle fibre influences its contractile behaviour. Mature fast-twitch fibres (FG or FOG) have a well developed SR network, which in turn forms many triads with the t-tubules at the A-I junctions of sarcomeres. The abundance of these triads permits the rapid sequestering of Ca^{++} for short contraction times. Conversely, slow-twitch (SO) muscle fibres contain fewer triads (Vrbova *et al.*, 1978).

E. Muscle Development

Skeletal muscle arises from the mesoderm. As the embryo develops, the mesoderm segregates into blocks called somites, which can be divided into two parts, the ventral sclerotome and the dorsal dermomyotome. The dermomyotome further differ-

entiates into a dermatome and a myotome, and it is from the latter that precursor muscle cells migrate into the limb buds or body wall. *En route*, the muscle precursor cells become further committed to the myogenic lineage.

The most primitive muscle cells are the myoblasts. These mononucleated cells undergo several cycles of division before the ability to mitose is lost. Myoblasts then undergo differentiation, which is characterized by the synthesis of many proteins specific to striated muscle and the fusion of myoblasts to form multinucleated myotubes (Hilfer, Searls and Fonte, 1973; Vrbova *et al.*, 1978). The stimulus which triggers the cessation of mitosis and leads to the differentiation of the myoblasts is not fully understood. However, a group of proteins called the muscle regulatory factors have been proposed to play an important role in the determination and differentiation of myogenic cells (Davis, Weintraub and Lassar, 1987; Wright, Sassoon and Lin, 1989; Rhodes and Konieczny, 1989).

Myotubes are characterized by a chain of centrally placed nuclei, which move to the periphery of the myotube around the time of innervation. At this point, a muscle cell is referred to as a muscle fibre. The maturation of the myotube into a muscle fibre is characterized by (i) movement of the nuclei to the periphery, (ii) development of the SR and t-tubule membrane systems and (iii) expression of different isoforms of the MHCs, MLCs, Tm, and Tn (Vrbova *et al.*, 1978).

The first muscle-specific protein to appear in the myoblast is the muscle regulatory factor myf-5 followed by the

appearance of the enzyme MCK. This is followed by the synthesis and assembly into myofibrils of molecules of actin, myosin and other related proteins, as well as the synthesis and incorporation of AChR's into the sarcolemma. The myoblasts then elongate and fuse to form myotubes (Hilfer *et al.*, 1973). Just prior to fusion of the myoblasts, a number of metabolic changes occur, which include an increase in the number of mitochondria, the appearance of enzymes for utilizing glucose and glycogen to produce energy and the development of the rER. All of these changes are made in preparation for contractile activity. Synthesis of the machinery and enzymes necessary for energy production continues and increases further when the myotubes begin contracting.

There are at least two distinct populations of myoblasts (Harris, Duxson, Fitzsimons and Reiger, 1989). Primary myoblasts are the first to arise and these fuse with each other to produce primary myotubes, which appear as described for typical myotubes. Secondary myoblasts arise later and fuse with each other to form binucleate myotubes, which then fuse with the preexisting smaller diameter primary myotubes. Since primary and secondary myoblasts do not fuse with each other, because of temporal differences, they give rise to two distinct populations of myotubes. It is not known whether they would be capable of fusing with each other if they were present at the same time.

Myoblasts and myotubes have several cell adhesion molecules (CAM) on their surfaces, some of which are muscle

specific. Neural cell adhesion molecules (N-CAMs), which mediate adhesion between nerve and muscle, are located on the surface of myotubes. When the developing myofibre becomes innervated, the N-CAM's become localized to the neuromuscular junction (Grumet, Rutishauser and Edelman, 1982; Covault and Sanes, 1985; Covault and Sanes, 1986). Once an axon has contacted a muscle fibre, it serves as a guide for other growing axons to find the muscle. Other CAMs contribute to muscle tissue remodelling (Kelly and Rubenstein, 1978). The protein $\alpha_5\beta_1$ integrin, which is expressed along the periphery of the myoblast before becoming localized into plaques, acts as an adhesive structure to anchor the developing myotube to the extracellular matrix. As such, it acts to promote the organization of the myotube and the extracellular matrix (Lakonishok, Muschler and Horwitz, 1992).

The proteins integrin VLA-4 (very late activation antigen 4) and VCAM-1 (vascular cell adhesion molecule 1) may also play a role in muscle tissue modelling (Rosen, Sanes, LaChance, Cunningham, Roman and Dean, 1992). VLA-4 is expressed on primary and secondary myotubes and VCAM-1 is expressed on secondary myoblasts and on secondary myotubes in those regions adjacent to primary myotubes. Based on their ability to interact with each other and their patterns of expression, these two proteins may interact to influence: (i) the alignment of secondary myoblasts along primary myotubes, and/or (ii) the fusion of secondary myoblasts.

F. Muscle Regeneration

Muscle regeneration refers to the process by which a muscle replaces those muscle fibres damaged by an insult sufficient to induce degeneration and therefore, the loss of the fibre. Degeneration of muscle fibres can be induced experimentally by mechanical or chemical means. Following injury to the muscle, satellite cells are activated to proliferate and differentiate into myoblasts. The course of muscle regeneration resembles muscle development with respect to the ordered sequence of myoblasts, myotube and mature muscle fibre (Carlson, 1973). The fully regenerated muscle fibre structurally resembles a typical adult muscle fibre, except that it maintains centrally placed nucleii. The same contractile proteins are expressed, although there may be differences in the particular isoforms accumulated compared to the original damaged muscle fibre (Whalen, Harris, Butler-Browne and Sesodia, 1990). Some of these differences may be due to the fact that regenerating muscle fibres are derived from satellite cells whereas developing fibres originate from stem cells. In addition, the different environments in which the two processes take place likely play a role (Mauro, 1961).

II. GENE EXPRESSION

A. Muscle Gene Expression

The expression of structurally distinct protein isoforms is characteristic of differentiation, development and physiological adaptation. Myogenesis provides an excellent example of such gene expression, in that many of the genes which encode the proteins required for muscle activity are expressed in a stage- and tissue-specific manner. Among these are the components of the contractile apparatus (actin, myosin, tropomyosin, troponin) and enzymes for muscle metabolism (e.g. MCK). Coordinated expression of these genes is required for proper assembly of the sarcomere. Just prior to their fusion into myotubes, myoblasts begin to express the genes for MHC (embryonic and slow isoforms), MLC (fast and slow isoforms), α actin (cardiac isoform followed by skeletal isoform), Tm and Tn-I, -C and -T (Matsuda, Bandman and Strohman, 1983; Sassoon, Garner and Buckingham, 1988; Lyons, Ontell, Cox, Sassoon and Buckingham, 1990). With continued development, the expression of some of these genes is substituted by that of another (e.g. embryonic MHC by slow or neonatal MHC), or a single gene may be alternatively spliced to produce a different isoform.

B. Muscle Gene Regulation

Gene expression may be regulated during transcription, post-transcription, or during translation.

1. Regulation during Transcription

Regulation of gene expression during transcription involves cis-acting elements and trans-acting factors. Cis-acting elements are DNA sequences which are part of the gene itself and are usually located 5' of the transcription start site in a region called the enhancer. Trans-acting factors are "external" factors, which are able to influence the expression of a gene by interacting with it directly or through another factor. Cis-acting elements act as positive or negative regulatory elements, which respectively enhance or suppress gene transcription when activated by the binding of a trans-acting factor. A given gene may have several positive and negative regulatory regions associated with it, and the level of transcription will depend on the balance between the activation of these opposing regions by the various trans-acting factors. Tissue-specific expression of genes is likely due to the availability of different trans-acting factors, which vary from one tissue to another, or within a given tissue over time (e.g. the muscle regulatory factors in developing muscle).

The muscle-specific genes for which cis-acting elements have been identified include: MCK (Sternberg, Spizza, Perry, Vizard, Weil and Olson, 1988), α and β cardiac MHC, and IIB MHC, (Rottman, Thompson, Nadal-Ginard and Mahdavi, 1990; Takeda,

North, Lakich, Russell and Whalen, 1992), MLC 1/3 (Periasamy, Strehler, Garfinkel, Gubits, Ruiz-Opazo and Nadal-Ginard, 1984), Tn-C, Tn-I and Tn-T (Mar and Ordahl, 1988; Yutzey, Kline and Konieczny, 1989; Parmacek, Vora, Shen, Barr, Jung and Leiden, 1992), and α-actin (Muscat and Kedes, 1987).

For example, the slow/cardiac form of the Tn-C (cTnC) gene contains an enhancer site located within the first intron of the gene, which is activated for gene expression in skeletal muscle myotubes (Parmacek *et al.*, 1992). On the other hand, cTn-C expression in cardiac myocytes is accomplished by activation of a different promoter and enhancer located in the 5' flanking region of the gene. The 5' cardiac-specific enhancer contains binding sites for several nuclear protein complexes, but if the binding sites are mutated to prevent binding of such proteins, cTn-C enhancer activity in cardiac myocytes is abolished. This indicates the importance of such binding sites in tissue-specific gene expression and it provides evidence for the role of trans-acting factors in modulating transcription (Parmacek *et al.*, 1992).

Other trans-acting factors include the muscle regulatory factors myf-5, MyoD, myogenin and MRF4 (Davis *et al.*, 1987; Wright *et al.*, 1989; Rhodes and Konieczny, 1989), and the myocyte-specific enhancer-binding factor (MEF-2) (Gossett, Kelvin, Sternberg and Olson, 1989). These proteins interact with specific DNA sequences to act as muscle-specific transcriptional regulators. The various muscle regulatory factors are differentially expressed during development, and they may

interact with different sets of genes and thus contribute to myogenic cell diversification (Yutzey, Rhodes and Konieczny, 1990; Block and Miller, 1992). For example, it has been shown that Tn-I and the MCK promoter are activated by MyoD and myogenin but not MRF4 (Yutzey *et al.*, 1990).

MEF-2 was initially found to bind to a specific site upstream of the MCK enhancer in differentiated myocytes. Subsequent analysis revealed that it also interacts with several muscle specific genes, including the MLC 1/3 enhancer (Gossett *et al.*, 1989). Therefore, MEF-2 binds in a stage- and site-specific manner to alter transcriptional activity.

Another example of a muscle-specific DNA regulatory sequence is the thyroid response element (TRE) found in the 5' region of the α cardiac and β MHC genes (Rottman *et al.*, 1990). The TRE responds differently in each of these genes: the α cardiac MHC gene is up-regulated, whereas the β MHC gene is down-regulated upon binding of the thyroid hormone/receptor complex to the TRE.

2. Post-Transcriptional Regulation

Regulation of gene expression can also occur after transcription. This may involve mRNA stability or processing of the mRNA, such as splicing and shortening or removal of the poly(A)+ (see Nadal-Ginard, 1990; Medford, Wydro, Nguyen and Nadal-Ginard, 1980). For example, mRNA for the contractile proteins MLC, Tm and Tn-T have been shown to undergo splicing, which generates multiple isoforms of each of these

proteins (Periasamy *et al.*, 1984; Breitbart and Nadal-Ginard, 1987; Wieczorek, Smith and Nadal-Ginard, 1988). The pattern of splicing varies depending on the stage of development and tissue type. The use of a particular splice site may be dictated by cis-acting elements or trans-acting factors.

3. Regulation during Translation

Regulation during translation may occur via several different mechanisms, which include: i) specific factor(s) that permit the initiation of protein synthesis on certain mRNAs (Heywood, Kennedy and Bester, 1974), ii) translational control RNAs (tcRNA), which may inhibit translation (Bester, Kennedy and Heywood, 1975), iii) inhibitory RNAs (iRNA), which prevent the mRNA from binding to ribosomes (Sarkar, Eller, Raychowdhury, Stedman and Wu, 1989) and iv) sequestering the mRNA in an inactive form within messenger ribonucleo-protein (mRNP) particles (Dym, Kennedy and Heywood, 1979; Sarkar *et al.*, 1989).

III. THYROID HORMONE

A. Introduction

Thyroid hormone is produced in the thyroid gland and secreted into the blood stream, via which it reaches many

tissues. Its production occurs in the presence of thyroid stimulating hormone (TSH), which is secreted from the thyrotroph cells of the anterior pituitary gland. Thyroid hormone in the serum in turn has a negative feedback effect on the cells producing thyroid stimulating hormone.

The basic units of the thyroid hormone molecule are produced in the colloid of the thyroid follicle. This occurs via the union of an iodide radical with the tyrosine amino acid of the thyroglobulin molecule. There are two basic forms of iodinated thyroglobulins, the mono- and di-iododtyrosines, which then combine to form tri-iodothyronine (T3) and thyroxin (T4). These are taken up into the follicular cells of the thyroid gland, from which they are secreted into the blood stream (Gorbman, Dickhoff, Vigna, Clark and Ralph, 1983).

Once in the blood stream, thyroid hormone may become associated with one of several proteins. In humans, thyroxine-binding globulin binds to both T3 and T4; but with a much stronger affinity for the latter. Albumin and thyroxine-binding pre-albumin are two other serum proteins which bind thyroid hormone. In humans, albumin is found in larger quantities than thyroxine-binding pre-albumin and although it binds both T3 and T4, it does so with a low affinity. In contrast, thyroxine-binding pre-albumin binds only T4. In rats there is no thyroxine-binding pre-albumin, and albumin binds most of the thyroid hormone (Gorbman *et al.*, 1983).

The relative binding affinities for T3 and T4 of the various plasma proteins and of the thyroid hormone receptors in the

nuclei of the target tissue, accounts for the differences in their biological activities. T4 has the same binding affinity for its nuclear receptor as it does for the plasma proteins, thus it moves slowly into the target tissue. On the other hand, T3 binds more strongly to the target tissue nuclear receptor than to plasma proteins, thereby favoring its movement out of the blood and into the tissue. As a result, T3 plays a greater role than T4 in altering biological activity (Krenning and Docteur, 1986).

In general, thyroid hormone acts to ensure proper growth and development. It principally affects the central nervous system, integument, skeleton, muscle and gonads. Absence of thyroid hormone during development results in abnormal growth and deformaties. For example, an adult rat, which had been thyroidectomized at birth, has a broad, round skull and thickened ears. Also, ossification of the bones is reduced and the animal is generally smaller in size. Early supplementation of a thyroidectomized animal with thyroid hormone results in normal growth.

Thyroid hormone acts on metabolic functions and in particular increases nitrogen, lipid and carbohydrate metabolism. It also controls the basal metabolic rate by increasing the rate of oxidative phosphorylation, resulting in the production of heat (Gorbman *et al.*, 1983; van Hardeveld, 1986). Many of the effects of thyroid hormone involve increased protein synthesis, especially with respect to its effects on growth and differentiation. T3, upon entering a cell, may bind to a cytoplasmic protein (of unknown nature) before passing to the

nucleus where it binds to a specific receptor. In contrast, T4 is either first converted to T3 within the cytoplasm or it binds to a protein in the cytoplasm before being transported to the nucleus, where it binds to a thyroid hormone receptor (O'Malley and Means, 1974). The thyroid hormone/receptor complex then binds to the TRE sequence on the DNA, through which it alters transcription.

T3 also binds to non-nuclear sites, such as the inner and outer mitochondrial membranes. This results in a configurational change of the mitochondrial membranes and a subsequent increase in oxidative phosphorylation (Sterling, Milch, Brenner and Lazarus, 1977; Sterling, Lazarus, Milch, Sakurada and Brenner, 1978).

B. Influence of Thyroid Hormone on Muscle

Thyroid hormone has profound effects on muscle metabolism. In muscle the overall effect of thyroid hormone is to increase the rate of metabolism of compounds like glycogen and phosphocreatine in order to provide energy (van Hardeveld, 1986). Mitochondria, which are the site of oxidative phosphorylation, are found to increase in number after administration of thyroid hormone to animals (Rubenstein, Lyons, Gambke and Kelly, 1983).

The profound effect of the hypo- and hyper-thyroid states on muscle metabolism in turn affects muscle physiology. In the hyperthyroid condition, a slow-twitch muscle begins to develop

properties of a fast-twitch muscle, such as faster speeds of contraction and greater fatiguability. This is due to increased activities of myosin ATPase and oxidative enzymes, increased calcium uptake by the SR and changes in the contractile proteins from the slow to fast isoforms (Ianuzzo, Patel, Chen, O'Brien and Williams, 1977; Nwoye, Mommaerts, Simpson, Seraydarian and Marusich, 1982; Leijendekker, van Hardeveld and Kassenaar, 1985).

Insufficient or excess amounts of thyroid hormone will alter the accumulation of the various myosin proteins. The hyperthyroid state in developing fast-twitch muscle causes the precocious appearance of adult fast myosin but the effects are less pronounced than those on slow-twitch muscle (Hall-Craggs, Wines and Max, 1983; Russell, Cambon, Nadal-Ginard and Whalen, 1988). The hypothyroid condition in a fast-twitch muscle changes myosin ATPase activity, Ca^{++} uptake by the SR, and myosin content to be more like those found in a slow-twitch muscle. A normally fast-twitch muscle in a hypothyroid animal will exhibit precocious accumulation of slow myosin and have low activities of myosin ATPase and SDH, resulting in slower contractile characteristics (Nwoye *et al.*, 1982; Butler-Browne, Herlicoviez and Whalen, 1984).

IV. NEURAL INFLUENCES ON MUSCLE

A. Role of Innervation on Muscle

The importance of innervation for the normal development and maintenance of skeletal muscle is evidenced by the severity of the changes which ensue when the nerve supply is removed. Denervation of skeletal muscle results in many structural, metabolic and physiological changes in the component myofibres. If the fibres do not become reinnervated, they will eventually degenerate to the point where they are refractory to reinnervation.

The most obvious structural change in a denervated muscle is atrophy or shrinkage of the component muscle fibres. This results in decreased muscle size and weight (Engel and Stonnington, 1974). Atrophy is caused by a loss of myofilaments and other muscle proteins, and reduced content of mitochondria and SR (Leung, Jeffrey and Rostas, 1984). The decrease in contractile protein content results in a reduced tetanus tension of the muscle. At the same time as myofilaments are being lost, there is a transformation in the expression of some of the isoforms of the contractile proteins, including MHC, MLC, Tm and Tn (Matsuda, Spector and Strohman, 1984; Rubenstein and Kelly, 1978; Butler-Browne, Bugaisky, Cuenound, Schwartz and Whalen, 1982).

Despite the atrophy of the muscle fibres, there is a transient increase in the twitch tension, probably due to a decreased speed of reuptake of Ca^{++} by the SR, which allows more time for tension development. The temporal characteristics of the isometric twitch (time-to-peak and half-relaxation time) slow upon denervation and its post-tetanic potentiation is lost. The longer half-relaxation time is possibly due to the loss of sarcoplasmic parvalbumin, which plays a role in transferring Ca^{++} to the SR for storage (Thesleff, 1974; Gerday and Gillis, 1976; Webster and Bressler, 1985).

Denervation results in increased sensitivity to iontophoretically applied ACh, which is due to the appearance of many ACh receptors over the entire sarcolemma. This is in contrast to innervated muscle where they are localized only at the motor end-plate (Thesleff, 1974; Pestronk, Drachman and Griffin, 1976). Denervation also results in a decreased AChE activity.

B. Mechanism of Neural Influences on Muscle

The neural influence on muscle may be mediated by two possible mechanisms: (i) the electrical activity, which brings about muscle contractile activity and (ii) the presence of myotrophic factors. Experiments to determine the relative role of these two influences are usually of two types. Either attempts are made (i) to delete the action of one neural influence from an innervated muscle in an attempt to create denervation-like

changes or (ii) attempts are made to replace one of the neural influences in a denervated muscle to see if changes are prevented.

1. Evidence for the Role of Electrical Activity

The role of electrical activity in the maintenance of muscle fibres has been examined using (i) various models of disuse of innervated muscle to see if denervation-like changes result or (ii) electrical stimulation of denervated muscle to see changes resulting from denervation are prevented.

Disuse may be created in an innervated muscle by immobilization, pharmacological blockade of neural conduction (e.g. tetrodotoxin), pharmacological blockade of neural transmission presynaptically (e.g. α-bungarotoxin) or postsynaptically (e.g. curare, β-bungarotoxin), and spinal isolation. All these result in the removal of contractile activity while leaving the nerve-muscle connection structurally intact and presumably not interfering with the supply of putative myotrophic substances. In almost all cases, the changes in disused muscle are not as severe as those found after denervation, indicating that electrical activity is not solely responsible for the maintenance of a healthy muscle fibre (Fischbach and Robbins, 1969; Pestronk *et al.*, 1976; Davis, 1988).

This is further supported by the fact that artificial electrical stimulation of denervated muscle fails to completely prevent any of the post-denervation changes (Salmons and Sréter, 1976). However, the pattern of stimulation may

influence the isoforms of myosin expressed in a denervated muscle, as it does in an innervated muscle (Ausoni, Gorza, Schiaffino, Gundersen and Lomo, 199O). For example, stimulation of a denervated slow-twitch soleus in a pattern resembling its normal motor unit activity, results in accumulation of slow MHC but high frequency stimulation results in fast MHC accumulation. Low frequency stimulated fast-twitch EDL, which had been denervated, has reduced fast IIB MHC protein. Denervated EDL stimulated with a high frequency mimicking normal motor unit activity, accumulates fast MHCs but not in the identical pattern to that in the normal control EDL. This suggests other factors, in addition to electrical activity, play a role in determining the myosin content, and therefore the contractile characteristics of fast-twitch muscle.

2. Evidence for the Role of Myotrophic Influences

It is now generally accepted that loss of electrical activity is not solely responsible for all of the changes seen in denervated muscle and that changes result at least partially from the loss of trophic factors that are normally provided to a muscle by its innervating neuron. These putative myotrophic factors are thought to be transported by axonal transport and released at the nerve terminal, where they are either taken up by, or in some other way interact with, the muscle fibres. Evidence for the existence of myotrophic influences by such neurogenic substances comes from studies involving, (i) blockade of axonal

transport to prevent their delivery to innervated muscle and (ii) addition of neural extracts to denervated muscle.

Blockade of axonal transport by the application of colchicine or vinblastine to the nerve, partially mimics the post-denervation changes despite the fact that contractile activity is not affected (Younkin, Brett, Davey and Younkin, 1978).

The identification of a specific myotrophic substance has been difficult to obtain. However, Davis and co-workers (see Davis, 1988) showed that administration of a crude or partially purified extract of peripheral nerve could ameliorate many of the changes resulting from denervation, including (i) atrophy, (ii) ultrastructural alterations, such as the losses in volume of mitochondria, SR and t-tubule, (iii) loss of parvalbumin, (iv) the prolongation of time-to-peak and half-relaxation time of the twitch, and (v) reduced tetanus tension. The recovery in tetanus tension was likely due to prevention of atrophy, and that of the half-relaxation time may be related to the prevention of the loss of parvalbumin. Nerve extract had no effect on atrophy of immobilized muscles, indicating that the myotrophic factor(s) acted on that component of denervation atrophy which was not due to disuse. These results lend support for the presence of a myotrophic factor in the nerve which acts on several parameters of the mature muscle fibres.

A neurally derived protein ARIA has been identified which acts to stimulate the synthesis of AChR on uninervated myotubes. It acts by binding to a receptor tyrosine kinase,

which leads to an increase in AChR mRNA (Falls, Rosen, Corfas, Lane and Fischbach, 1993).

Recently, it has been shown that ciliary neurotrophic factor partially prevents many changes in denervated muscle in a manner similar to that with nerve extracts (H. Davis, personal communication). Ciliary neurotrophic factor is synthesized by Schwann cells and is found in axons of peripheral nerve.

V. SKELETAL MUSCLE REGENERATION

A. Introduction

When a muscle fibre is damaged, it degenerates and is replaced by a new muscle fibre, which is produced by a process known as regeneration. The sequence of events during muscle regeneration is not influenced by the cause of the degeneration, although their temporal appearance may be influenced by the severity of the trauma.

Crucial to the process of regeneration is the presence of satellite cells, as well as the revascularization and reinnervation of the regenerating muscle fibres (Carlson, 1986). The success of regeneration is influenced by the severity of injury, especially if it disrupts the vascular supply or the integrity of the endomysial framework of the damaged fibres. In the case of very severe injury (e.g. complete mincing of the muscle), connective tissue

scarring may physically impede subsequent regeneration of the muscle fibres. If the endomysial tubes are intact, they provide a framework to guide the growth of the new fibres.

The initial 96 hours of the degenerative/regenerative process may be divided into three phases, which consistently occur regardless of the mechanism of injury (Mauro, 1970).

Stage 1: 24-72 hours after injury.

This stage is characterized by degeneration and necrosis of the damaged muscle fibres, following which phagocytic cells digest and remove the necrotic material (Hudson and Field, 1973). At the same time, satellite cells of the damaged muscle fibres are activated to divide, and some of the daughter cells differentiate into mononucleated myoblasts (Muir, 1970; Roth and Oron, 1985; Schultz, 1989). The origin of regenerating muscle fibres was determined from experiments showing the incorporation of radio-labelled satellite cell nuclei into regenerating muscle fibres (Hall-Craggs, 1974; Schultz, Jaryszak and Valliere, 1985). The proliferation and differentiation of satellite cells is under the influence of a number of factors, including several growth factors (e.g. fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor) and hormones (e.g. adrenocorticotropin stimulates proliferation; prostaglandin stimulates differentiation and fusion) (Grounds, 1991).

There is evidence that two different populations of satellite cells exist, one which expresses only fast MHC and one which expresses both fast and slow MHC. Subsequent maturation of the regenerating muscle fibres leads to an adult population of fast,

fast/slow or slow containing muscle fibres (Stockdale and Miller, 1987).

Stage 2: 72-96 hours after injury.

The newly formed myoblasts fuse with each other to form multinucleated myotubes, which are characterized by a large nucleus, loosely scattered chromatin and a prominant nucleolus (Carlson, 1973). Organelles required for protein synthesis (e.g. rER and Golgi) are much more prominant than in mature muscle and a new basement membrane is produced to surround the myotube (it may persist from the original fibre depending on the technique used to induce degenerqtion of the muscle fibres) (Allbrook, 1962). Owing to the high energy demands at this time, numerous mitochondria with well defined cristae are evident. Myofibrillogenesis commences at this stage.

Stage 3: greater than 96 hours.

The final stage of regeneration involves the gradual maturation of myotubes into myofibres. Myofibrillogenesis is more prominant than in younger myotubes and as the myofilaments become organized into sarcomeres, the cross-striations typical of mature skeletal muscle become visible. The diameter of a muscle fibre increases by the addition of new myofibrils to the periphery of previously formed myofibrils. Consequently, there is a gradient of myofibril size, with the thickest and oldest fibrils located centrally and the thinnest and most newly formed ones located at the periphery (Allbrook, 1962).

Complete differentiation of the regenerating myofibre requires motor innervation. Severed axons regenerate at a rate of approximately 3 mm/day, and synapse formation occurs in the late myotube stage (Barker, Scott and Stacey, 1986). As the fibre completes maturation, the nuclei decrease in size, the nucleoli fade and the nuclear chromatin condenses. The new muscle fibre closely resembles a typical mature fibre, except that the nuclei remain centrally placed.

The initial pattern of expression of contractile proteins in regenerating muscle is similar to that found during early stages of development. In regenerating muscle fibres, as in developing muscle fibres, there is an expression of the fast forms of Tn-I, -T, and -C (Dhoot and Perry, 1982), cardiac Tn-T (Saggin, Gorza, Ausoni and Schiaffino, 1990), cardiac and embryonic C-protein (Saad, Obinata and Fischman, 1987), β Tm, and a fast and slow Tm in fast and slow-twitch muscle, respectively (Matsuda *et al.*, 1983). The embryonic and neonatal MHC and MLC isoforms are also expressed, followed quickly by expression of adult isoforms (d'Albis, Couteaux, Janmot, Roulet and Mira, 1988; Whalen *et al.*, 1990). By approximately three weeks after injury, the regenerating myofibre expresses the contractile proteins typical of an adult myofibre.

B. Mechanism of Muscle Regeneration

Prior to the discovery of satellite cells, it was thought that newly regenerating muscle fibres arose from surviving nuclei,

which budded off the damaged fibre with adjacent portions of sarcoplasm (Mauro, 1961; Hall-Craggs, 1974). It is now generally accepted that none of the original nuclei survive and all nuclei in the regenerating fibres arise from division of satellite cells within the basement membrane. Evidence for this comes from both *in vivo* and *in vitro* studies using radio-labelling of newly formed nuclei, which are then followed over time to determine their fate (Konigsberg, Lipton and Konigsberg, 1975; Schultz *et al.*, 1985; Bischoff, 1980). However, it has been shown that with partial excision of a muscle, myoblasts derived from satellite cells can fuse with the stumps of the severed muscle fibres (Roth and Oron, 1985). This differs from the earlier theory of muscle regeneration where it was thought that the myoblasts fusing with the injured muscle fibres originated by dedifferentiation of nuclei from the original muscle.

In summary, all myoblasts in regenerating muscle originate from satellite cells and these may fuse with each other to form an entirely new muscle fibre or they may fuse with the end of a surviving portion of a damaged muscle fibre.

C. Experimental Models of Muscle Regeneration

Various physical or chemical procedures may be used to produce degeneration of muscle fibres and each of these techniques has certain advantages and disadvantages as a model for studying muscle regeneration.

1. Physical Methods

a) Muscle mincing involves separating the muscle from its neurovascular supply and its tendonous insertions and chopping it into small pieces, which are then grafted back into the original muscle bed. This procedure results in complete degeneration of the entire muscle, thus analysis of the regenerating muscle is not confused by the presence of co-existing mature, muscle fibres. However, mincing results in considerable scarring, which can interfere with functional and morphological analyses. Autologous grafting, without mincing, is useful for smaller muscles although some original fibres may persist and complicate subsequent analysis.

b) Crushing or freezing of the muscle also induces degeneration followed by regeneration. Their advantages are that they leave the basement membrane and neurovascular supply intact. However, it is difficult to induce degeneration of the whole muscle with these techniques, and the persisting normal muscle fibres may influence interpretation of the results.

2. Chemical Methods

The application of the anaesthetic bupivicaine (Marcaine), which causes chronic depolarization of the sarcolemma, may be used to induce degeneration in muscles, but it is difficult to affect the entire muscle. Several toxins derived from snake venoms (taipoxin, cardiotoxin and notexin) have been injected into muscle to produce degeneration. They have proven to be very useful because the neurovascular supply and basement

membranes are left intact, and it is possible to destroy all fibres in the muscle with minimal scarring. As a result, the ensuing regeneration is faster and more homogenous than that following the use of bupivicaine or any of the various physical methods.

Cardiotoxin which is derived from the venom of the snake *Naja mossambica mossambica*, and acts by irreversibly depolarizing the sarcolemma, is very effective in causing a homogenous degeneration and regeneration of relatively small muscles such as in the mouse, but lethal doses are required to be effective in larger animals (d'Albis *et al.*, 1988). Notexin from the snake *Notechis scutatus scutatus* can be used effectively in larger animals such as the rat (Harris, Johnson and Karlsson, 1975). Although notexin acts at the neuromuscular junction to prevent ACh release, it also causes muscle degeneration through its phospholipase A activity (Harris *et al.*, 1975). Satellite cells are not affected by exposure to the toxin and subsequently they differentiate to give rise to a population of myoblasts which then fuse and mature to form adult muscle fibres.

VI. MYOSIN

A. The Structure of Myosin

The myosin molecule (480 kDa), which is the major protein component of the thick myofilaments, is a hexamer composed of

two MHCs and four MLCs. The two MHCs coil about each other to form a rod, then at the neck, or hinge region, they separate to form two globular heads. Two MLCs are associated with each myosin head.

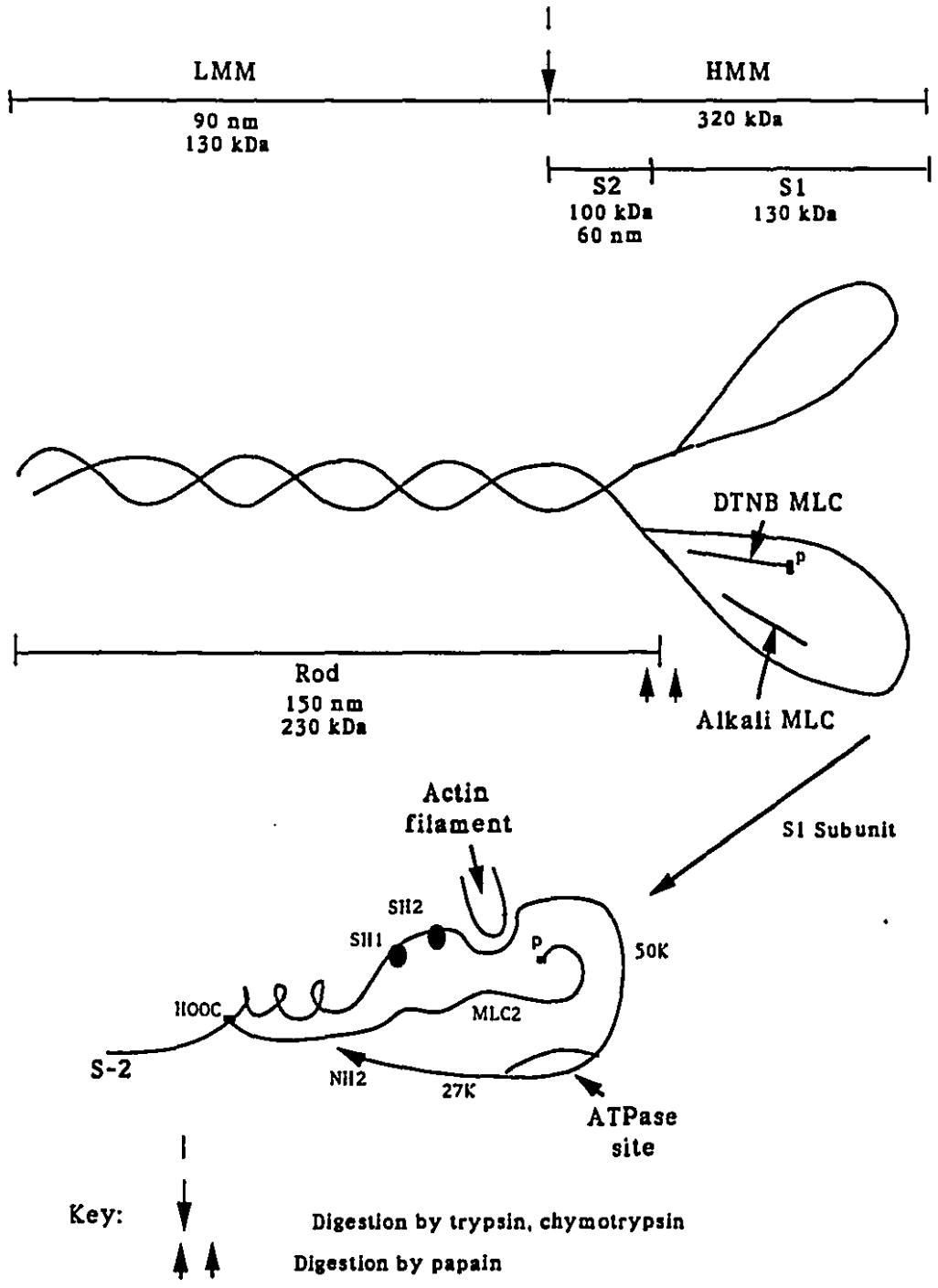
Enzymatic digestion of the myosin molecule results in its breakdown into several subunits which lend themselves to structural and functional analysis (Fig. 2) (Huxley, 1963; Lowey, Slayter, Weeds and Baker, 1969; Weeds and Lowey, 1971). Cleavage with trypsin or chymotrypsin separates myosin into two fragments: light meromyosin (LMM) and heavy meromyosin (HMM). The light meromyosin fragment contains most of the rod region and as such is a coiled-coil α-helical structure. The heavy meromyosin fragment can be further cleaved to produce three domains: two S1 domains which are the head regions, and an S2 domain that consists of the hinge region.

The S1 domain of the heavy meromyosin fragment is the most complicated, structurally and functionally. It is pear-shaped with the narrow end being the site of attachment near the hinge (Elliott and Offer, 1978). The S1 domain contains both actin-binding and myosin ATPase sites and it alone is sufficient to move the myosin filament relative to the actin filaments (Ebashi, 1963; Mornet, Pantel, Audemard and Kassab, 1979;

Figure 2. The structure of myosin. Digestion of myosin with trypsin or chymotrypsin produces two fragments, heavy meromyosin (HMM) and light meromyosin (LMM). Heavy meromyosin can be further divided into two domains, S1 (consisting of the MHC and MLC) and S2 (the hinge region).

The lower portion of the diagram illustrates the myosin ATPase and actin filament binding sites and the location of MLC2 on the MHC head.

Figure 2. The Structure of Myosin



Toyoshima, Kron, McNally, Niebling, Toyoshima and Spudich, 1987). The S2 domain, or hinge region, normally links the filamentous rod to the globular heads. This region is flexible and thus allows for movement of the myosin head relative to the rod (Elliott and Offer, 1978), as was initially proposed by Huxley (1969) in his sliding-bridge theory for the mechanism of muscle contraction.

1. Myosin Heavy Chains (MHC)

The α helical coiled-coil rod formed by the two MHC's is structurally suited to carry out its role in muscle contraction. The MHC component (95 kDa) of the S1 subunit can be digested using trypsin into three fragments of 27, 50, and 20 kDa (Mornet *et al.*, 1979). The 27 kDa fragment contains the myosin ATPase/ATP-binding site (Szilagyi, Balint, Sreter and Gergely, 1979), and the 50 and 20 kDa fragments are involved in actin binding (Mornet, Bertrand, Pantel, Audemard and Kassab, 1981). The actin-binding site is located towards the C-terminal region of the S1 subunit which extends to meet the S2 hinge domain. The myosin ATPase site is at the N-terminal end. The 20 kDa fragment has two sulfhydryl (SH1, SH2) groups, which serve to link the actin-binding site to the myosin ATPase site. Evidence also suggests a link between the thiol groups and MLC at the S1-S2 junction (Mornet *et al.*, 1981).

The actin-binding and myosin ATPase sites are essential to muscle contraction. The myosin ATPase activity is proportional to the force and speed of contraction (Barany, 1967).

2. Myosin Light Chains (MLC)

The two MLCs associated with each of the two heads can be divided into two categories based on the method used to isolate them: (i) the DTNB (5,5'-dithiobis-2-nitrobenzoic acid) light chains and (ii) the alkali light chains (see Barton and Buckingham, 1985). One of each type of light chain is associated with each head.

The DTNB MLCs (20 kDa) are localized to the neck region, in close proximity to the head/tail junction where they may interact with the MHC (Fig. 2). They can bind Ca^{++} and can be phosphorylated (Wagner and Giniger, 1981). Phosphorylation of the DTNB MLC is not necessary for ATPase activity but is thought to be associated with the post-tetanic twitch potentiation in a fast-twitch muscle. The DTNB MLC's are also frequently referred to as "regulatory MLC's" (MLC2) since they are thought to have a regulatory function on muscle contraction, although their exact mechanism of this is unknown.

The alkali light chains (MLC1 and MLC3) are not essential for actin binding or ATP hydrolysis but may serve to stabilize myosin conformation (Wagner and Giniger, 1981).

The relative positions of the MLCs and the MHCs vary with the functional state of the muscle. The regulatory MLC is capable of binding with the MHC and its ipsilateral alkali MLC. At rest, the C-terminal of the regulatory MLC is linked to both the MHC and the alkali MLC, but the N-terminal region is free. However, in the contracted state the N-terminal region of the regulatory

MLC moves closer to the ipsilateral alkali MLC (Hardwicke, Wallimann and Szent-Gyorgyi, 1983).

B. Myosin Isoforms

There are many different isoforms of MHC and MLC which may be assembled in numerous combinations to form a myosin molecule. The various isoforms are expressed in a stage- and tissue-specific manner. The different combinations of MHCs and MLCs found together may be evaluated with the use of techniques such as polyacrylamide gel electrophoresis, immunocytochemistry and molecular cloning.

1. MHC Isoforms

There are a number of distinguishable MHC isoforms, which have been identified to date (Table 1). Nine of these are specific to skeletal muscle: β (slow), slow tonic, embryonic, neonatal, fast IIA, fast IIB, fast IIX(IID), extraocular and superfast (Whalen, Schwartz, Bouveret, Sell and Gros, 1981; Swynghedauw, 1986; Pette and Staron, 1990). The mouse downstream MHC (MdM) was recently identified as a new isoform; however, based on its pattern of expression and its DNA sequence it is probably the same isoform as the IIX/IID (Parker-Thornburg, Bauer, Palermo and Robbins, 1992). Each of these skeletal muscle isoforms is expressed in a tissue-specific and/or stage-specific manner. Stage-specific expression is evidenced by the chronological appearance of embryonic, neonatal and adult isoforms. Examples

of tissue-specific expression are (i) extraocular MHC, which is found only in extraocular muscle fibres and (ii) super fast MHC which is found in jaw closing and tensor tympani muscles.

The particular MHC isoform accumulated imparts different physiological characteristics for that muscle fibre. For example, fibres in the rat soleus, which contain β (slow) MHC have low myosin ATPase activity, resulting in slow contractile behaviour. In addition, they use oxidative metabolism, which renders them fatigue resistant.

The fast MHCs can generally be divided into IIA (fast-oxidative glycolytic; FOG), IIB (fast glycolytic; FG) and IIX (also FOG). Of these three MHC isoforms, the IIB MHC imparts the fastest contractile properties, the IIA the slowest, and IIX-containing fibres exhibit contractile characteristics intermediate to those of IIB- and IIA-containing fibres.

The IIX/IID fast MHC isoform was identified simultaneously in two separate studies. This isoform, which predominates in diaphragm and masseter but is also found in several hindlimb muscles, was termed IIX by Schiaffino, Gorza, Sartore, Saggin, Ausoni, Vianello, Gundersen and Lomo (1989) and IID by Bar and Pette (1988), although it is generally believed that IIX and IID are the same isoform. In keeping with current popular practice, the term IIX will be used hereafter to refer to this isoform.

More recently, another potential new fast isoform, the MdM MHC was described, whose mRNA is found to be expressed in mouse diaphragm, tongue, soleus and masseter muscles

(Parker-Thornburg *et al.*, 1992). Its pattern of expression is similar to that of IIX MHC, except that no IIX is detected in the soleus. Despite this difference, the IIX and MdM isoforms are thought to be the same based on the strong homologies between the known nucleotide sequences of the MdM and IIX genes (S. Schiaffino, personal communication). In the 3' non-coding region, the sequence of which is known to be unique to each isoform of MHC, the first 32 nucleotides after the stop codon are exactly the same with the exception of the presence of a guanine nucleotide in position ten of the MdM sequence. Based on this, it is thought that the MdM and IIX genes are the same.

It has long been thought that a given mature muscle fibre contains only one type of myosin, composed of two MHC's of the same isoform. However, during periods of myosin transition, as occurs during development or after perturbation of the muscle, this may not be the case. Using electrophoretic analysis of single myofibres, several different hybrid myosin molecules containing MHC's of two different isoforms were found (Aigner, Gohlsch, Hamalainen, Staron, Uber, Wehrle and Pette, 1993). These were designated IIC (IIA with β), IIDA (IIA with IID) and IIBD (IIB with IID). Muscle fibres containing these hybrids have contractile characteristics between those possessed by fibres containing homodimers of either isoform. It is most likely that these muscle fibres are in transition from containing one MHC type to another.

2. MLC Isoforms

There are six MLC isoforms: MLCemb, MLC2s (slow), MLC2f (fast), MLC1f, MLC1s and MLC3f (Barton and Buckingham, 1985).

The MLC1f and 3f proteins are products of the same gene, with the particular isoform expressed being determined by the activation of either its MLC1f promotor or its MLC3f promotor, which occurs in a stage-specific manner (Periasamy *et al.*, 1984; Cox, Garner and Buckingham, 1990). Expression of MLCemb is also stage-specific. Early in myogenesis, MLCemb/MLC1f are expressed, and this is followed by MLC1/MLC3f expression (Barton and Buckingham, 1985).

Many different combinations of isoforms of MLC are possible but for a given myosin molecule the two regulatory MLCs are always of the same isoform whereas the two alkaline MLCs may be different from each other and in any combination. Fast-twitch muscles predominately contain MLC1/3 and MLC2f but some MLC1s is found in IIA MHC-containing muscle fibres. A mixture of MLC1s, MLC2s, and MLC1/3f are found in slow-twitch muscles (Table 1). The fast myosins may contain homodimers or heterodimers of MLC1f or MLC3f (Hoh and Yeoh, 1979; Kirschbaum, Simoneau, Bar, Barton, Buckingham and Pette, 1989; Termin and Pette, 1990). Homodimers of MLC1f predominate in type IIA muscle fibres, whereas homodimers of MLC3f predominates in type IIB muscle fibres (Table 1).

Several of the skeletal MHC and MLC isoforms are also expressed in cardiac muscle tissue. The MLC1s, MLCemb and MHCslow are equivalent to the cardiac muscle MLC1ventricular

(MLC1v), MLC1atrial (MLC1a) and β cardiac MHC respectively (Lompré, Nadal-Ginard and Mahdavi, 1984; Barton, Robert, Fiszman, Leader and Buckingham, 1985; Barton, Cohen, Robert, Fiszman, Bonhomme, Guenet, Leader and Buckingham, 1985).

The number of possible combinations of isoforms of the MHCs and MLCs provides a very broad spectrum of myosin molecules, which determine the contractile characteristics offibres containing them. The expression of these isoforms is influenced by several factors including innervation, load and thyroid hormone.

3. Factors Influencing Myosin Expression

Although expression of myosin in developing muscle follows an intrinsic sequence of transitions from embryonic to neonatal to adult isoforms, extrinsic factors such as altered innervation, thyroid hormone and load can alter this pattern (Table 2). In general, both MHC and MLC components are affected similarly. The results presented in Table 2 concern protein accumulation since, with the exception of IIB MHC mRNA (Russell *et al,* 1988), analysis of mRNA content has not been conducted. For example, chronic low frequency stimulation of fast-twitch muscle results in an increased expression of both slow MHC and MLC isoforms (Kirschbaum *et al.*, 1990).

Although denervation does not affect the myosin transitions in developing fast twitch muscle; the full expression of slow myosin is nerve-dependent and fails to occur if the slow-twitch muscle is denervated during development (Butler-Browne

Table 1: Isoforms of MHCs and MLCs in Skeletal Muscle [1]

Fibre Type	MHC [2]	MLC [3]
Embryonic	MHCemb	MLCemb/MLC1f
Neonatal	MHCneo	MLC1f, MLC2f, MLC3f
Slow/β	MHCslow/β	MLC2s, MLC1s, MLC1f, (MLC3f)
IIA	MHCIIA	MLC2f, MLC1f, (MLC3f)
IIB	MHCIIB	MLC2f, MLC1f, MLC3f
IIC (transitional IIA-IIB)	MHCIIA +slow	MLC2f/s, MLC1f, MLC3f, MLC1s

[1] Barton and Buckingham (1985)

[2] Additional MHC isoforms:
Mammalian: IIX/IID/MdM, extraocular, superfast, slow tonic
Avian: two slow isoforms, SM1 and SM2, and two embryonic isoforms (early and late)

[3] Brackets indicate minor form

et al., 1982; Gambke, Lyons, Haselgrove, Kelly and Rubenstein, 1983; Harris, Fitzsimons and McEwan, 1989; Condon, Silberstein, Blau and Thompson, 1990).

The exact individual roles played by electrical contractile activity and by putative myotrophic influences in determining myosin expression are unknown, but the former likely plays the greater role since electrical stimulation of innervated muscle alters myosin expression in a predictable manner. That is, high frequency stimulation of slow-twitch muscle fibres induces fast myosin expression and low frequency stimulation of fast-twitch muscle induces the expression of slow myosin (Jolesz and Sreter, 1981; Ausoni *et al.*, 1990; Termin and Pette, 1992).

The response of skeletal muscle to thyroid hormone is highly variable and is determined in a muscle-type-specific manner (Izumo, Nadal-Ginard and Mahdavi, 1986). Generally, the hyperthyroid condition favors fast myosin, whereas hypothyroidism favors slow. As such, the hyperthyroid condition causes an increase in fast myosin content in adult slow-twitch muscles and a precocious expression of fast myosin in developing fast-twitch muscles. Conversely, the hypothyroid condition results in an increased slow myosin content in fast-twitch muscle and there is a subtle precocious appearance of slow myosin in developing slow-twitch muscle (Butler-Browne *et al.*, 1984; d'Albis, Lenfant-Guyot, Janmot, Chanoine, Weinman and Gallien, 1987). An interaction between thyroid hormone and neuronal signals is suggested by the results of Nwoye *et al* (1982) (see Table 2: hyper/hypothyroid denervated).

Table 2: Factors Affecting Expression of Myosin Isoforms

Experimental Manipulation	Fast Muscle [1]	Slow Muscle [1]
Hyperthyroid [2]	early appearance of fast myosin	++fast myosin
Hypothyroid [2]	delayed appearance of fast myosin and ++slow myosin	early and ++slow myosin
Denervated [3]	no effect	++fast myosin
Denervated/ hyperthyroid [3]	early appearance of fast myosin	++fast myosin
Denervated/ hypothyroid [3]	fast myosin	+fast myosin
Load (use) [4]	++slow myosin	slow myosin
Chronically stimulated [5]		
a) high frequency	fast myosin	++fast myosin
b) low frequency	++slow myosin	slow myosin

[1] The symbol ++ denotes an increased myosin content
[2] Izumo *et al.* (1986)
[3] Nwoye *et al.* (1982)
[4] Ianuzzo et al. (1991)
[5] Kirschbaum *et al.* (1990)

It would appear that when thyroid levels are low, neural activity plays a greater role than normal in determining myosin expression. Thus, it is possible that there is a hierarchy of influences, which determine the exact myosin expression.

The load on a muscle also influences myosin accumulation, and this effect is influenced by thyroid hormone (Ianuzzo, Hamilton and Li, 1991). Compensatory hypertrophy results in an increase in slow myosin expression, however, this increase can be attenuated by the addition of thyroid hormone.

In summary, evidence indicates that there is a strong interrelationship between neuronal and hormonal factors and load in influencing myosin expression and therefore, in influencing the contractile characteristics of the muscle.

VII. MYOSIN HEAVY CHAIN

Transitions between isoforms of MHC occur not only in developing skeletal myofibres but also in adult muscle under various pathological and experimental conditions. Various factors are involved with these transitions, including the state of innervation, certain hormones and contractile activity. Two such factors, innervation and thyroid hormone, will be focused on to outline their role in influencing MHC expression in developing and regenerating muscle.

A. MHC Transitions in Developing Muscle

Developing muscle has a characteristic order of appearance of the different isoforms of MHC. This order is embryonic MHC, then neonatal MHC, then the adult isoforms (IIA, IIB, IIX/D, or β) (Whalen, Schwartz, Bouveret, Sell and Gros, 1979; Whalen *et al.*, 1981).

Analysis of MHC expression in the slow-twitch soleus indicates that two separate myoblast populations, which differ in their potential pattern of MHC expression, contribute to the formation of muscle fibres. In the rat soleus seven days after birth, β MHC is present in large diameter fibres whereas embryonic MHC and neonatal MHC are detected in small diameter fibres (Butler-Browne *et al.*, 1984). The diameter of the muscle fibre is an indication of its age (i.e. older fibres have larger diameters). With time, the expression of embryonic MHC is lost and that for neonatal MHC is reduced with a concomitant increase in adult fast MHC. However, the majority of the fibres contain β MHC. Based on these results it would appear there are two muscle cell populations which differ in their MHC expression: (i) an early appearing population which gives rise to the β MHC-containing fibres (large diameter fibres), and (ii) a later appearing population which gives rise to the fast MHC-containing muscle fibres (small diameter fibres).

The existence of two distinct populations of myoblasts has been confirmed by analysis of MHC protein and mRNA in developing muscle prenatally. One population is present at 9.5 d

of embryonic development and gives rise to the primary muscle fibres. The other, which is present at 16-18 d, gives rise to the secondary muscle fibres. These two myoblast populations form myotubes which initially express different MHC isoforms.

Myoblasts in the 9.5 d myotome fuse into myotubes which first produce β and embryonic MHC mRNA and proteins. Neonatal MHC mRNA can be detected by 10.5 d, although its protein is not detected until 16 d (Harris *et al.*, 1989). Embryonic MHC is the predominant isoform at 10.5 d but it is largely replaced by neonatal MHC, which predominates by 15 d (Weydert, Barton, Harris, Pinset and Buckingham, 1987; Lyons *et al.*, 1990). Until 16 d of development only primary myotubes are present, thus they are capable of expressing the genes for embryonic, neonatal and β MHC. The primary myotubes of the soleus are an exception since they express only the genes for embryonic and β MHC (Narasuwa *et al.*, 1987; Condon *et al.*, 1990).

The secondary myotubes, which are present at 18 d, contain embryonic and neonatal MHC in all types of muscle fibres. As development continues the neonatal MHC in primary and secondary myotubes is gradually replaced by one of the adult isoforms. In the mouse, IIB mRNA is detected at day 18 of gestation; however, in the rat it is not detected until seven days after birth (Weydert *et al.*, 1987; Periasamy, Wydro, Strehler-Page, Strehler and Nadal-Ginard, 1985). IIA mRNA and protein accumulates between five and six days after birth (Wieczorek, P iasamy, Butler-Browne, Whalen and Nadal-Ginard, 1985).

The soleus gradually accumulates β MHC until it is expressed in almost 85% of the muscle fibres. The remaining fibres contain IIA MHC, presumably originating from the secondary myotube population (Narasuwa *et al.*, 1987).

In some muscles, there is regional specialization of MHC expression. For example, in the tibialis anterior (TA) and extensor digitorum longus (EDL), all primary myotubes express embryonic and β MHC (Narasuwa *et al.*, 1987). As development progresses, the expression of β MHC gradually becomes localized to those muscle fibres in the deep region of the muscle whereas those of the superficial region begin to express the fast MHCs. As a result, different parts of an adult muscle are adapted for particular functional roles.

During avian myogenesis, there are three separate embryonic myoblast populations, which give rise to myotubes containing (i) fast MHC, (ii) fast and slow MHC or (iii) slow MHC, respectively (Stockdale and Miller, 1987). The adult muscle fibres arising from each of these "lineages" continue to express the same MHC as the original myoblasts. In regenerating avian muscle the satellite cells are committed to one of two cell lineages: (i) fast or (ii) mixed fast/slow. Both of these lineages of satellite cells, upon differentiation into myoblasts, express neonatal MHC. With further maturation however, those myofibres arising from "fast" satellite cells express fast MHC, whereas those muscle fibres arising from "mixed" satellite cells express fast MHC, slow MHC or mixed fast/slow MHC (Feldman and Stockdale, 1991).

In summary, skeletal muscle development involves a progressive committment of cells arising from the mesoderm, first to a skeletal myogenic lineage, and then to a specific fast or slow lineage. The associated developmental changes may be influenced in early stages by environmental cues and the relative position within the muscle mass, and at later times by innervation and other external signals such as load or hormones.

1. The Role of Innervation on MHC Transitions in Developing Muscle

Denervation of fast-twitch muscle at birth does not alter the normal accumulation of adult fast MHC, but does prevent the accumulation of β MHC (Butler-Browne *et al.*, 1982). In slow-twitch muscle, β MHC is detected early in myogenesis regardless of the state of innervation. However, its continued synthesis requires innervation (Narasuwa *et al.*, 1987; Fredette and Landmesser, 1990).

To explain the differing effects of innervation on fast and slow MHC expression, Crow and Stockdale (1986) suggested that myofibre development occurs in two phases: (i) an embryonic phase during which primary muscle fibres form independently of innervation, although probably dependent on intrinsic properties of the muscle fibres and/or environmental factors and (ii) a fetal phase, which is characterized by hypertrophy of the primary myofibres and the formation of secondary myofibres, during which innervation is necessary for sustained development, including the continued synthesis of β MHC. This

theory has since been supported by evidence from studies in which the appearance of primary or secondary myotubes was analysed in muscles denervated at different times prenatally (Hoh and Hughes, 1988; Harris *et al.*, 1990a).

The accumulation of different MHC isoforms can also be altered by experimentally manipulating the innervation of the muscle. High frequency stimulation of the slow-twitch soleus and low frequency stimulation of the fast-twitch EDL leads to an increased content of IIA and IIX MHC mRNA and protein. Therefore, soleus contains more fast myosin and EDL contains myosin more characteristic of slow-contracting muscle than their respective unstimulated controls (Ausoni *et al.*, 1990; Kirschbaum, Kucher, Termin, Kelly and Pette, 1990; Termin and Pette, 1992).

2. The Role of Thyroid Hormone on MHC Transitions in Developing Muscle

The effects of hypo- and hyper-thyroid conditions on MHC expression in developing and adult fast-twitch EDL and slow-twitch soleus were presented in Table 2. The hyperthyroid condition causes a precocious appearance of fast myosin, whereas a hypothyroid condition causes increased accumulation of slow myosin (Butler-Browne *et al.*, 1984; Russell *et al.*, 1988). Analysis of MHC expression in other muscles indicates that all of the MHC isoforms respond to altered levels of thyroid hormone. However, the particular response is not intrinsic to the isoform of

MHC but rather is determined in a muscle-specific manner (Table 3) (Izumo *et al.*, 1986).

Table 3: The Effect of Thyroid Hormone on MHC Expression [1]

Muscle	Hyperthyroid [2]	Hypothyroid [2]
SOL	-β +IIA +IIB	+β -IIA -IIB
EDL	-IIA N.A.	N.A.IIA N.A.IIB
TA		+IIA

[1] Izumo *et al.*, (1986)
[2] N.A.=no affect

B. MHC Transitions in Regenerating Muscle

The pattern of MHC expression in regenerating skeletal muscle shares some similarities with that in developing muscle, but there are both temporal and kinetic differences. Whereas in developing fast-twitch muscle, there is a sequential appearance of embryonic, neonatal and adult fast isoforms of MHC, in regenerating muscle, the neonatal and adult fast forms appear much earlier and may even occur concomitantly with embryonic MHC (Matsuda *et al.*, 1983; Saad *et al.*, 1987; d'Albis *et al.*, 1988;

Whalen *et al.*, 1990). Thus, the adult fast isoform appears much earlier in regenerating than developing muscle. In regenerating slow-twitch muscle, the synthesis of β MHC, which is nerve dependent, coincides with the timing of reinnervation, and thus occurs later than for the adult fast forms of MHC (Whalen *et al.*, 1990).

1. Effect of Innervation on MHC Transitions in Regenerating Muscle

Innervation plays an important role in determining MHC expression in regenerating muscle. Both innervated and denervated regenerating myotubes have similar initial patterns of MHC expression (i.e. embryonic and neonatal, then fast). As regeneration continues, innervated fibres gradually lose their embryonic and neonatal forms of MHC, which are replaced predominantly by β MHC and to a limited extent fast MHC. However, denervated regenerating muscle fibres synthesize neonatal MHC for a longer time than innervated regenerates, and this is then eventually replaced by fast MHC (Cerny and Bandman, 1987; d'Albis *et al.*, 1988; Whalen *et al.*, 1990). Thus, like in developing muscle, innervation is necessary for β MHC synthesis in regenerating muscle. In addition, innervation plays a role in suppressing the production of neonatal MHC.

2. Effect of Thyroid Hormone on MHC Transitions in Regenerating Muscle

In regenerating adult fast-twitch muscle, a hyperthyroid condition causes precocious appearance of adult fast MHC in association with an early disappearance of neonatal MHC (d'Albis, Weinman, Mira, Janmot and Couteaux, 1987). Conversely, a hypothyroid condition delays the transition from neonatal to fast MHC.

A hyperthyroid condition in the regenerating slow-twitch soleus results in a slight increase in content of fast MHC, however, β MHC continues to be strongly expressed (Sesodia, Bockhold, Laurent-Winter and Whalen, 1993).

C. MHC Gene Family

1. Members and Organization

The MHCs are encoded for by a family of genes, which are located on chromosome eleven and fourteen of the mouse and chromosome seventeen of the human (Weydert, Daubas, Caravetti, Minty, Bugaisky, Cohen, Robert and Buckingham, 1985). There are nine known members of the MHC gene family, each of which is between 23 and 28 kb in length (Parker-Thornburg *et al.*, 1992). Sequence analysis indicates large regions of homology flanked by gene-specific regions of divergent DNA sequences (Wydro, Nguyen, Gubits and Nadal-Ginard, 1983). Sequence divergence is particularly prevalent in the 3'-non-coding regions of the MHC genes.

Although the MHC genes have been localized to specific chromosomes, their exact organization is still unknown. In the mouse, the genes for the embryonic, neonatal and IIB MHC are localized to within 370 kb of each other. However, despite this close physical association, they do not appear to be activated in a coordinate fashion (Cox, Weydert, Barlow and Buckingham, 1991). The position of several of the MHC genes on chromosome seventeen of the human has revealed the following organization of the MHC genes (in order from 5' to 3'): embryonic, neonatal, IIA, IIB and IIX (Soussi-Yanicostas and Whalen, 1993).

2. Gene Structures

The structure of a gene determines the influence of cis-acting sequences and trans-acting factors on its expression. The sequences of the 5' promotor regions of some of the MHC isoforms have been determined, providing information on specific sequences required for activation of transcription in a tissue- and stage-specific manner. Considerable detail is known for some of these genes, which are outlined below.

a) The rat embryonic MHC gene has been isolated and analyzed for the presence of cis-acting regulatory sequences. In addition to its introns, the gene contains 41 exons, which are spread over 24 kb (Mahdavi, Strehler, Periasamy, Wieczorek, Izumo, Grund, Strehler and Nadal-Ginard, 1986). It is the first MHC gene to be induced in developing muscle, under both *in vitro* and *in vivo* conditions. *In vivo*, its expression is independent of innervation and it is deinduced around the time

of birth. The sequences in 1.4 kb of the 5'-flanking DNA are sufficient to direct the expression of the embryonic MHC gene in a tissue-specific manner (Bouvagnet, Strehler, White, Strehler-Page, Nadal-Ginard and Mahdavi, 1987). Using MHC mini-gene constructs, which contained deletion mutations and were coupled to a CAT reporter gene, three regulatory domains have been identified as being necessary for its expression: (i) the upstream sequences from -1413 to -174 bp, which contain three positive and two negative regulatory elements, are responsible for determining the level of expression of the gene and (ii) a muscle-specific regulatory element from -173 to -142 bp, which restricts expression of the MHC gene to muscle cells. These upstream sequences have been shown to function in an orientation-, position- and promoter-dependent fashion (Bouvagnet *et al.*, 1987). Two additional positive cis-regulatory elements have been identified: one extending from -93 to -84 bp and another extending from -80 to -74 bp (Yu and Nadal-Ginard, 1989).

b) The β MHC gene has a 781 bp enhancer- and promoter-containing region and 5'-flanking sequences which are required for tissue- and stage-specific transcription. Muscle-specific expression requires the sequences extending 294 bp upstream from the transcription initiation (cap) site (Cribbs, Shimizu, Yockey, Levin, Jakovcic, Zak and Umeda, 1989). Results from deletion analysis and linker-scanner mutations demonstrated that transcription of the β MHC gene is regulated by at least two cis-acting regulatory regions, which are located 184

and 275 bp upstream from the cap site. Both of these cis-acting regulatory regions are required for expression of β MHC since neither is capable of acting alone.

The more distal cis-acting regulatory region appears to exert a positive effect on transcription, because if it is deleted, the promoter of β MHC is inactive in differentiating skeletal muscle (Cribbs *et al.*, 1989). On the other hand, the proximal region contains a sequence resembling a CCAAT box (ATGCCATAACAAT), which in other genes is known to bind proteins (Jones, Kadonga, Rosenfeld, Kelly and Tjan, 1987). Therefore, it is possible that this region interacts with general transcription factors. The proximal region also encompasses another sequence that is present in the regulatory regions of several genes. This sequence resembles the binding site for the AP-2 protein, which is an enhancer binding protein involved in the regulation of a number of cellular genes. It is possible that AP-2 or an AP-2-like protein may bind to this region to mediate the effects of other cis- and trans-acting elements.

The regulatory sequences of the β MHC gene were examined using linker-scanner mutants between -295 bp and the TATA box (Shimizu, Prior, Umeda and Zak, 1992). Five positive cis-acting sequences that were necessary for transcription were identified and named elements A to E. Element A, consisting of the sequences between -275 and -263, contains an MCAT' motif in an inverted orientation. Element B is located between base pairs -207 and -180, and element C is found between -136 and -127. Element D (-91 to -80) appears to play

a role in transcription since mutations in this region block promoter activity. This element contains the sequence 5'-CTAAAT-3' at -88 to -83 bp. This is very similar to the CCAAT box found at -50 to -100 bp in many eukaryotic genes. Finally, element E consists of a TATA consensus sequence at -28 bp. Elements A and B were shown to work cooperatively in an orientation-dependent manner in determining muscle- and stage-specific expression (Shimizu *et al.*, 1992). The spacing between these two elements is important; normally separated by 55 bp, the insertion of 99 bp or more, results in reduced promoter activity. Located between elements A and B is an SP-1 binding site. Mutation of this region decreases promoter activity to 40% of normal; indicating that the binding of SP-1 or an SP-1-like factor is necessary for full promoter activity. Also located within the 5' regulatory region of the β MHC gene is a negative thyroid response element (TRE), although its exact location and sequence have not yet been determined (Rottman *et al.*, 1990).

c) The IIB gene enhancer region contains a TATAA box at -27 bp, and a CArG box at -99 bp. In addition, a MEF1 MyoD-binding site is located at -942 bp and three regions rich in nucleotides A and T (mAT1, 2, 3) are located between -140 and -220 bp (Takeda *et al.*, 1992). The identification of a MEF1 site was unexpected since MyoD acts to induce myogenesis (i.e. occurs very early in differentiation) and the IIB gene is an adult MHC gene.

The AT-rich region in mouse and chicken skeletal MHC genes were found to be homologous. Such evolutionary

conservation suggests that they may serve a regulatory role (Takeda *et al.*, 1992). Two of the three AT-rich regions have been found to contribute to the muscle-specific transcriptional activity of the IIB MHC gene.

Based on information known about the various MHC genes, positive regulatory elements must overcome the effects of negative elements in order for the gene to be transcribed. The presence of multiple positive and negative cis-acting elements in an enhancer indicates that there may be an interplay of many transcription factors in the regulation of stage- and tissue-specific expression of the MHC gene family during myogenesis.

There is little evidence for post-transcriptional or translational control of MHC expression, although post-transcriptional regulation of embryonic MHC is indicated by the finding of a high level of transcription of the embryonic gene but low levels of cytoplasmic mRNA accumulation (Cox *et al.*, 1991). A delay in the appearance of neonatal MHC protein following the transcription of its gene also suggests post-transcriptional or translational control of neonatal MHC (Harris *et al.*, 1989).

The stability of mRNA may also be a factor in regulating MHC expression. Over the course of myogenesis there is increased synthesis of mRNA as well as increased stability of the mRNA produced (Buckingham, Caput, Cohen, Whalen and Gros, 1974; Medford, Nguyen and Nadal-Ginard, 1983). The increased mRNA stability commences at the time of withdrawal from the cell cycle (Medford *et al.*, 1983).

A number of trans-acting factors modulate transcription of various cell types. These include thyroid hormone, muscle regulatory factors and innervation. With respect to MHC expression, it has been shown that innervation plays a role, although the molecular mechanism by which it exerts its influence is not known. Thyroid hormone binds to the thyroid hormone receptor in muscle cells, then this complex binds to the TRE of the MHC genes to affect transcription. No relationship has yet been found between known muscle regulatory factors and the expression of MHC (Miller, 1990; Lyons *et al.*, 1990).

In summary, the members of the MHC gene family possess both positive and negative regulatory sequences, which together determine transcription of the genes. There are also post-transcriptional influences on expression of MHC genes. The combined effect of the transcriptional and post-transcriptional influences results in a tightly controlled stage- and tissue-specific expression of the MHCs.

PURPOSE

Physical or chemical trauma to muscle results in its degeneration followed by regeneration. When studying muscle regeneration, one can study several parameters, including ultrastructural and contractile characteristics. However, I propose to study the expression of different isoforms of MHC, which determine some of the physiological characteristics of a muscle fibre. Regenerating muscle is characterized by the successive appearance of different MHC isoforms as is seen in developing muscle. The appearance of these isoforms appears to be under the influence of thyroid hormone and innervation individually or possibly through an interaction between them. The role of thyroid hormone and innervation on MHC expression in regenerating muscle is yet to be fully determined.

To induce a cycle of muscle degeneration and regeneration, venom from the snake *Notechis scutatus scutatus*, which contains the toxin notexin, was injected into the vicinity of the rat soleus. Use of this toxin is advantageous because it causes degeneration of the muscle while leaving the basal laminae and neurovascular supply intact. MHC expression in regenerating muscle was studied in terms of (i) MHC protein content, using monoclonal antibodies to the different isoforms of MHC and (ii) accumulation of MHC mRNA, using PCR (qualitative analysis) and slot blot hybridization (quantitative analysis), which should provide an indication of gene expression.

The results of these experiments should help to elucidate the individual or combined roles of innervation and thyroid hormone on MHC isoform expression during muscle regeneration. This could be particularly important when considering muscle pathology. Muscle regeneration, or lack of, is characteristic of several muscle pathologies, including polymyositis.

MATERIALS AND METHODS

I. ANIMALS AND EXPERIMENTAL GROUPS

All experiments were carried out on 100 g male Wistar rats (Iffa Credo, Lyon, France), which were given food and water *ad libitum*. Animals were randomly placed in one of four experimental groups:

(1) Innervated regenerates: one soleus muscle was induced to undergo a cycle of degeneration and regeneration by treatment with notexin. No other manipulation was performed.

(2) Denervated regenerates: degeneration was induced as for group (1) and in addition, the soleus was chronically denervated.

(3) Hyperthyroid Regenerates: one soleus muscle was induced to degenerate and during the subsequent regeneration period, the rats were maintained in a hyperthyroid state.

(4) Hyperthyroid/denervated regenerates: one soleus muscle was induced to degenerate and was denervated, and the rat was maintained in a hyperthyroid state.

Within each treatment group were 2 to 4 subgroups for which muscles were analysed at different times post-toxin injection: 4 d (groups 1, 2), 7 d and 14 d (all groups) and 21 d (groups 1, 2, 3).

Table 4: Number of Animals/Group/Time Point

Group	Days Post -Notexin Injection			
	4	7	14	21
1	8	8	8	14[2]
2	15	15	15	15
3[1]		8	8	8
4[1]		20	20	

[1] Blank spaces indicate no group was examined at this time point.

[2] This was divided into two groups: one (n=8) from which the muscles were used for immunohistochemistry and a pooled RNA preparation and the second (n=6) from which a RNA preparation was made for each muscle.

The 21 d time point for the hyperthyroid/denervated group was omitted since these muscles had a large amount of connective tissue and only sparse muscle fibres and thus, a very large number of muscles would have been required to obtain a useful quantity of RNA.

(5) Control Muscles: Muscles of normal rats at two ages were also examined. A group of 30 day-old rats (n=8) were used to determine the mRNA levels of several different muscles: soleus, EDL, diaphragm, tibialis anterior, masseter, gastroc-

nemius. Also, a two-day rat was used to obtain bulk hindlimb muscle for mRNA analysis.

II. EXPERIMENTAL PROCEDURES

A. Notexin-Induced Degeneration

Venom from the *Notechis scutatus scutatus* snake (Latoxan, Rosans, France) was injected subcutaneously into the dorsolateral aspect of the right hindlimbs of ether anaesthetized rats to induce degeneration of the soleus muscle. The snake venom, which contains notexin, a toxic component, was injected in two separate injections (200 μl of 0.2 μg/μl in PBS), which were given three hours apart. The injections were given subcutaneously along the line of demarcation between the gastocnemius and soleus (SOL) muscles, using a 20 g needle (Microlance, Becton Dickinson, Ireland).

B. Denervation

Denervation of the right soleus was accomplished by removal of one centimetre of the sciatic nerve just below the sciatic notch. This was carried out during the interval between the two injections of toxin. Successful denervation was

confirmed, when the rats had recovered from the anaesthetic, by the loss of the flexor response in the denervated right hindlimb.

C. Thyroid Hormone Treatment

A hyperthyroid condition was attained by daily intra-peritoneal injection of L-3', 5-triiodothyronine (L-T3; Sigma, France) (100 µl of 0.15 mg/ml L-T3 in PBS), starting on the day of notexin injection, following the protocol of Russell *et al.* (1988).

D. Necropsy and Preparation of Tissues

Rats were killed by cervical dislocation and the solei were removed immediately from both hindlimbs. The proximal thirds of each of the regenerating and contralateral control muscles were placed side by side between two pieces of liver and then were frozen in isopentane cooled to -150°C by liquid nitrogen. These were subsequently sectioned on a cryostat and stained histologically with toluidine blue, or hematoxylin and eosin, or by immunohistochemistry for the presence of different isoforms of MHC.

The distal two-thirds of each muscle pair were frozen in liquid nitrogen and stored at -80°C until used for preparation of RNA.

E. Histological Evaluation

Cryostat sections, 20 μm thick, were cut at -20°C from each of the muscle samples and immediately stained (i) for 30 sec with 0.5% toluidine blue or (ii) for 2 hr in stabilized hematoxylin (Rhone-Poulenc, France), rinsed in water, then stained for 30 min with 1% eosin. Stained sections were dehydrated in graded alcohols, rinsed in xylene and mounted in Mowiol (Hoechst, Germany).

The presence of centrally placed nuclei was used as an indicator of muscle degeneration/regeneration. Only those muscles in which at least 95% of the muscle fibres contained centrally placed nuclei were included in the study.

F. Immunofluorescence

1. Experimental Procedure

Serial cryostat sections, 10 μm thick, were cut at -20°C and collected on ethanol-cleaned slides and air dried for 20-30 min. Muscle sections were incubated with the primary antibody (Table 5) in a humid chamber at 37°C for one hour, then rinsed three times with PBS for 15 min each. Sections were then incubated with a rhodamine-labelled goat anti-mouse secondary antibody (Nordic Immunological Laboratories, Netherlands) diluted to 1:20 in PBS, at 37°C in a humid chamber for 30 min. After rinsing 3 times for 15 min in PBS, sections were mounted

directly in Mowiol (Hoechst, Germany) and stored in the dark at 4°C.

Two control groups were included: a) sections were incubated only with the secondary antibody and then stored in a humid chamber, washed and mounted and b) sections were processed as described above except no antibodies (primary or secondary antibodies) were used .

2. Specificity of Antibodies

a) MY32 is a monoclonal antibody produced using rabbit fast muscle myosin as the immunogen. It detects neonatal and fast IIA, IIX and IIB isoforms of MHC. The specificity of the antibody was tested using immunohistochemical analysis and immunoblot assays (Havenith, Visser, Schrijvers-van Schendel and Bosman, 1990). MY32 can be used for immunoperoxidase and immunofluorescence labeling of formalin-fixed or frozen sections of skeletal muscle (Sigma, France).

b) WBslow is a monoclonal antibody raised in mice against adult rabbit slow muscle myosin. The specificity of the antibody to β MHC was determined using western blotting. WBslow is effective on frozen but not paraffin sections (Davis, Harris and Brown, 1989; Franchi, Murdoch, Brown, Mayne, Elliott and Salmons, 1990).

c) RNMy2/9D2 is a monoclonal antibody produced using native myosin extracted from hind limb muscle of 7 day old rats. The specificity of the antibody to embryonic and

neonatal MHC was determined using western blotting. RNMy2/9D2 is effective on frozen but not paraffin sections

d) SC-71 is a monoclonal antibody directed against IIA MHC. Its specificity was tested using SDS PAGE, immunoblots and immunohistchemistry (Schiaffino *et al.*, 1989).

e) MF20 is produced using adult chicken pectoralis myosin (the immunogen) injected into mice. It is directed against the rod domain of the MHC molecule. The specifcity of MF20 was determined using immunoblotting and immuno-fluorescence (Bader, Masaki and Fischman, 1982).

Table 5: Primary Antibodies used in Immunohistochemistry

Antibody Name	Anti-X	Dilution[1]	Source
WBslow	slow/β	1:100	Davis *et al.* (1989)
MY32	fast (neonatal/IIA /IIB/IIX)	1:1000	Sigma, France Havenith *et al.* (1990)
RNMy2/9D2	embryonic/ neonatal	undiluted	Davis *et al.* (in press)
SC-71	IIA	1:500	Schiaffino *et al.* (1989)
MF20	all MHC	undiluted	Bader *et al.* (1982)

[1] Dilutions were done in sodium phosphate buffered saline (1X- 137 mM NaCl, 2.7 mM KCl, 3.2 mM $Na_2HPO_4 \cdot 12H_20$, 1.5 mM KH_2PO_4, pH 7.3)

G. Total RNA

Within a treatment group, those muscles which exhibited centronucleation of at least 95% of their myofibres (as determined by examination of sections stained with toluidine blue) were pooled and used for preparation of total RNA using a guanidinium thiocyanate/CsCl gradient method (Chirgwin, Przybyla, MacDonald and Rutter, 1979). As a precaution against

RNase activity, all solutions were prepared with 0.1% diethyl pyrocarbonate (DEPC)-treated H_2O. In brief, muscles were homogenized in 4 M isothiocyanate (10 ml/g muscle) using a Polytron homogenizer (Kinematica GmbH). Cesium chloride (0.15 g/ml) and sarcosyl (final concentration 0.5%) were added to the homogenate which was then stirred for 10 min. This was then centrifuged at 16,000 g for 5 min at 25°C. The supernatant was removed and layered onto a CsCl cushion (4.0 ml of 5.7 M CsCl) in 10 ml centrifuge tubes. The tubes were centrifuged for 19 hr at 180,000 g at 25°C. The pellet of RNA was re-dissolved in 400 µl DEPC-H_2O and transferred to Eppendorf tubes. Forty µl of 3 M $NaC_2H_3O_2$ and 1 ml absolute alcohol were added and the RNA was allowed to precipitate at -20°C overnight. The RNA was recovered by centrifugation at 15,000 g for 30 min at 4°C, and the pellet was washed in 70% ethanol, followed by a similar centrifugation protocol. The pellets were dried in a Speed Vac Concentrator (Prosciences, France) for 10 min, then were re-dissolved in 50 to 100 µl DEPC-H_2O, depending on the initial amount of tissue used (ie. pellets from larger amounts, or >0.50 g, of tissue were re-dissolved in larger volumes of DEPC-H_2O). The concentration of RNA was determined by measuring the optical density at 260 nm (OD260)(Hitachi U-1100 Spectrophotometer).

For an additional 6 rats, 21 d post-toxin injected soleus muscles were prepared exactly as described above, except instead of pooling muscles, RNA was prepared from individual soleus muscles.

H. Qualitative Analysis of RNA using the Polymerase Chain Reaction

Complementary DNA was synthesized, using the total RNA preparation according to the protocol of J. Chamberlain (Baylor College of Medicine, Houston, Texas; personal communication), which is a modification of the standard procedure described by Sambrook, Fritsch and Maniatis (1989). A mixture containing 10 μg of total RNA, 18 U RNasin, 10 μg random hexamers (Pharmacia LKB, Sweden), 1X buffer (250 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, 150 mM KCl, 50 mM DTT) and DEPC-H_2O to a final volume of 16.4 μl, was heated at 95°C for 1 min and then chilled on ice. To this mixture was added 2.0 μl of dNTP (which is a mixture of 25 mM each of dATP, dTTP, dCTP, dGTP), 18 U RNasin, and 20 U AMV reverse transcriptase (Appligène, France). The mixture was incubated for 1 hr at 42°C, then 30 μl of a solution containing 0.7 M NaOH and 40 mM EDTA were added and the mixture was incubated for a further 10 min at 65°C. After incubation, the DNA was precipitated by adding 5 μl of 2 M ammonium acetate (pH 4.5) and 130 μl of 100% ethanol and recovered by centrifugation at 15,000 g for 30 min, at 4°C. The pellet was washed in 70% ethanol, then dried in a speed vac (approximately 10 min). The cDNA recovered was used as the template for the PCR reaction.

Two μl (approximately 200 ng) of the cDNA template were combined with 100 ng of each primer (sense and anti-sense) in 30 μl of sterile H_2O. The primers used were chosen from the 3'

end of the DNA sequences for α-skeletal actin or one of five different isoforms of MHC (neonatal, β, IIA, IIB, or MdM). The sequences of the forward primers were taken from coding regions, whereas those of the reverse primers were taken from non-coding regions, with the exception of that for α-skeletal actin, where both primers were taken from the coding region (Table 6). The sequences of the 3' non-coding regions are known to be specific for each isoform of MHC (Nguyen, Gubits, Wydro and Nadal-Ginard, 1982; Wydro, Nguyen, Gubits and Nadal-Ginard, 1983).

Table 6: Primer Sequences for PCR Amplification of cDNA of Isoforms of Rat MHC

Neonatal MHC[1] (271 bp)	
Sense:	5'-GAGGACCGCAAGAACGTGCTGAGGC-3'
Anti-sense:	5'-TGAGGAGGCCGCCAAGTGGCTGA-3'
Type I (β) MHC[2] mRNA (199 bp)	
Sense:	5'-CCAAGTTCCGCAAGGTGCAGCACGA-3'
Anti-sense:	5'-CCTAAGGTGCTGTTTCAAAGGCTCC-3'
Type IIA MHC[3] mRNA (302 bp)	
Sense:	5'-TCCTCAGGCTTCAAGATTTGGTGG-3'
Anti-sense:	5'-AATAGAATCACATGGGGGCATGACC-3'
Type IIB MHC[3] mRNA (220 bp)	
Sense:	5'-CTACAGACTAAAGTGAAAGCCTAC-3'
Anti-sense:	5'-CACCTTTCAACAGAAGGAATTGAGC-3'
MdM/IIX MHC[4] (230 bp)	
Sense:	5'-CGGGTGAAGAGCCGGGAGTTCACAC-3'
Anti-sense:	5'-CTCTCCTGATGTACAAATGATCGGC-3'

[1] Neonatal MHC; Periasamy, Wieczorek and Nadal-Ginard (1984).

[2] β MHC; Kraft, Bravo-Zehnder, Taylor and Lenawand (1989).

[3] IIA and IIB MHC; Nadal-Ginard, personal communication.

[4] MdM/IIX; Parker-Thornburg *et al.* (1992).

[1-4] The bracketed figures are the expected sizes of the amplified fragments.

The primer/DNA mixture was denatured by heating to 94°C for 5 min, then 20 µl of PCR buffer was added. The buffer contained 25 mM dNTP, 335 mM Tris-Cl, 83 mM $(NH_4)_2SO_4$, 33.6 mM $MgCl_2$, 50 mM β-mercaptoethanol, 0.85 mg/ml BSA and 0.75 U Taq DNA polymerase (250 U/ml) (AmpliTaq, Cetus). The PCR reaction was carried out for 40 cycles each of which consisted of the following sequence: 1 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. The PCR amplification was completed by an elongation step of 3 min at 72°C (Hybaid Intelligent Heating Block).

The resultant cDNA was separated by electrophoresis as follows: 20 µl of the PCR reaction product plus 4 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) were loaded on a 2% agarose gel (1.4% NuSieve agarose-FMC, Bioproducts; 0.6% agarose, Sigma) and run for 2.5 hr at 100 V. The gel was then stained with ethidium bromide for 20 min, and photographed under UV light (Polaroid film). The sizes of resultant cDNA fragments were determined by comparison to those of a standard marker (pBR322, Appligène, France).

The PCR products were transferred from the gel onto a nylon membrane (Gene Screen Hybridization Transfer Membrane, NEN Research Products, Dupont, USA) for hybridization with a probe whose sequence was taken from the MHC sequence at a position internal to those of the related PCR primers (Table 7). The DNA transfer procedure was carried out according to the standard protocol as described by Sambrook *et al.* (1989). The initial step of this procedure was the depurination of DNA in 0.25 M HCl for 20 min. The gel was then

immersed in a solution of 0.5 M NaOH/1.5 M NaCl two times for 15 min each in order to denature the DNA. The gel was placed on top of a long piece of Whatman 3MM paper so that the ends of the filter paper extended into the transfer buffer (0.5 M NaOH/1.5 M NaCl). On top of the gel were stacked in order a Gene Screen membrane, 5 pieces of Whatman 3MM paper, and a stack of absorbant pads. The entire stack on the support was placed in 300 ml of denaturing solution and left for 16 hr at RT. The membrane was then rinsed in a solution containing 0.2 M Tris-Cl (pH 7.5) and 2X SSC (1X SSC = 0.15 M NaCl and O.15 M sodium citrate) and then left to air dry.

I. Northern Blot Hybridization to Test Slot Blot Probe Specificity

1. Preparation of Northern Blot

Prior to their use on the slot blots, the probes (Table 7) were tested for their specificity to one of the MHCs or to α-cardiac or α-skeletal actin. This was done by hybridizing the probe, in the case of the MHCs, to a Northern blot containing RNA prepared from various muscles (adult rat soleus, gastrocnemius, tibialis anterior, diaphragm, heart and neonatal bulk hindlimb) and to a slot blot containing these same samples. Hybridization of the probe to RNA samples known to contain a particular MHC was used as an indicator of probe specificity.

The RNA (100 ng/μl) was denatured by dissolving it in a solution of 50% formamide, and 6% formaldehyde in MOPS buffer

(0.2M Morpholinopropanesulfonic acid sodium salt, 0.05 M sodium acetate, 0.01 M Na_2EDTA, pH 7.0). The mixture was incubated for 15 min at 60°C and then cooled on ice. Loading dye was added to each RNA sample and these were electrophoresed on a 1.5% agarose gel containing 6% formaldehyde.

The RNA was then transferred to a Gene Screen Hybridization Transfer Membrane, according to the capillary blot procedure recommended by the manufacturer. The gel was placed on top of a long piece of filter paper (Whatman 3MM) so that the ends of the filter paper extended into the transfer buffer (0.025 M sodium phosphate, pH 6.5). On top of the gel were stacked in order a gene screen membrane, 5 pieces of Whatman 3MM paper, and a stack of absorbant pads. The transfer continued at RT until the gel was reduced to a thin layer (approx. 17 hr-3 mm thick). The membrane was then washed in 0.025 M sodium phophate (pH 6.5) and baked at 80°C for 2 hr.

2. Preparation of the Probes

Sequences taken from the 3' end of the α-actin (skeletal or cardiac) or MHC (β, IIA, IIB, MdM) genes (Table 7) were used to synthesize probes with a Gene Assembler (Pharmacia, LJB, Sweden). The probes were labelled at the 5' end to a specific activity of 2 X 10^7 dpm/pmol using (γ-^{32}P)ATP (Amersham, UK) and T4 polynucleotide kinase (Biolabs, New England), then cleaned on a Chroma Spin-10 column (Clontech, California).

The membranes were prehybridized, as well as hybridized, at 42°C for 24 hr in 50% formamide, 0.04% polyvinyl-pyrrolidine, 0.04% bovine serum albumin, 0.04% ficoll, 5X SSC, 1.0% SDS and 100 μg/ml salmon sperm DNA (boiled for 5 min before added). For the hybridization, the probe was boiled for 5 min and then added to the hybridization solution. After hybridization was complete, the membrane was washed as follows: i) twice in 2X SSC for 5 min at RT, ii) once in 2X SSC with 1.0% SDS for 30 min at 65°C, and iii) once in 0.1X SSC at for 15 min at RT. The membrane was then placed in a Cronex cassette (Dupont, USA) containing an intensifying screen and Kodak Scientific Imaging Film (Eastman Kodak, New York) and stored at -80°C. Three exposures were taken at 24 hrs, 4 d, and 14 d, respectively. The specificity of the probe was determined by its hybridization to a sample of muscle known to strongly express the MHC of interest.

Table 7: Probe Sequences for Slot Blot Hybridizations

β MHC[1] (50 bp)

5'-TC CAG GTC TCA GGG CTT CAC AGG CAT CCT TAG GGT TGG GTA GCA CAA GAT-3'

IIA MHC[2] (51 bp)

5'-TAG GAT TTA ATA GAA TCA CAT GGG GGC ATG ACC AAA GGC TTC ACA TTT TGT-3'

IIB MHC[2] (51 bp)

5'-CAG GAC AGT GAC AAA GAA CGT CAC ATT GTG TGA TTT CTT CTG TCA CCT TTC-3'

MdM MHC[3] (60 bp)

5'-GAG CTC TTG GCA GAT AAA TTT TTT ATT CTC CCA AAG TTA TGA GTA CAA AAC AGA GTG ACA-3'

α-skeletal actin[4] (50 bp)

5'-GAG GAT GGG GCC ACC CTG CAA CCA TAG CAC GAT GGT CGA TTT GTC GTC CT-3'

α-cardiac actin[4] (49 bp)

5'-GGA GAT GGG AGA GGG CCT CAG AGG ATT CCA AGC ACA ATA CTG TCG TCC T-3'

[1] Kraft *et al.* (1989) [2] Nadal-Ginard, personal communication
[3] Parker-Thornburg *et al.* (1992)
[4] Zakut, Shani, Givol, Neuman, Yaffe and Nudel (1982)

J. Analysis and Quantification of MHC mRNA

The MHC mRNA was analysed and quantified by slot blot hybridization. In preparation, 4 μg of RNA was incubated in 50% formamide, 6% formaldehyde at 50°C for 1 hr to denature the RNA. The RNA was then applied onto a nylon membrane (Gene Screen Hybridization Transfer Membrane, Dupont, USA), using a Schleicher & Schuell Minifold II Slot-Blot System. Four membranes were prepared; (i) two membranes each using RNA from the groups of regenerating muscle (one with serial dilutions starting with 2.0 μg RNA and the other starting with 0.5 μg RNA), (ii) one membrane containing RNA from the 30 d samples, and (iii) one membrane containing RNA prepared from individual 21 d post-toxin injected muscles. For all membranes RNA samples were loaded in two-fold serial dilutions (approx. 6 dilutions in total). The membranes were prehybridized, hybridized and washed as described above for the Northern blots, with the probe being labelled to a specific activity of 2×10^7 dpm/pmol using (γ-^{32}P)ATP (Amersham, UK) and T4 polynucleotide kinase (Biolabs, New England). The membranes were placed in a PhosphorImager cassette (Molecular Dynamics) for 20 hrs. The cassette screen was then scanned using a PhosphorImager system equipped with Image Quant software.

The membrane was stripped by incubating it at 75°C for 2 hrs in 5 mM Tris-Cl (pH 8.0), 0.2 mM Na_2EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinyl-pyrrolidine, 0.002% bovine

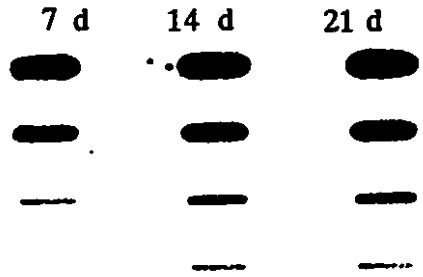
serum albumin and 0.002% ficoll. It was then ready to be prehybridized/hybridized with the next probe of interest.

The method of normalizing MHC mRNA in the different RNA samples is detailed below. Values of radioactive counts/μg total RNA obtained for each of the MHC mRNAs were normalized to the counts/μg total RNA of α-skeletal actin or of α-skeletal and α-cardiac actin combined. Low quantities of α-cardiac actin mRNA were detected in the 4 d innervated and denervated, and 7 d hyperthyroid/denervated regenerates, but not in any other experimental group. In these cases, statistical analyses (using a two-tailed t-test, $p<0.01$) were not different if values were normalized to only α-skeletal actin mRNA or to total α-actin mRNA (α-skeletal plus α-cardiac actin mRNA).

The method used to quantify the different isoforms of MHC mRNA is shown in Table 8, which uses the hybridization results of the probes to α-skeletal actin mRNA and β MHC mRNA to innervated regenerates as an example. The counts above background as determined by the PhosphorImager are listed for the 7, 14, and 21 d innervated regenerate samples after hybridization with α-skeletal actin and β MHC probes.

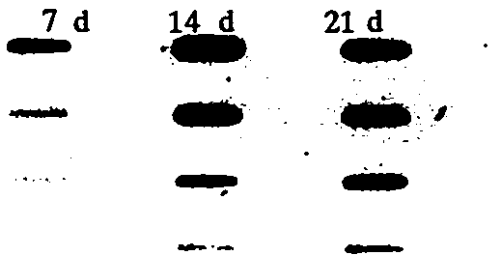
Table 8: Quantification of MHC mRNA: Determination of MHC mRNA[1](counts/μg total RNA)

i) Probe: α-skeletal actin



RNA	Innervated	Regenerates	(counts[2])
(μg)	7 d	14 d	21 d
0.500	518356	721154	741459
0.250	189944	316654	349250
0.125	42596	83669	101910
0.063	13678	37028	34290

ii) Probe: β MHC

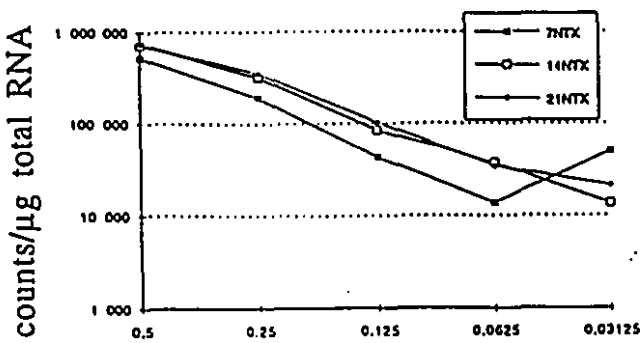


RNA	Innervated	Regenerates	(counts[2])
(μg)	7 d	14 d	21 d
0.500	24632	160648	72984
0.250	7819	86662	68110
0.125	3426	27137	34193
0.063	1747	10177	11297

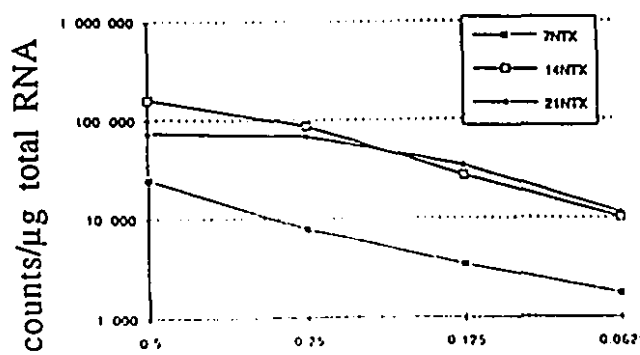
[1] Using the results of 7 d, 14 d and 21 d innervated regenerate samples hybridized with a probe to α-skeletal actin (i) or β MHC (ii).

[2] Counts: The radioactive filter is placed in a PhosphoImager cassette which is scanned by laser, resulting in the emmission of light. This is eventually converted to pixel values or counts.

The content of MHC or α-skeletal actin mRNA (counts/μg total RNA) were plotted versus total RNA (μg) (Fig. 3). A linear regression analysis was done to determine the y-intercept, slope and correlation coefficient of the graph. Using the equation y=ax+b, where x=1, the value in counts/μg total RNA was determined for each probe used with each sample. In two cases, with α-skeletal actin and β MHC, a plateau was reached at the higher concentrations of RNA, suggesting saturation of the mRNA signal, and these higher values were not used in the linear regression analysis.



α-skeletal actin
y=ax+b
7 d NTX $1.1x10^5$
14 d NTX $1.5x10^6$
21 d NTX $1.5x10^6$



β MHC
y=ax+b
7 d NTX $5.0x10^4$
14 d NTX $3.4x10^5$
21 d NTX $1.5x10^5$

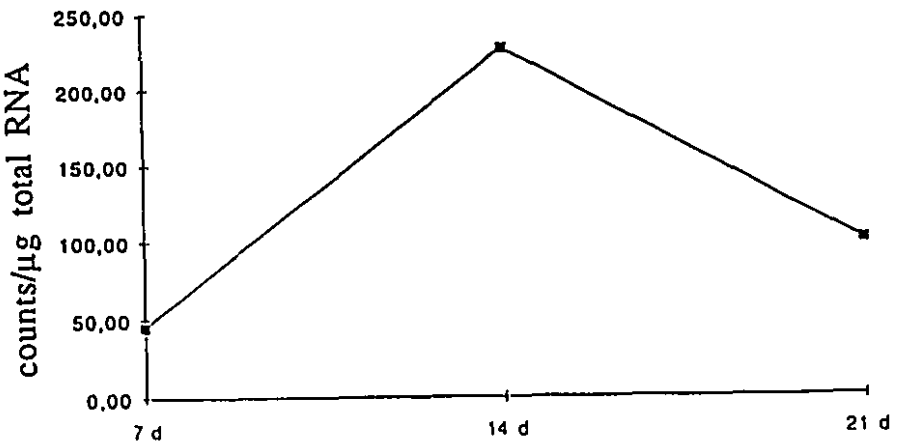
Figure 3. Graphing of the Quantity of α-Skeletal Actin mRNA and β MHC mRNA for Different Amounts of RNA.

The values (counts/μg total RNA) for each isoform of MHC mRNA were normalized to those of α-skeletal actin mRNA using the equation:

$$\frac{\text{counts/μg MHC mRNA}}{\text{counts/μg α-skeletal actin mRNA}} \times 1000$$

Data was presented graphically by plotting the normalized values in counts/μg total RNA (cts/μg) versus time of regeneration (days post-notexin treatment).

Figure 4. Expression of β MHC mRNA in Innervated Regenerating Soleus



RESULTS

I. HISTOLOGICAL EVALUATION

The morphological development of the regenerating innervated muscles, as seen in cross-sections stained with hematoxylin and eosin, was the same as that found in a previous study, which also used notexin to induce regeneration (Whalen *et al.*, 1990) (Table 9). At 4 d post-toxin injection, the innervated regenerating soleus appeared to be composed predominately of multinucleated myotubes, but with some mononucleated myoblasts still present (Fig. 5). By 7 d, the muscles consisted mostly of small-diameter muscle fibres with centrally placed nuclei. Between 7 d and 21 d, the myofibres of innervated regenerates gradually increased in diameter, reaching a similar size as contralateral controls by 21 d post-toxin injection.

The denervated regenerates resembled the innervated regenerates at 4 d and 7 d of regeneration, but at 14 d and 21 d, the fibres in denervated regenerating muscles were smaller and more disorganized than those in innervated regenerates and normal control muscles.

The hyperthyroid condition did not affect the morphological appearance of innervated regenerating muscle. However in denervated regenerating muscle, the hyperthyroid condition resulted in a greater amount of connective tissue at 7 d and 14 d than was present in denervated regenerates at the same time points.

Figure 5. Morphological appearance of innervated (a-d) and denervated (f-h) regenerating rat soleus stained with hematoxylin and eosin. The time points shown are: 4 d (a), 7d (b, f), 14 d (c, g), and 21 d (d, h) post-toxin injection. Normal control rat soleus is shown in (e).

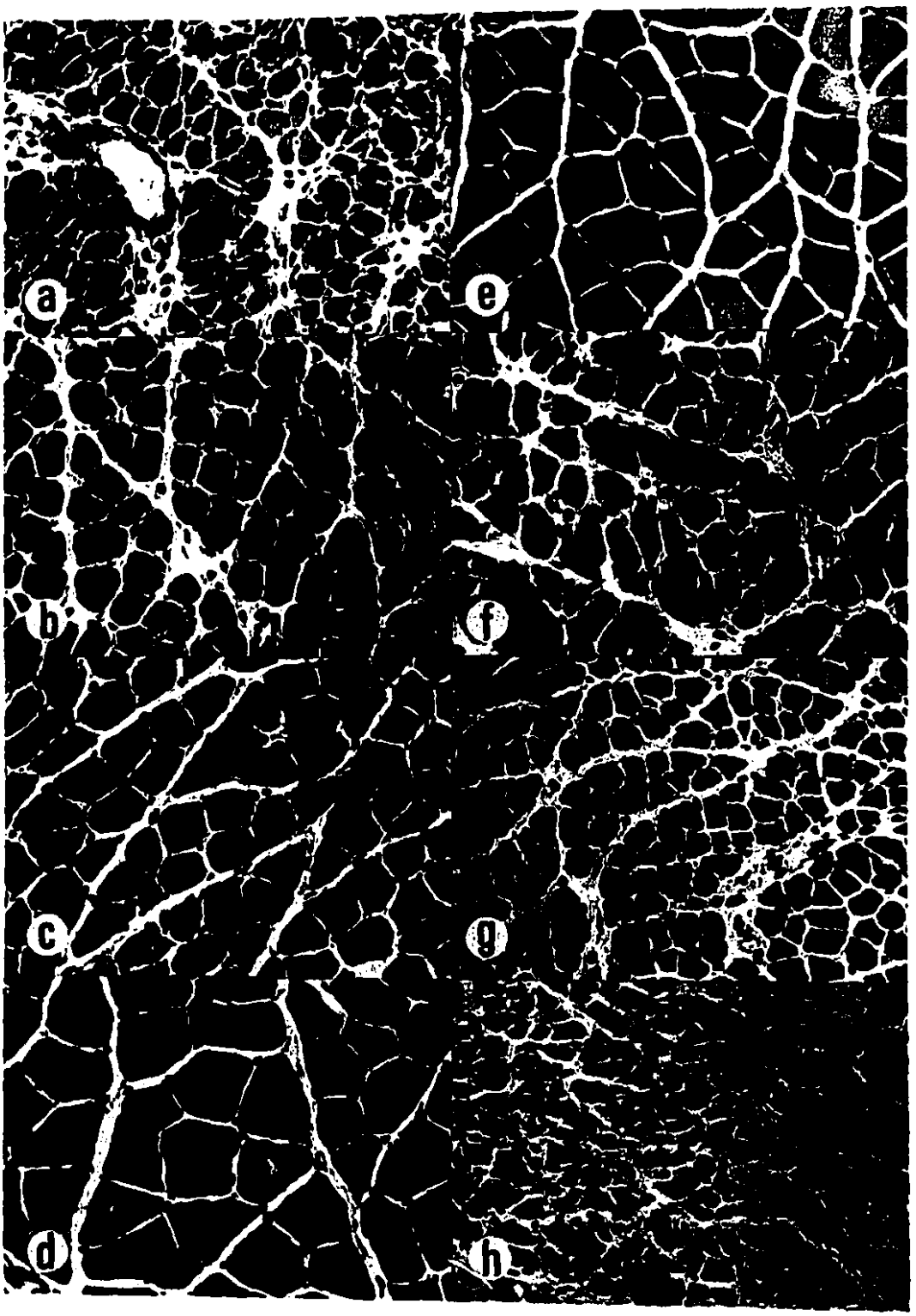

a
b
c
d
e
f
g
h

Table 9: Time Course of Muscle Regeneration Following Notexin-Induced Degeneration (days post-toxin injection)[1]

Characteristics	Days Post-Toxin Injection
degeneration and phagocytosis	1
myoblasts appear	2-3
formation of myotubes	3-4
reinnervation begins	4-5
increase in fibre size	7-21
regeneration complete	21-28

[1] Whalen, Harris, Butler-Browne and Sesodia (1990)

II. IMMUNOHISTOCHEMISTRY

All of the antibodies have been tested for their specificity using immunoblotting (see Materials and Methods). In addition, all of the antibodies have previously been used with immunofluorescence on different muscle samples, which provides an indication of their reactivity and sensitivity to different isoforms of MHC (LaFramboise *et al.*, 1989; Ecob-Prince, Hill and Brown, 1989; Havenith *et al.*, 1990; Davis *et al.*, 1993).

The isoforms of MHC detected in regenerating solei muscles varied depending on the time post-notexin injection, the state of innervation of the muscle and the presence of a hyperthyroid state. The results of the immunohistochemical staining are summarized in Table 10.

All control cryostat sections which were incubated without primary or secondary antibodies or were incubated with secondary antibodies only were not stained.

A. Normal Soleus

Normal contralateral solei muscles consisted of a large proportion of β MHC-containing muscle fibres (approximately 85%). The remaining muscle fibres stained for neonatal/fast and IIA MHC but not embryonic/neonatal MHC, thus these muscle fibres predominately contained IIA MHC (Fig. 6).

B. Innervated Regenerating Soleus

In the early period of regeneration (4 d and 7 d) all myofibres stained with antibodies against embryonic/neonatal and neonatal/fast MHC, but this was decreasing by 14 d (Fig. 7 and 17). By 21 d, no fibres stained for embryonic/neonatal MHC (Fig. 8 and 12) and only a small number (approximately 15%) stained for neonatal/fast MHC (Fig. 8).

β (slow) MHC was absent at 4 d but gradually increased between 7 d and 21 d, at which point approximately 95% of the myofibres contained it (Fig. 8, 16 and 18).

Although approximately 15% of the muscle fibres in contralateral control soleus muscles contained IIA MHC, no fibres in the regenerating solei stained with the antibody to IIA MHC (Fig. 19).

C. Denervated Regenerating Soleus

Denervation of the regenerating soleus resulted in a different staining pattern for the various isoforms of MHC relative to that seen in innervated regenerating muscles. The most striking difference was the complete lack of β MHC in denervated regenerates, whereas this isoform was in about 95% of the fibres in innervated regenerates (Fig. 9 and 18).

Table 10: Immunohistochemical Detection of MHC Proteins in Regenerating Rat Soleus Muscle

Antibody	Muscle Treatment[1]	Time Post-Toxin[2] (Days) 4	7	14	21
Emb/Neo MHC	Contralatera	-	+/-	-	-
	NTX	+	+	m	-
	NDEN	+	+	+	-
	NTH	nd	+	+/-	-
	NTD	nd	+	+	nd
β MHC	Contralatera	+	+	+	+
	NTX	-	+/-	+	+
	NDEN	-	-	-	-
	NTH	nd	+/-	m	+
	NTD	nd	-	-	nd
Neo/Fast MHC	Contralatera	m	m	m	m
	NTX	+	+	+	m
	NDEN	+	+	+	+
	NTH	nd	+	+	m
	NTD	nd	+	+	nd
IIA MHC	Contralatera	m	m	m	m
	NTX	-	-	-	-
	NDEN	-	-	-	+/-
	NTH	nd	-	m	m
	NTD	nd	-	+/-	nd

[1] Contralateral = soleus contralateral to the injected hindlimb
NTX = notexin treated (regenerating),
NDEN = denervated regenerates
NTH = hyperthyroid regenerates
NTD = hyperthyroid/denervated regenerates

[2] + = positive results (>90% of the muscle fibres were +)
- = negative results
+/- = odd spot (scattered fibres stained with the antibody, < 10%)
m = mosaic (approximately 25% of the fibres were +)
nd = not determined

The antibodies for embryonic/neonatal and neonatal/fast MHC stained the majority (approximately 95%) of muscle fibres through 14 d, after which staining for embryonic/neonatal MHC disappeared (Fig. 7 and 9). By 21 d denervated regenerates contained fast isoforms of MHC (approximately 95%) in contrast to innervated regenerates which had only 15% of fibres staining as fast. That a majority of myofibres stained with the neonatal/fast antibody and only a few stained faintly with the IIA antibody (and none with the embryonic/neonatal antibody) in 21 d denervated regenerates, suggests that the staining with neonatal/fast antibody at 21 d was mostly due to the presence of IIB and/or IIX MHC (Fig. 20).

D. Hyperthyroid Regenerating Soleus

The pattern of MHC accumulation in 7 d hyperthyroid regenerates was similar to that in innervated regenerates except there was an accelerated loss of fibres staining for the embryonic/neonatal antibody (Fig. 10, 12 and 15). Another difference was noted at 14 d and 21 d: some fibres stained for IIA MHC (approximately 25%), whereas no fibres in innervated regenerates accumulated IIA MHC. (Fig. 11 and 21).

E. Hyperthyroid/Denervated Regenerating Soleus

An antibody against all MHCs was used to identify myofibre-containing areas in the hyperthyroid/denervated regenerates, which had a large content of connective tissue In serial sections, it was found that embryonic/neonatal and neonatal/fast antibodies stained the hyperthyroid/denervated regenerating muscle fibres strongly at 7 d and 14 d (Fig. 13 and 14). Thus, the embryonic/neonatal isoform persisted longer in hyperthyroid/denervated regenerates compared to in innervated regenerates. β MHC was absent at least up until 14 d. IIA MHC was not detected at 7 d, but a small amount was seen at 14 d, similar to that found in 21 d denervated regenerates (Fig. 14). Four and 21 d hyperthyroid/denervated regenerates were not examined.

Figure 6. Immunofluorescence staining of serial sections of rat soleus muscle contralateral to 21 d innervated regenerate, using antibodies to β (a), embryonic/neonatal (b), neonatal/fast (c) or IIA (d) MHC. Scale bar =100 μm.

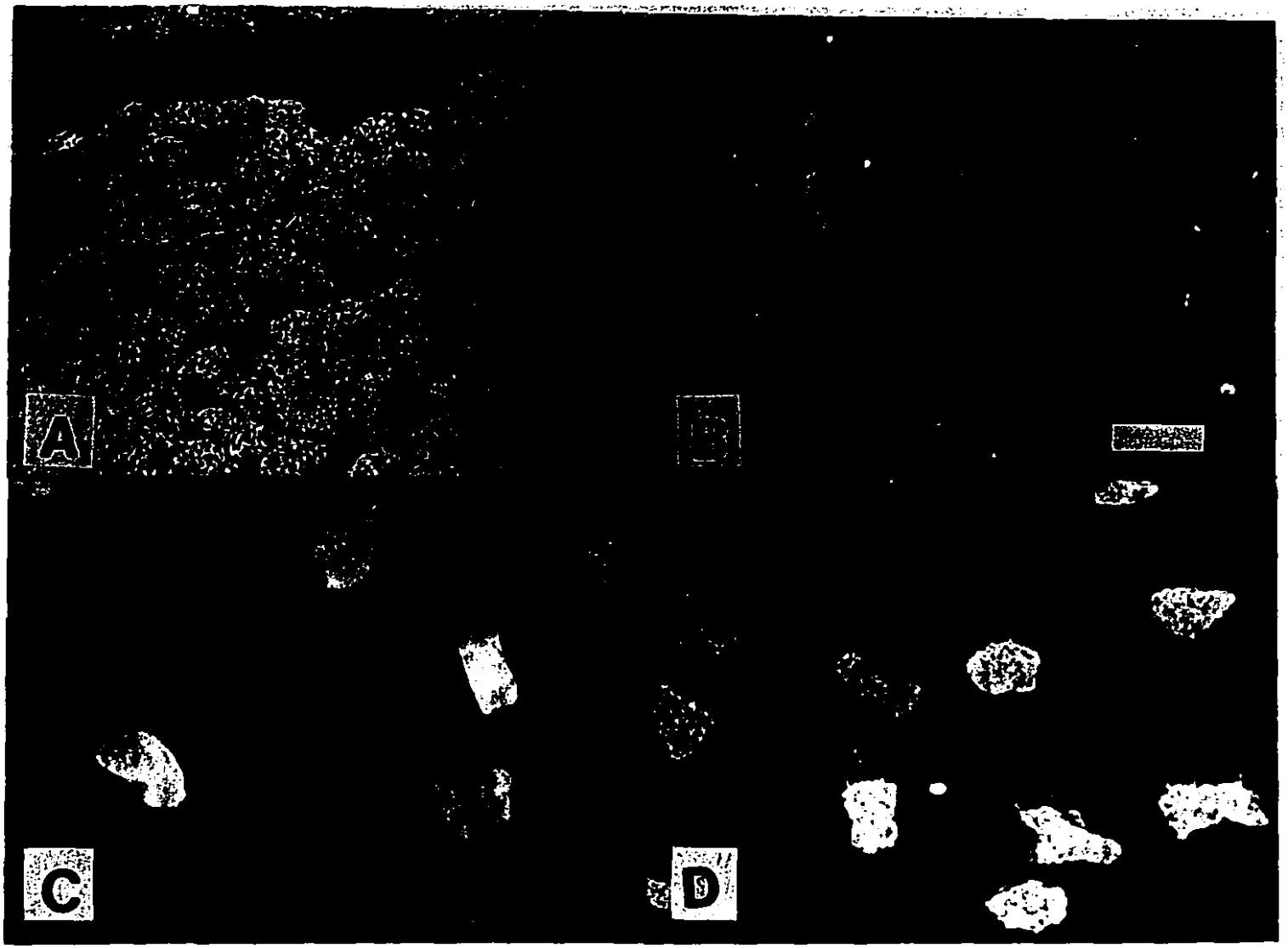

A
B
C
D

Figure 7. Immunofluorescence staining of serial sections of 4 d innervated (a-c) and denervated (d-f) regenerating rat soleus muscle, using antibodies to β (a, d), embryonic/neonatal (b, e), or neonatal/fast (c, f) MHC. Scale bar =100 μm.

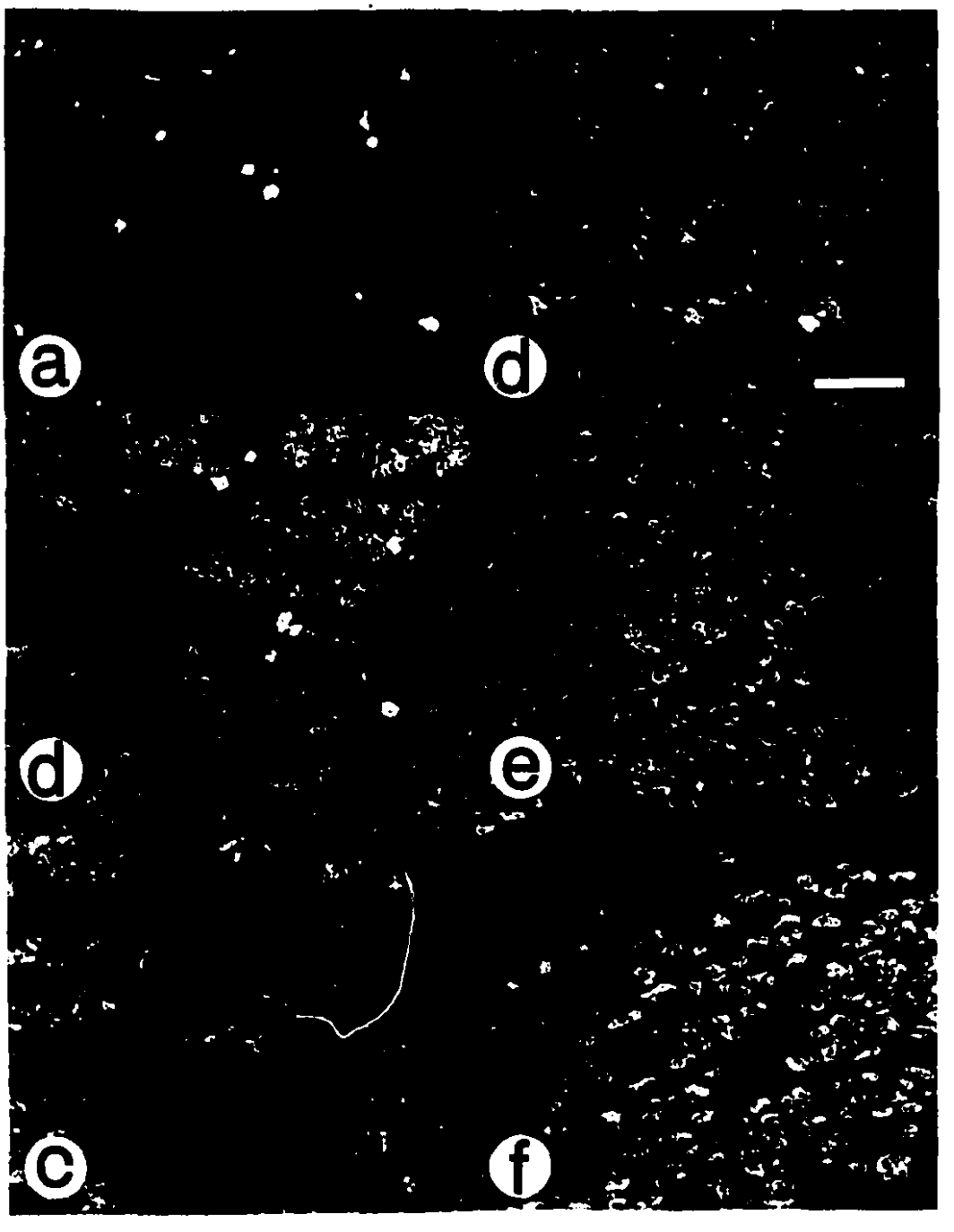

a
d
d
e
c
f

Figure 8. Immunofluorescence staining of serial sections of 7 d (a-c) and 21 d (d-f) innervated regenerating rat soleus, using antibodies to β (a, d), embryonic/neonatal (b, e), and neonatal/fast (c, f) MHC. Scale bar =100 μm.

a
b
c
d
e
f

Figure 9. Immunofluorescence staining of serial sections of 7 d (a-c) and 21 d (d-f) denervated regenerating rat soleus. The antibodies used stained for β (a, d), embryonic/neonatal (b, e), and neonatal/fast (c, f) MHC. Scale bar =100 μm.

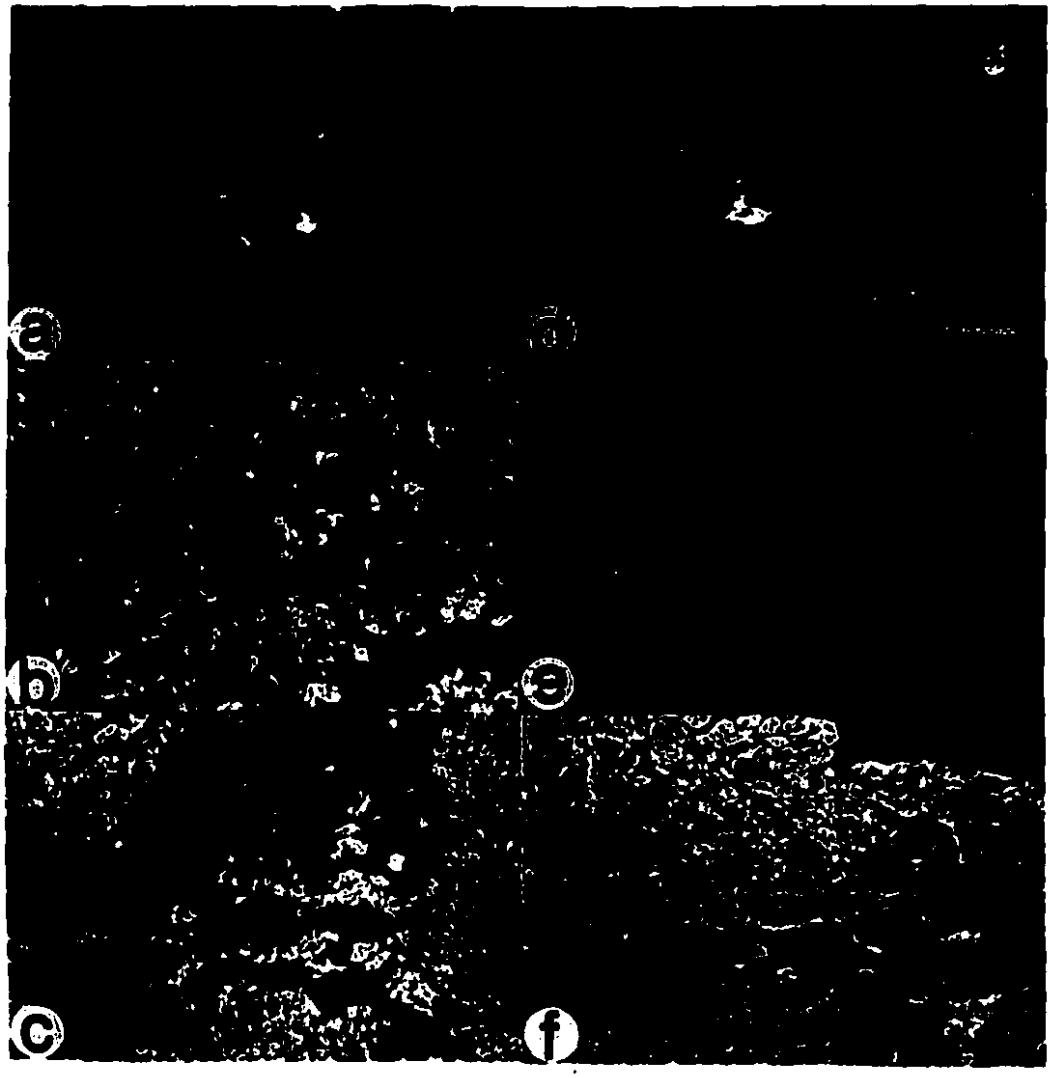

a
b
c
d
e
f

Figure 10. Immunofluorescence staining of serial sections of 7 d hyperthyroid regenerating rat soleus, using antibodies to β (a), embryonic/neonatal (b), IIA (c), and neonatal/fast (d) MHC. Scale bar =100 μm.

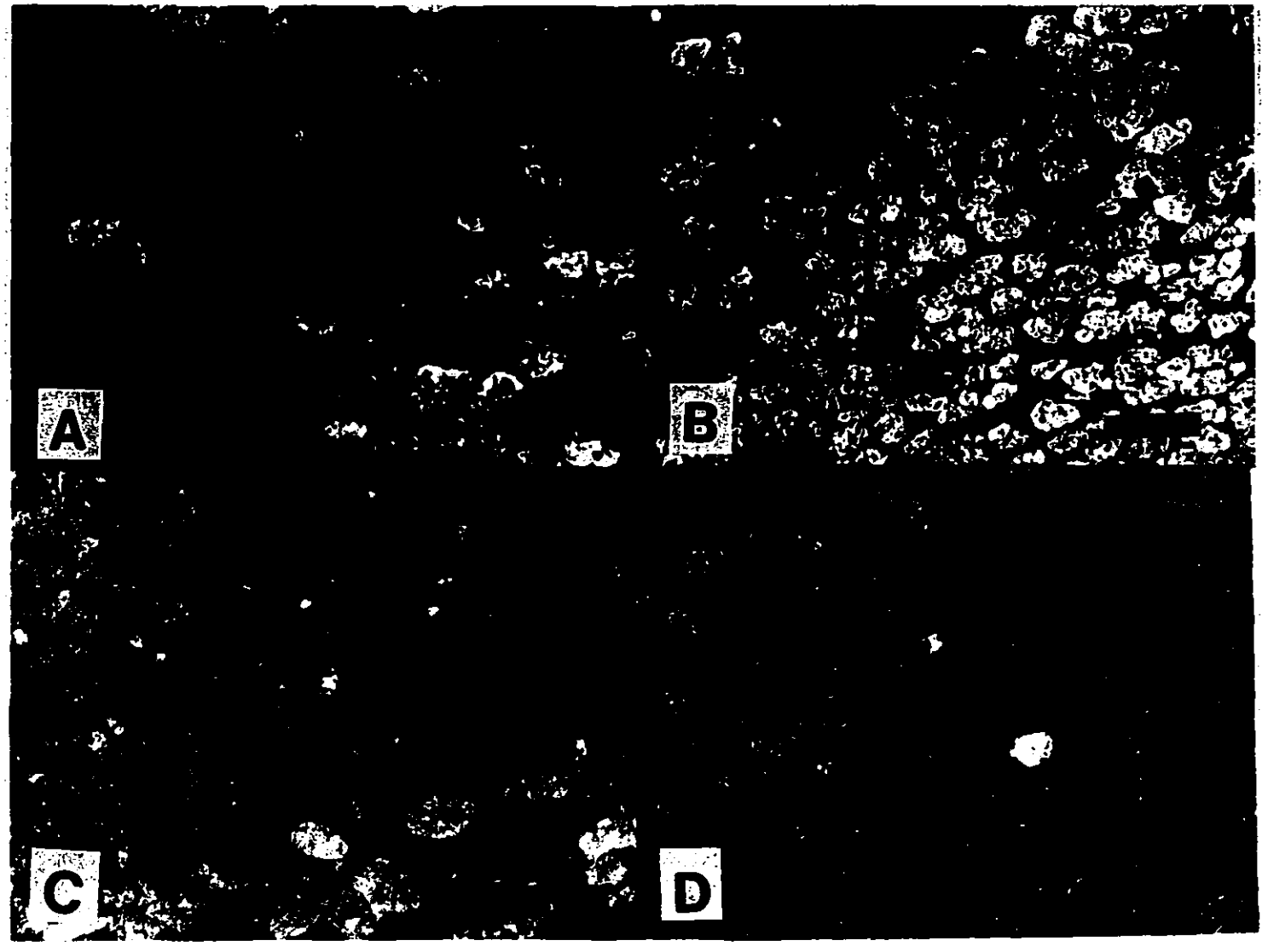

A
B
C
D

Figure 11. Immunofluorescence staining of serial sections of 21 d hyperthyroid regenerating rat soleus, using antibodies to β (a), embryonic/neonatal (b), IIA (c), and neonatal/fast (d) MHC. Scale bar =100 μm.

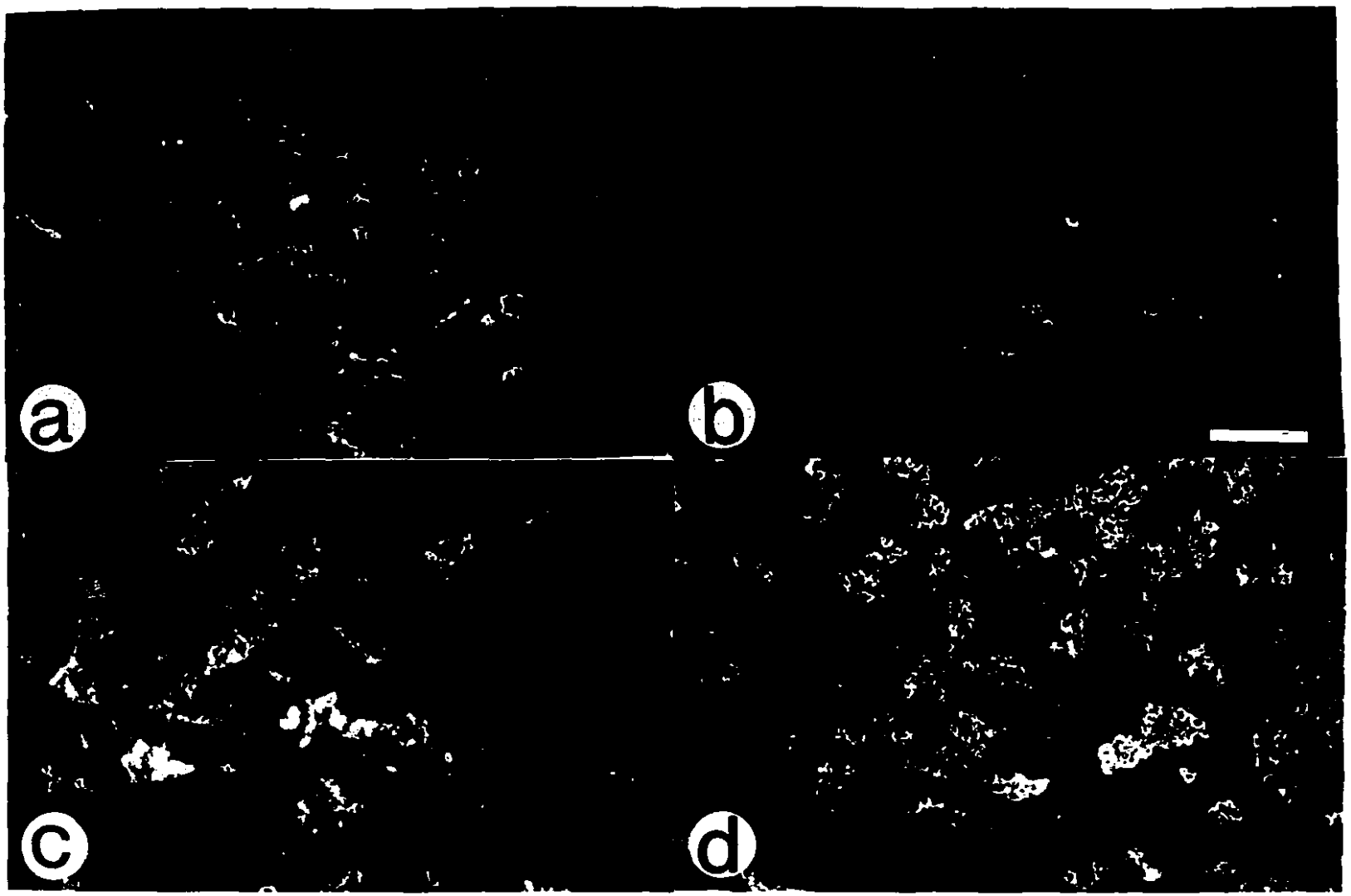

a
b
c
d

Figure 12. Detection of embryonic/neonatal MHC in 7 d (a), 14 d (b), and 21 d (c) innervated (a-c) and hyperthyroid (d-f) regenerating rat soleus. Scale bar =100 μm.

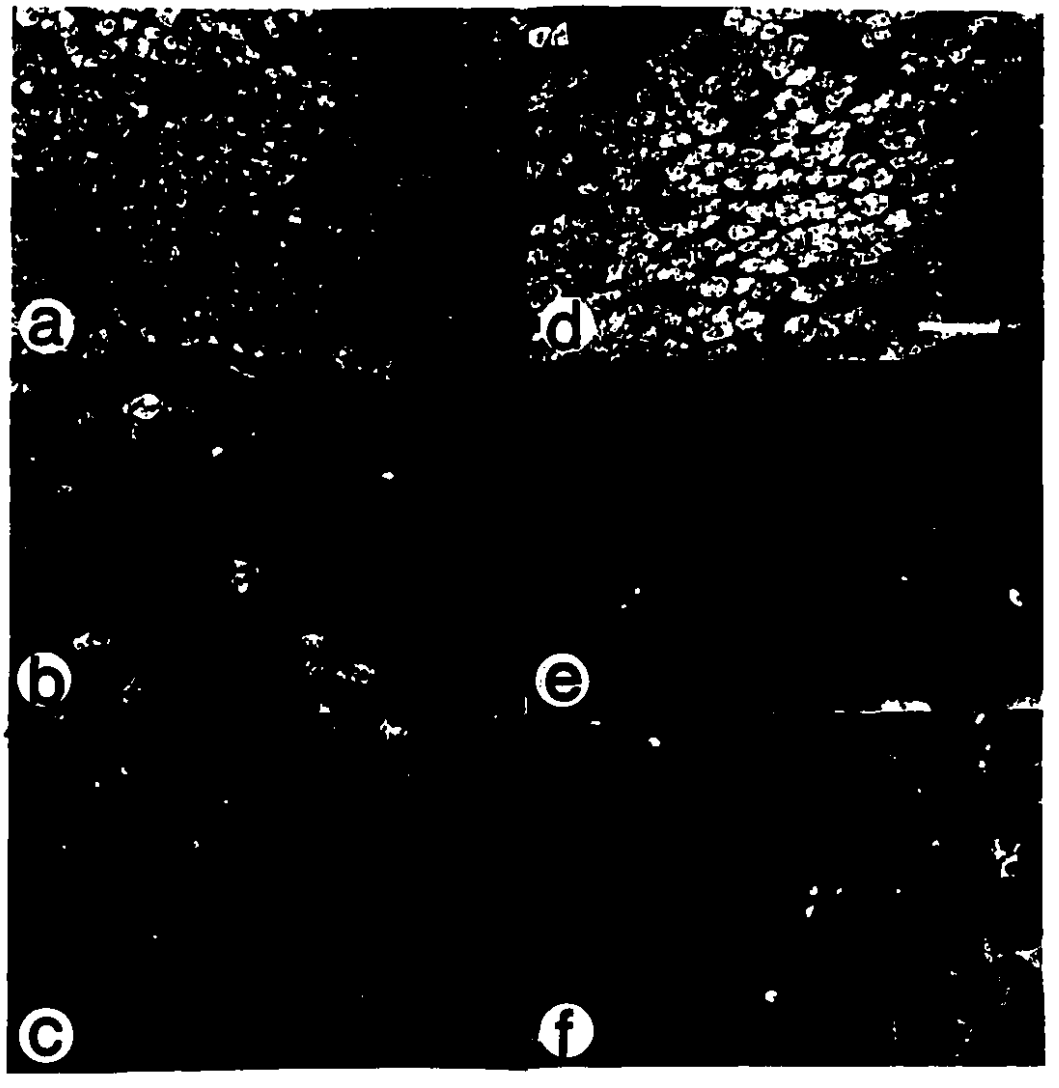

a
d
b
e
c
f

Figure 13. Immunoflurescence staining of serial sections of 7 d hyperthyroid/denervated regenerating rat soleus, using antibodies to β (a), embryonic/neonatal (b), IIA (c), and neonatal/fast (d) MHC. Scale bar =100 μm.

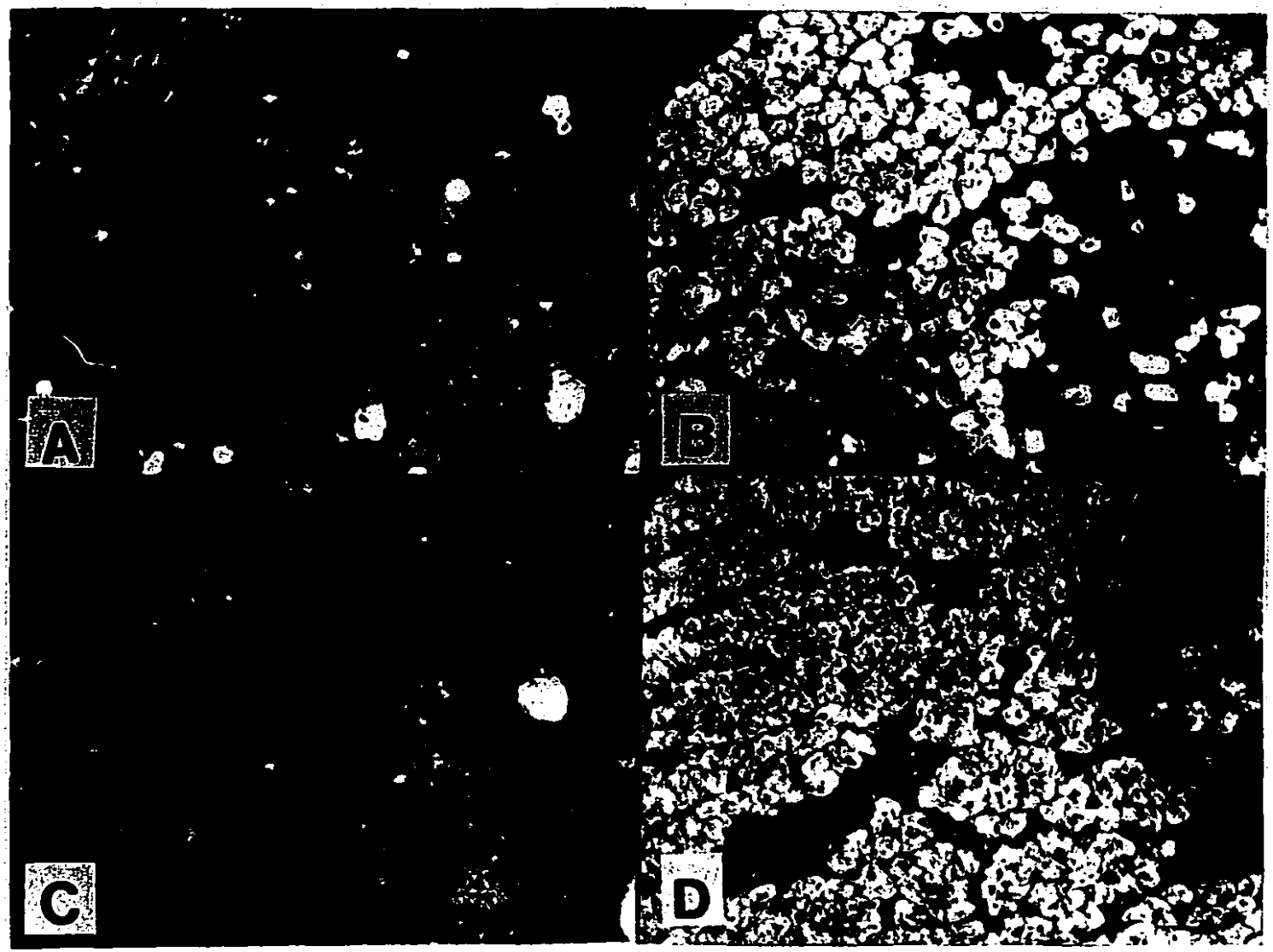

A
B
C
D

Figure 14. Immunofluorescence staining of serial sections of 14 d hyperthyroid/denervated regenerating rat soleus, using antibodies to β (a), embryonic/neonatal (b), IIA (c), and neonatal/fast (d) MHC. Scale bar =100 μm.

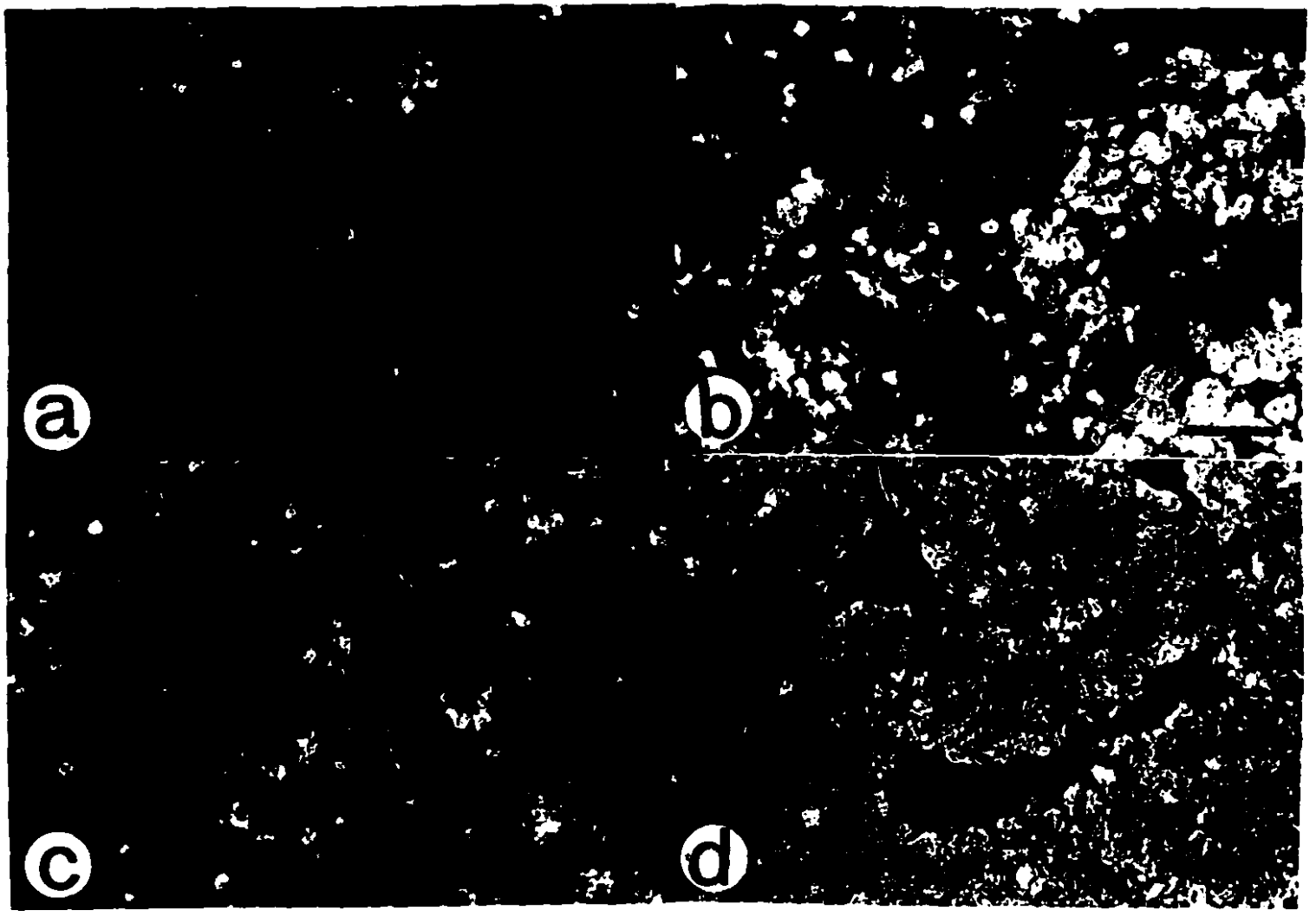

a
b
c
d

Figure 15. Immunofluorescence staining of 14 d innervated (a), denervated (b), hyperthyroid (c), and hyperthyroid/denervated (d) regenerating rat soleus, using an antibody to embryonic/-neonatal MHC. Scale bar =100 μm.

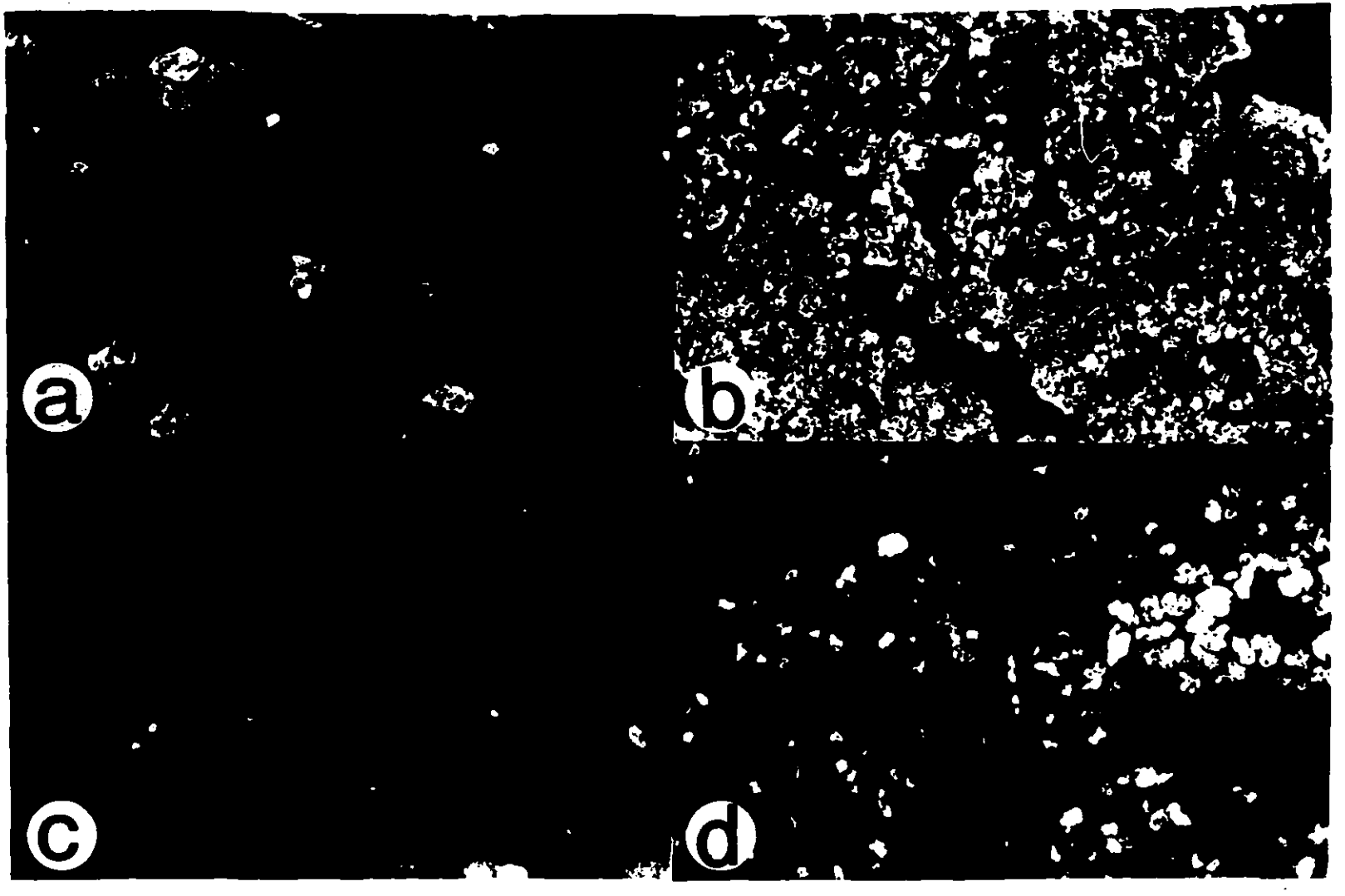

a
b
c
d

Figure 16. Immunofluorescence staining of 14 d innervated (a), denervated (b), hyperthyroid (c), and hyperthyroid/denervated (d) regenerating rat soleus, using an antibody to β MHC. Scale bar =100 μm.

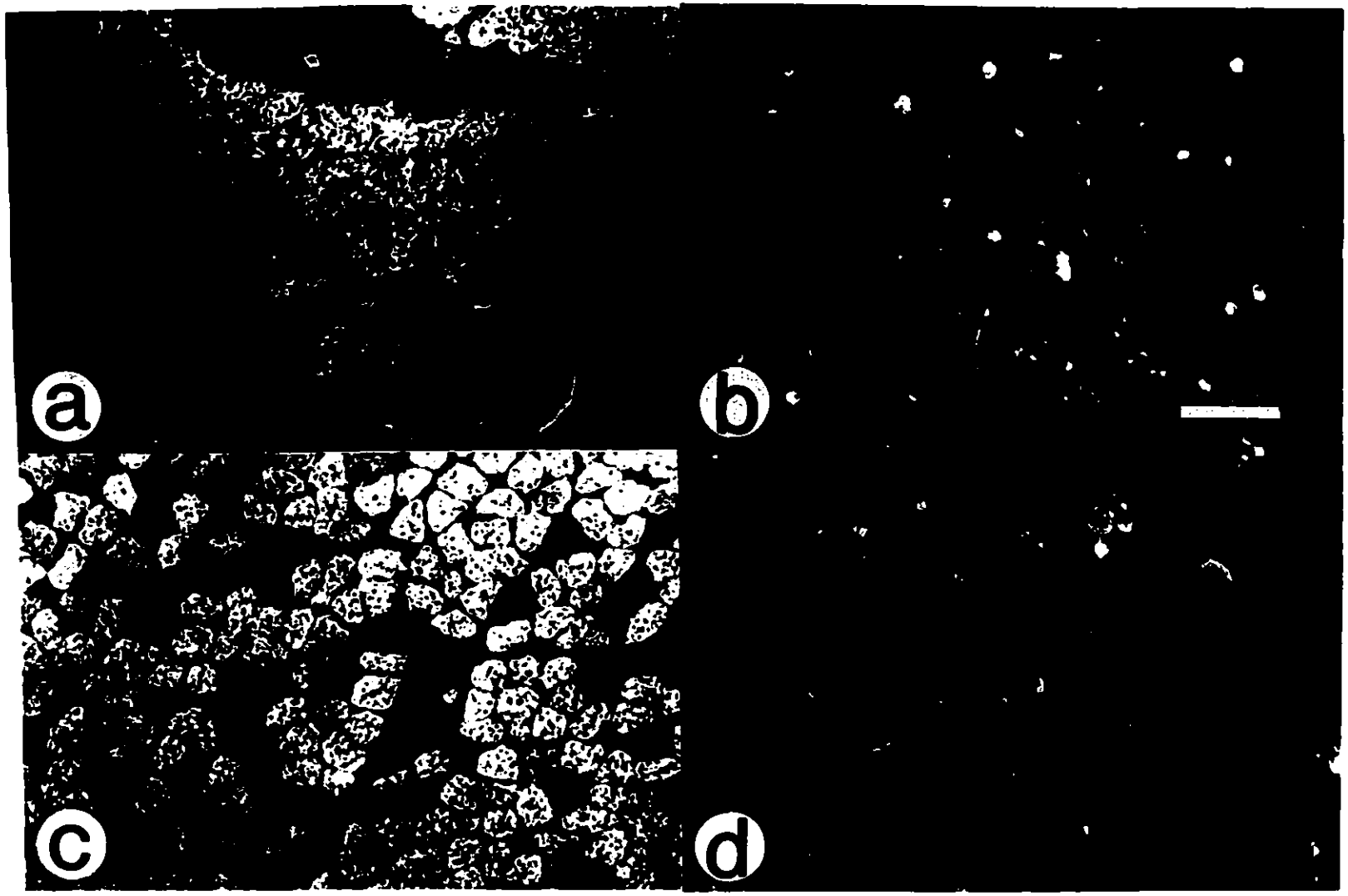

a
b
c
d

Figure 17. Immunofluorescence staining of 14 d innervated (a), denervated (b), hyperthyroid (c), and hyperthyroid/denervated (d) regenerating rat soleus, using an antibody to neonatal/fast MHC. Scale bar =100 μm.

EXPRESSION OF FAST MHC IN 14d REGENERATED MUSCLES

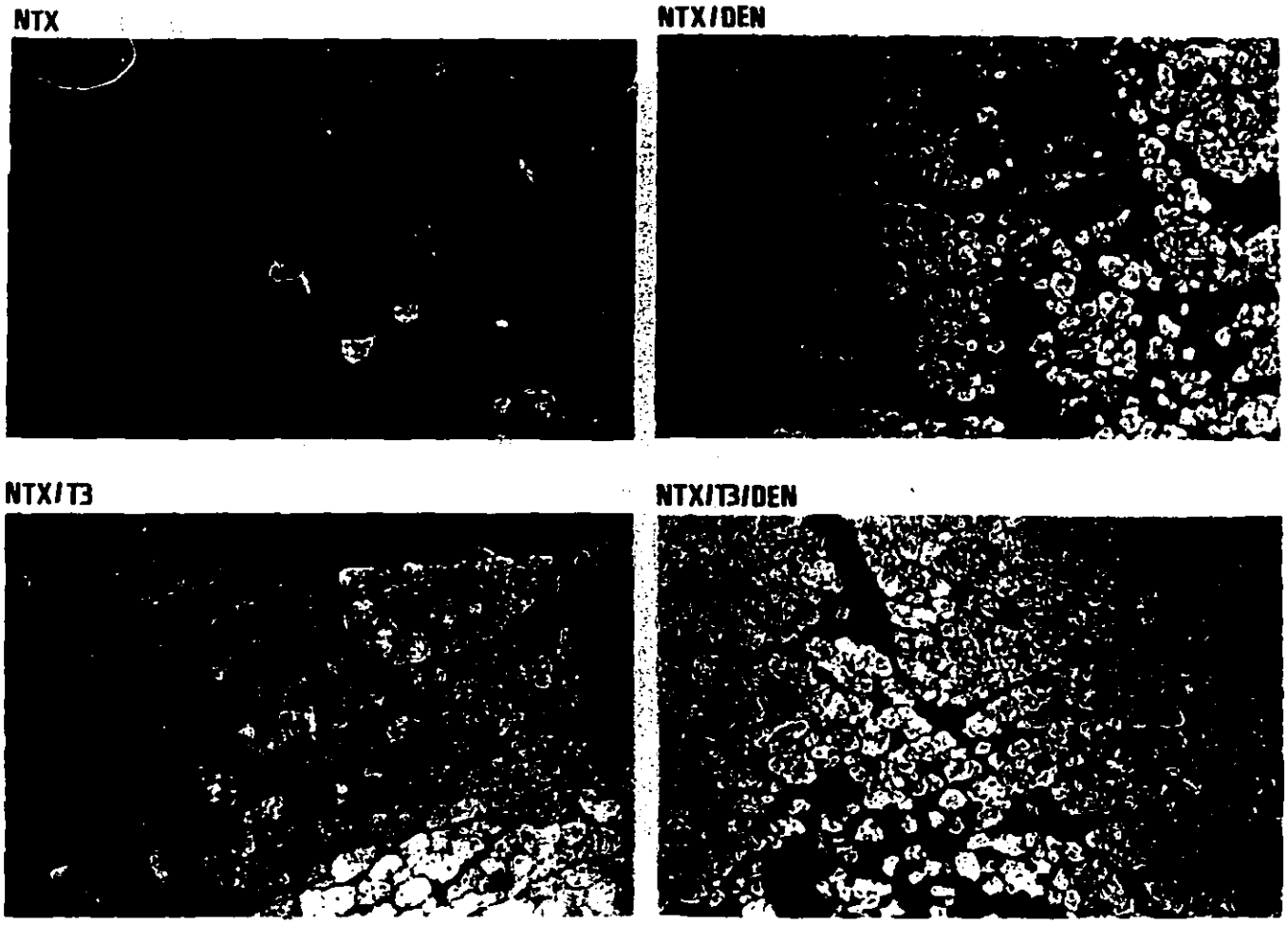


Figure 18. Immunofluorescence staining of 21 d innervated (a), denervated (b), and hyperthyroid (c) regenerating rat soleus, using an antibody to β MHC. Scale bar =100 μm.

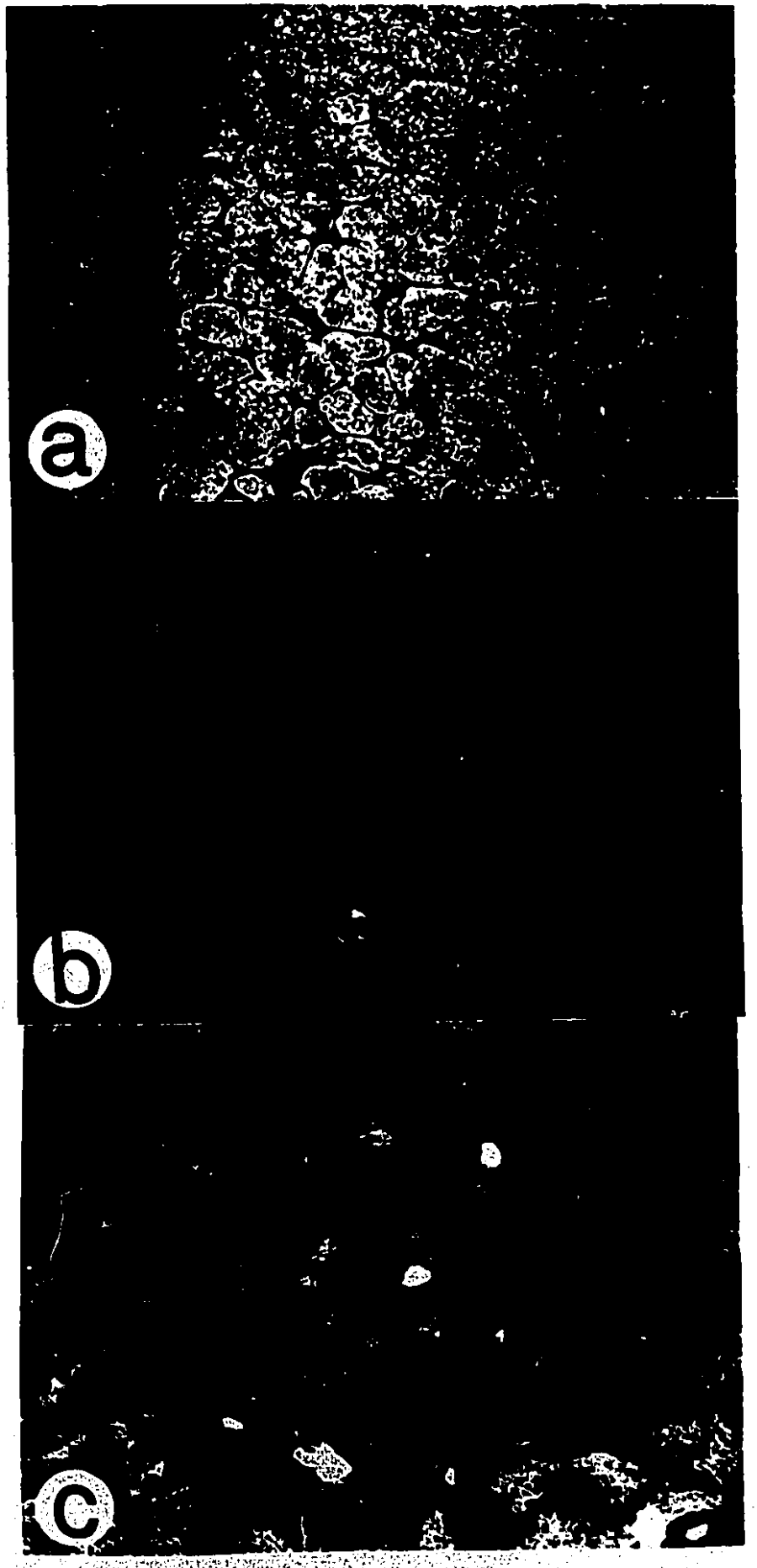

a
b
c

Figure 19. Immunofluorescence staining of 7 d (a), 14 d (b), and 21 d (c) innervated regenerating rat soleus, using an antibody to IIA MHC. The contralateral soleus to the 21 d regenerating soleus is shown in (d). Scale bar =100 μm.

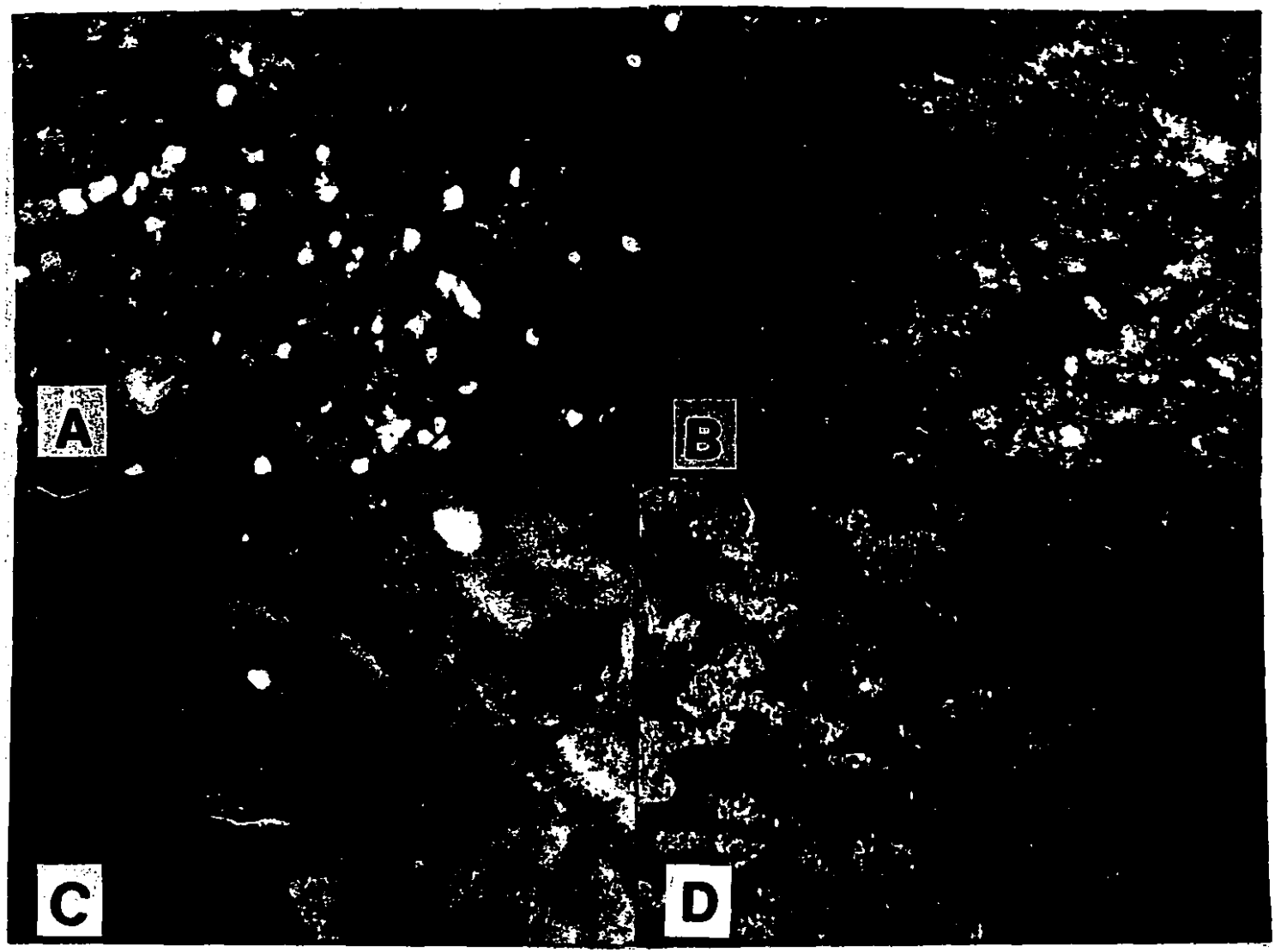

A
B
C
D

Figure 20. Immunofluorescence staining of 4d (a), 7 d (b), 14 d (c), and 21 d (d) denervated regenerating rat soleus, using an antibody to IIA MHC. Scale bar =100 μm.

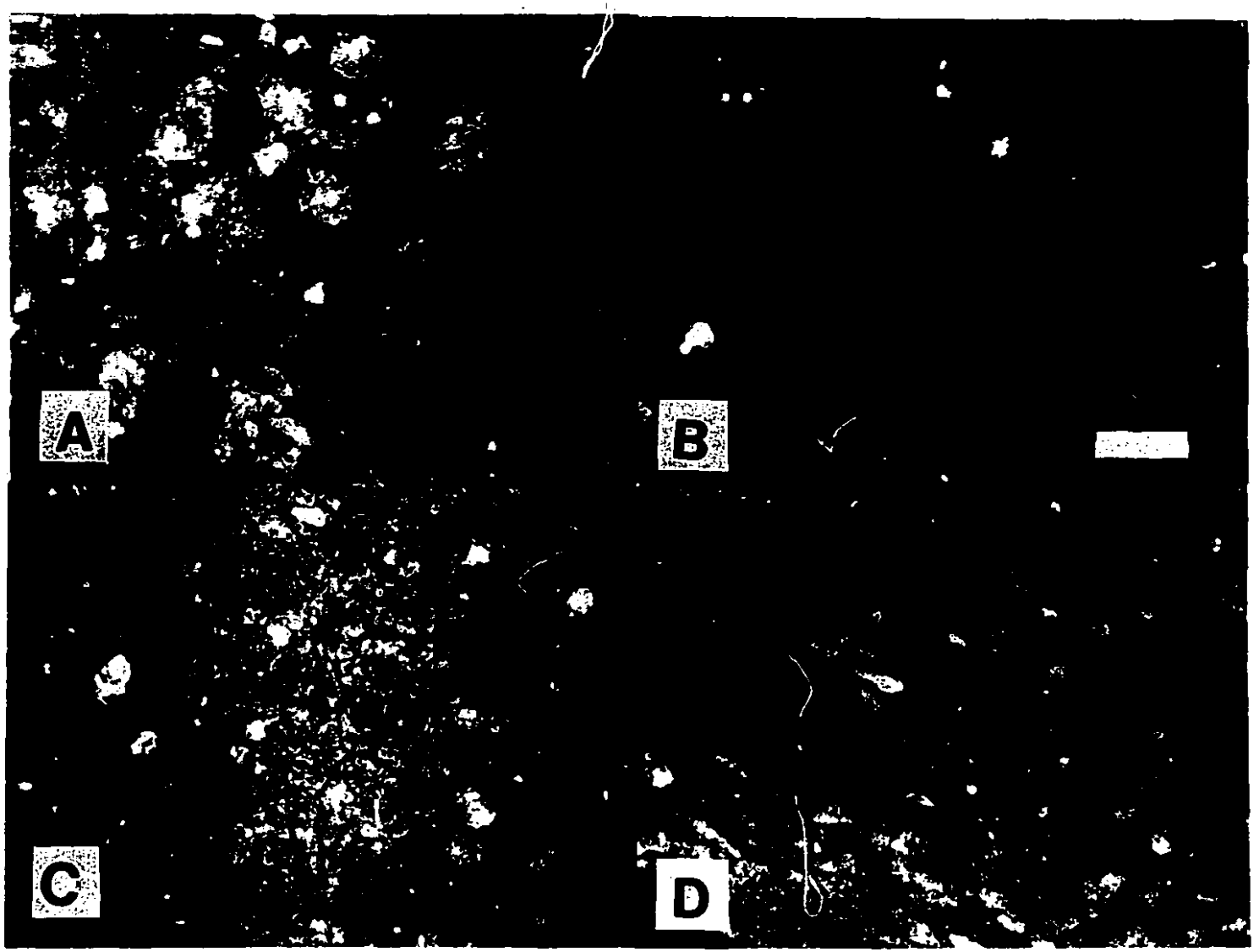

A
B
C
D

Figure 21. Immunofluorescence staining of 7 d (a), 14 d (b), and 21 d (c) hyperthyroid regenerating rat soleus, using an antibody to IIA MHC. Scale bar =100 μm.

a
b
c

III. DETECTION OF MHC mRNA IN REGENERATING MUSCLE

The presence of mRNA for the various isoforms of MHC was detected by PCR using primers which allowed amplification of an isoform-specific portion of the MHC sequence. This was confirmed since a single band at the expected size was seen when each PCR product was electrophoresed on a gel. The individual isoforms of MHC mRNA which were detected using these primers were: neonatal, β, IIB, IIA, IIX and α-cardiac.

For the purpose of this thesis, the MdM and IIX isoforms of MHC will be considered to be the same and results obtained using PCR primers which were based on the published mouse MdM MHC gene sequence will be attributed to IIX MHC.

A study was conducted to detect the presence of IIX MHC mRNA in different muscles. In all muscles analysed, except cardiac muscle, a single band at the expected size of 230 bp was seen (Fig. 22). Hybridization of a transfer blot from this gel, with a probe internal to the IIX MHC PCR primers, resulted in bands similar to those seen on the gel for the IIX MHC amplification (Fig. 22). No bands were seen in the samples where the β, IIA, IIB and neonatal MHC cDNA had been amplified, indicating that this probe did not hybridize to other PCR amplified products which represent other MHC isoforms.

Results of the presence of mRNA for the various isoforms of MHC in the different experimental groups are outlined in Table 11 and briefly summarized below.

A. Innervated Regenerating Soleus

Messenger RNA for all isoforms of MHC except α-cardiac were detected by PCR in innervated regenerating soleus. Those for neonatal, β and IIX MHC were present by 4 d, and those for IIA and IIB MHC by 7 d (Fig. 23-26). All persisted to at least 21 d except neonatal MHC mRNA, which had disappeared by 14 d. The β isoform was the strongest and increased with time, whereas the IIB isoform was the weakest.

B. Denervated Regenerating Soleus

The types of MHC mRNA in denervated regenerates differed from those in innervated regenerates in several ways: (i) the neonatal isoform persisted beyond 14 d, to at least 21 d, (ii) β MHC mRNA disappeared between 4 d and 7 d (Fig. 27), (iii) IIB MHC mRNA, which was also first detected at 7 d, continued to increase with time and (iv) IIA MHC mRNA was first detected at 14 d instead of 7 d (Fig. 24).

C. Hyperthyroid Regenerating Soleus

The presence of a hyperthyroid state changed the accumulation of mRNA for the MHC isoforms. It differed from innervated regenerates in the following ways: (i) the intensity of the band for neonatal MHC mRNA at 7 d was weaker in hyperthyroid regenerates, (ii) the intensity of the bands for β and IIB MHC mRNAs was stronger in the hyperthyroid regenerates and (iii) a faint band was detected at 14 d with the α-cardiac primers in the hyperthyroid regenerating soleus (Fig. 24-26).

D. Hyperthyroid/Denervated Regenerating Soleus

The types of MHC isoforms detected in denervated soleus regenerating in a hyperthyroid state differed from those in innervated regenerates in the following ways: (i) neonatal MHC mRNA persisted to at least 14 d at which time point it was not detected in innervated regenerates, (ii) β MHC mRNA was detected, but the intensity of its band was less in hyperthyroid/ denervated regenerates, (iii) IIB MHC mRNA was not detected until 14 d and (iv) the IIX MHC mRNA was detected at 7 d but not at 14 d (Fig. 28).

Table 11: Detection of mRNA Transcripts Coding for MHC Isoforms in Regenerating Rat Soleus Muscles as Determined by PCR

Probe	Muscle Treatment[1]	Time Post-Toxin[2] (Days) 4	7	14	21
Neo MHC	NTX	+	+	-	-
	NDEN	+	+	+	+
	NTH	nd	+/-	-	-
	NTD	nd	+	+	nd
β MHC	NTX	+	+	++	+++
	NDEN	+	-	-	-
	NTH	nd	+++	+++	+++
	NTD	nd	+	+	nd
IIB MHC	NTX	-	+/-	+	+
	NDEN	-	+	+	++
	NTH	nd	++	++	++
	NTD	nd	-	+	nd
IIA MHC	NTX	-	++	+	++
	NDEN	-	-	++	++
	NTH	nd	+	++	++
	NTD	nd	++	++	nd
MdM/IIX MHC	NTX	+	++	++	++
	NDEN	+	++	++	++
	NTH	nd	++	++	++
	NTD	nd	++	-	nd
α MHC	NTX	-	-	-	-
	NDEN	-	-	-	-
	NTH	nd	-	+/-	-
	NTD	nd	-	-	nd

1 NTX = notexin-treated (regenerating)
NDEN = denervated regenerates
NTH = hyperthyroid regenerates
NTD = hyperthyroid/denervated regenerates

2 + = a distinct band was detected,
++ more intense band detected
+++ = the most intense band detected
- = no band was detected
+/- = faint band detected
nd = not determined

Figure 22. Detection of IIX MHC mRNA in rat muscle. PCR amplification was conducted using primers chosen to detect mRNAs for IIX MHC from cDNA prepared from rat soleus (1), EDL (2), TA (3), plantaris (4), diaphragm (5), masseter (6), and heart (7). The expected size of IIX MHC mRNA band was 230 bp. Other lanes contain (9)-amplified product of soleus cDNA and β MHC primers (199 bp); (10)-soleus cDNA and IIA MHC primers (302 bp); (11)-gastrocnemius cDNA and IIB MHC primers (220 bp); (12)-neonatal cDNA and neonatal primers (271 bp); Lane 8 is the standard marker pBR322. Below the PCR gel is the result of the hybridization of the transfer of the gel with a IIX probe chosen from the sequence internal to the PCR primers.

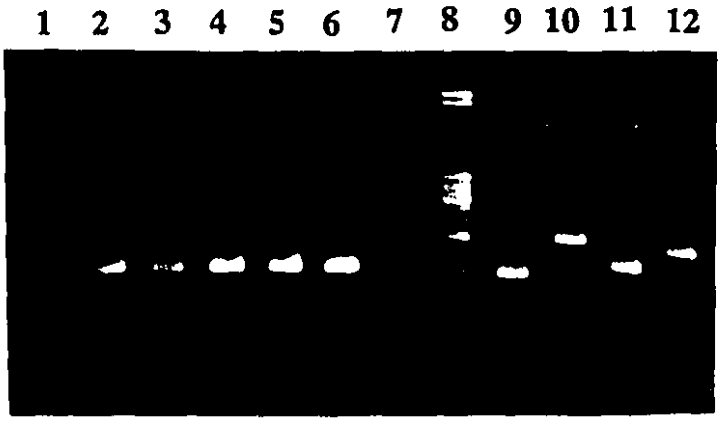

1 2 3 4 5 6 7 8 9 10 11 12

Figure 23. Detection of IIX MHC mRNA in regenerating rat soleus, using PCR. The results are shown in order from left to right: innervated (NTX: 7 d, 14 d, and 21 d), denervated (NDEN: 7 d, 14 d, and 21 d), hyperthyroid (NTH: 7 d, 14 d, and 21 d), and hyperthyroid/denervated (7 d and 14 d) regenerating soleus. Primers were chosen to detect the mRNAs for IIX MHC (230 bp). The control (ct) amplification used the same IIX primers and cDNA prepared from mouse hindlimb muscle. The standard marker (M) was pBR322.

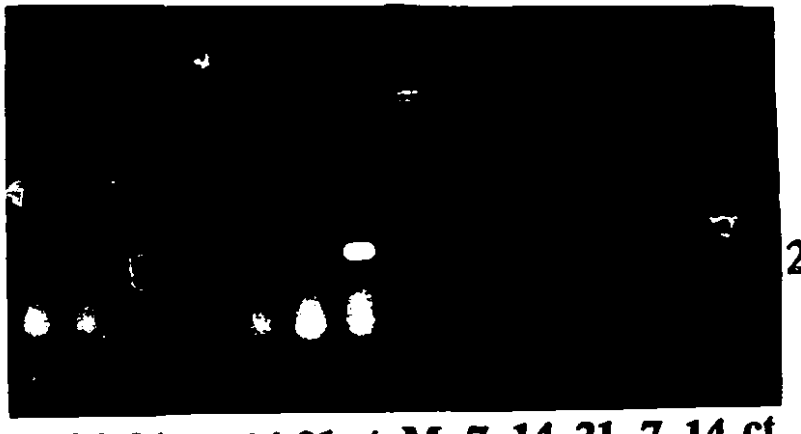
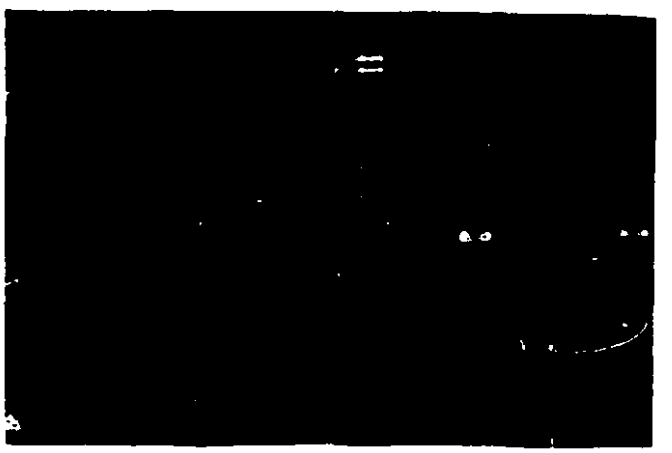

230 bp
7 14 21 7 14 21 ct M 7 14 21 7 14 ct
NTX NDEN NTH NTD

Figure 24. Detection of IIA MHC mRNA in regenerating rat muscle, using PCR. Complementary DNA was prepared from innervated (NTX), denervated (NDEN), hyperthyroid (NTH), and hyperthyroid/denervated (NTD) regenerating soleus. Primers were chosen to detect mRNAs for IIA MHC (302 bp). Results are presented for the different time points analysed (7 d, 14 d, and 21 d) in each group (except NTD for which there is no 21 d group) and for the contralateral muscle (c) of the latest time point in regeneration analysed. The control (ct) amplification used cDNA made from adult soleus.

NTX

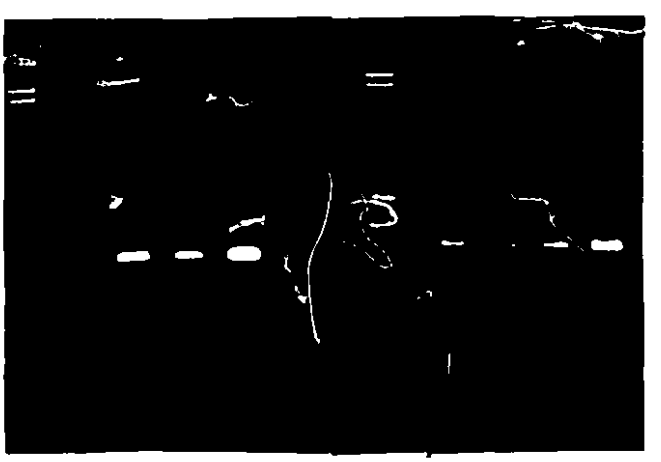


NDEN

302 bp

M 7 14 21 c ct M 7 14 21 c ct

NTH



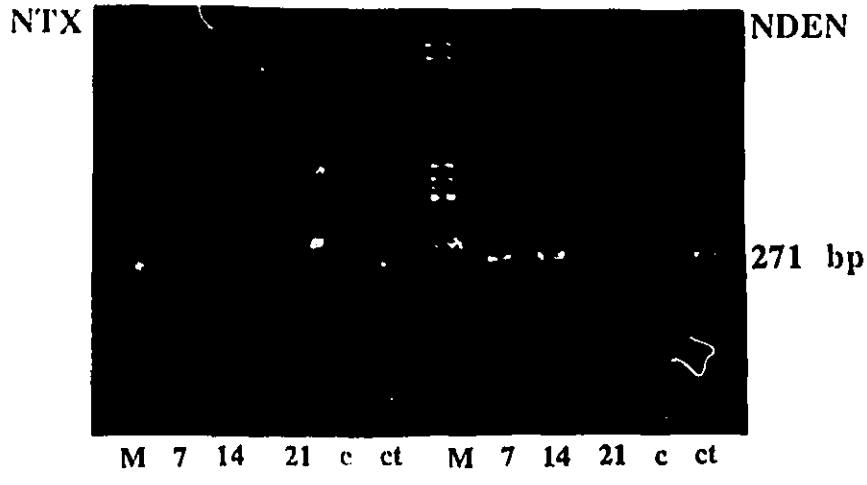
NTD

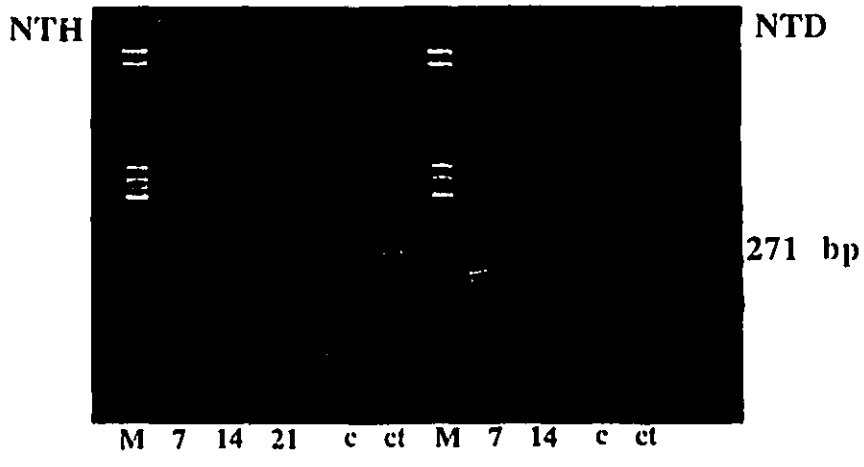
302 bp

M 7 14 21 c ct M 7 14 c ct

Days

Figure 25. Detection of neonatal MHC mRNA in regenerating rat muscle, using PCR. Complementary DNA was prepared from innervated (NTX), denervated (NDEN), hyperthyroid (NTH), and hyperthyroid/denervated (NTD) regenerating soleus. Primers were chosen to detect mRNAs for neonatal MHC (271 bp). Results are presented for the different time points analysed (7 d, 14 d, and 21 d) in each group (except NTD for which there is no 21 d group) and for the contralateral muscle (c) of the latest time point in regeneration analysed. The control (ct) amplification used cDNA made from rat neonatal bulk hindlimb muscle.


NTX
NDEN
271 bp
M 7 14 21 c ct M 7 14 21 c ct


NTH
NTD
271 bp
M 7 14 21 c ct M 7 14 c ct

Days

Figure 26. Detection of β MHC mRNA in regenerating rat muscle, using PCR. Complementary DNA was prepared from innervated (NTX), denervated (NDEN), hyperthyroid (NTH), and hyperthyroid/denervated (NTD) regenerating soleus. Primers were chosen to detect mRNAs for β MHC (199 bp). Results are presented for the different time points analysed (7 d, 14 d, and 21 d) in each group (except NTD for which there is no 21 d group) and for the contralateral muscle (c) of the latest time point in regeneration analysed. The control (ct) amplification used cDNA from adult soleus.

NTX

NDEN

199 bp

M 7 14 21 c ct M 7 14 21 c ct

NTH

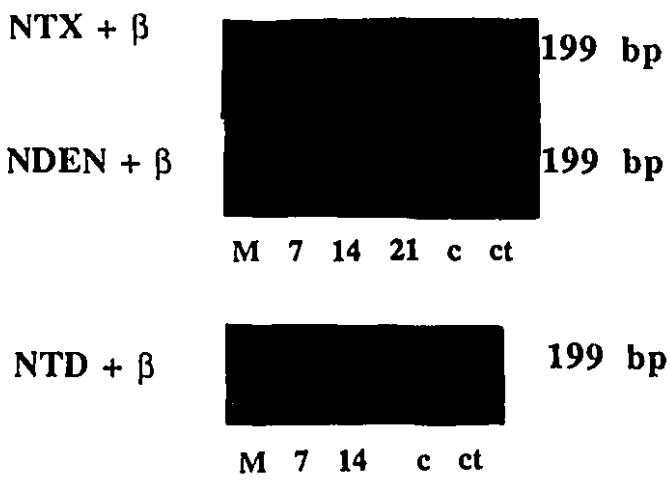
NTD

199 bp

M 7 14 21 c ct M 7 14 c ct

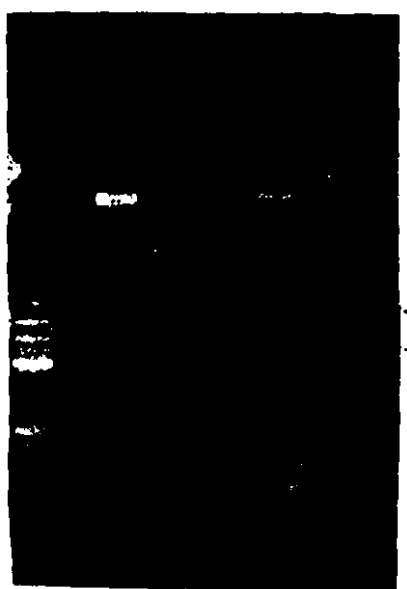
Days

Figure 27. The effect of innervation on the presence of β MHC mRNA. PCR amplification was conducted using primers chosen to detect mRNAs for β MHC (199 bp) and cDNA prepared from innervated (NTX: 7 d, 14 d, 21 d), denervated (NDEN: 7 d, 14 d, 21 d), and hyperthyroid/denervated (NTD: 7 d, 14 d) regenerating rat soleus. The control (ct) amplification used the same β MHC primers and cDNA from adult soleus. PCR amplification of the same primers and cDNA prepared from the contralateral muscle (c) of the latest time point is also shown. The standard marker (M) was pBR322.



Days

Fig. 28. Detection of IIX and IIB MHC mRNA in hyperthyroid/denervated regenerating rat soleus, using PCR. The amplified products of the IIX and IIB MHC mRNAs are shown for 7 d and 14 d hyperthyroid/denervated regenerate (NTD), the contralateral muscle to the 14 d hyperthyroid/denervated regenerates (c), and the normal control rat soleus. The standard marker (M) was pBR322.



Days

IV. SPECIFICITY OF SLOT BLOT PROBES

A. Specificity of Probes for α-Cardiac and α-Skeletal Actin as Determined by Slot Blot Hybridization

When a slot blot containing RNA samples from adult cardiac or adult or neonatal hindlimb muscles was hybridized with probes to α-cardiac and α-skeletal actin mRNA to determine their specificity, the probe for α-cardiac actin mRNA hybridized only to cardiac RNA whereas that for α-skeletal actin mRNA hybridized only to the skeletal muscle RNA (Fig. 29). This confirmed that the α-cardiac and α-skeletal actin probes were specific to the isoform of α-actin mRNA in cardiac and skeletal muscle respectively.

B. Specificity of MHC Probes as Determined by Northern Hybridization

The specificity of the probes for β, IIA and IIB MHC mRNA, which were used for slot blot hybridizations, was confirmed by hybridization to a membrane containing RNA from different muscle samples known to contain a predominance of one of the isoforms. The probe for β MHC mRNA hybridized strongly with RNA from soleus and weakly with RNA from diaphragm and

gastrocnemius, and not at all with that from tibialis anterior (TA) and heart (Fig. 30a). The IIB probe hybridized strongly with RNA from diaphragm, tibialis anterior and gastrocnemius muscles, but not with RNA from soleus, neonatal or heart muscle (Fig. 30b). The IIA probe hybridized with RNA from diaphragm, tibialis anterior and gastrocnemius muscles, but not with RNA from soleus or heart muscle (Fig. 30c). Therefore, each of these probes exhibited the pattern of hybridization predicted from the known pattern of protein accumulation.

An attempt was made to make a specific probe for neonatal MHC but two separate probes made from the 3' non-coding sequence failed to bind to any of the RNA samples, including those from two separate samples of neonatal muscle. Thus, there was no probe capable of detecting neonatal MHC mRNA.

Figure 29. Slot blot analysis of α-cardiac and α-skeletal actin probe specificity. A slot blot was made containing RNA samples from soleus (SOL), extensor digitorum longus (EDL), tibialis anterior (TA), plantaris (PLN), masseter (MASS), diaphragm (DIAP), heart or gastrocnemius (GAST) muscles of 30 d rat, or neonatal rat bulk hindlimb (NEO) muscle. A sample of RNA prepared from hepatoma cells (HEP) served as a control. The blot was hybridized to probes designed to detect α-cardiac actin mRNA (left) and α-skeletal actin mRNA (right).

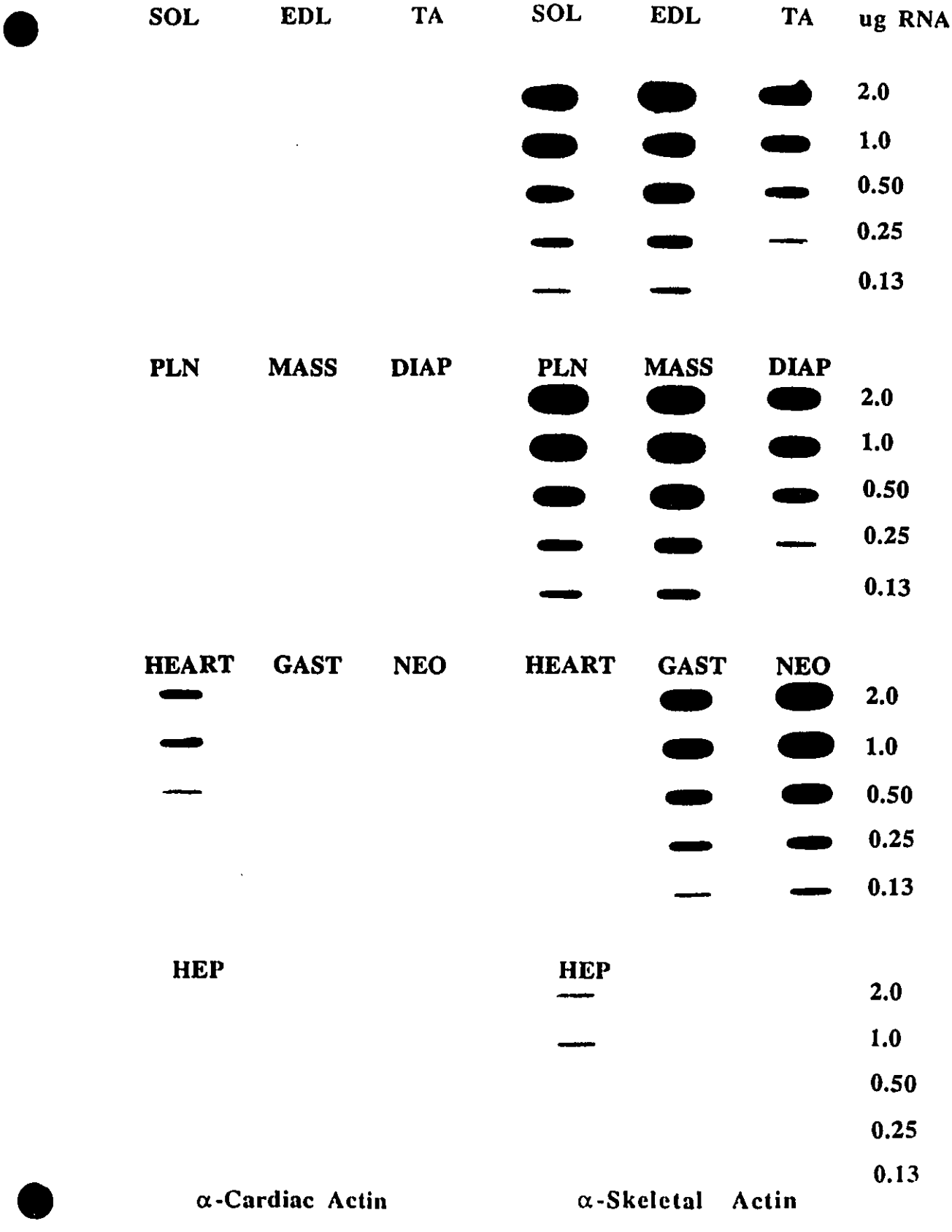
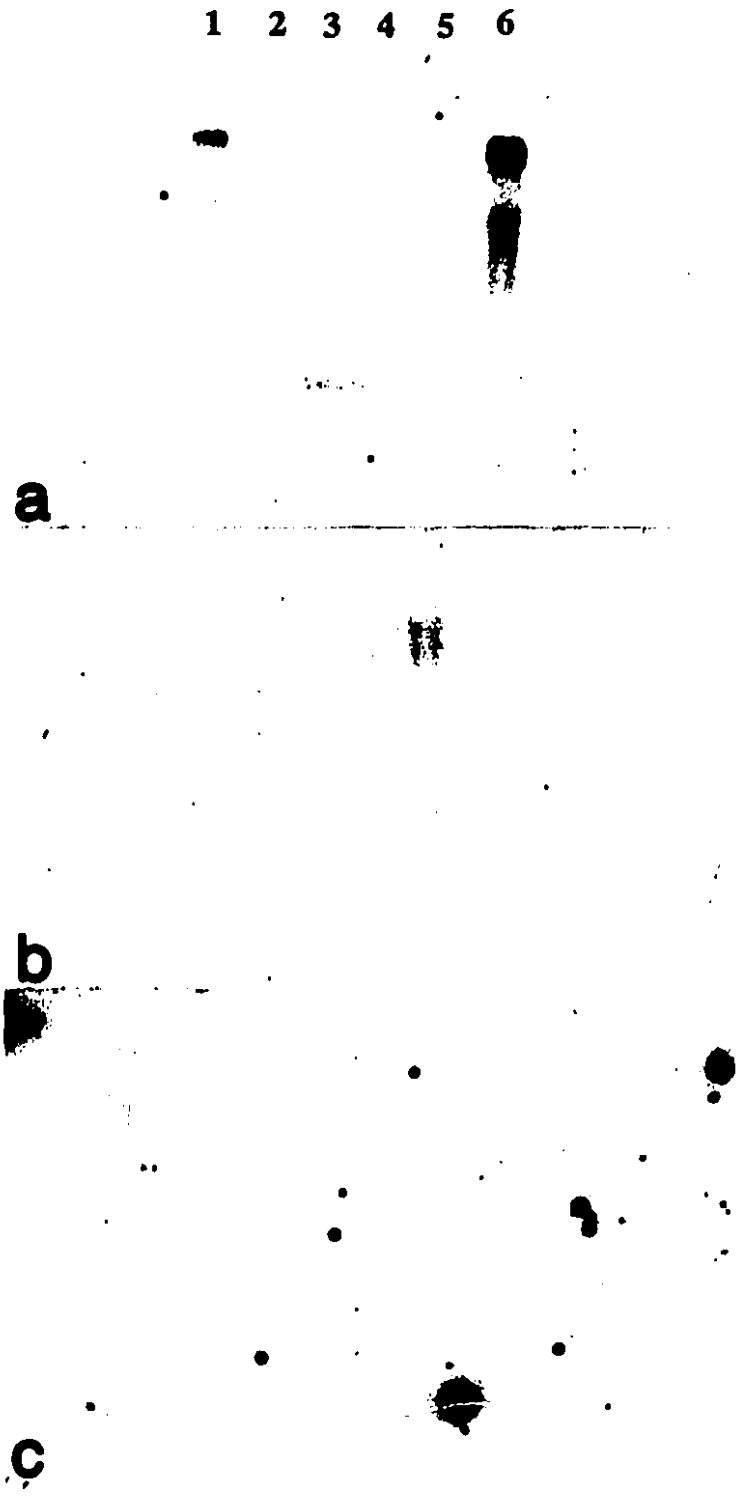

SOL
EDL
TA
SOL
EDL
TA
ug RNA
2.0
1.0
0.50
0.25
0.13
PLN
MASS
DIAP
PLN
MASS
DIAP
2.0
1.0
0.50
0.25
0.13
HEART
GAST
NEO
HEART
GAST
NEO
2.0
1.0
0.50
0.25
0.13
HEP
HEP
2.0
1.0
0.50
0.25
0.13
α-Cardiac Actin
α-Skeletal Actin

Figure 30. Northern blot analysis of MHC probe specificity. A Northern blot containing RNA made from diaphragm (lanes 1 a-c), TA (lanes 2 a-c), neonatal muscle (lanes 3 a-c), heart (lanes 4 a-c), gastrocnemius (lanes 5 a-c), and soleus (lanes 6 a-c), was hybridized to probes designed to detect β (a), IIB (b), and IIA (c) MHC mRNA.


1 2 3 4 5 6
a
b
c

V. QUANTITATIVE ANALYSIS OF α-SKELETAL AND α-CARDIAC ACTIN mRNA AND MHC mRNA IN REGENERATING MUSCLE

A. Validity of Pooling Muscles for Preparing RNA

In all of the experiments involving analysis of mRNA content, total RNA was prepared from muscle samples pooled from several rats. Therefore, before drawing conclusions from such data, it was necessary to prove that all the muscles treated in a given manner regenerated similarly with regards to the expression of α-skeletal actin and MHC. This was carried out by preparing RNA from individual solei muscles taken 21 d after toxin injection. A slot blot membrane containing serial dilutions of the individual 21 d post-toxin injected RNA samples was hybridized first to a (γ^{32}P)ATP-labelled α-skeletal actin mRNA probe and then to a (γ^{32}P)ATP-labelled β MHC mRNA probe. The content of each of these mRNAs in each of the muscle samples was determined by measuring the β radiation emitted per μg total RNA. There were no statistically significant differences, as determined using a two-way analysis of variance (ANOVA), in the content of α-skeletal actin mRNA or β MHC mRNA in the different 21 d post-toxin injected samples ($p<0.05$) (Table 12).

Table 12: Alpha-Skeletal Actin and β MHC mRNA Content in Individual 21 d Innervated Regenerating Solei

Muscle	Probe	
	α-skeletal actin mRNA (10^5 cts/μg total RNA)	β MHC mRNA (10^5 cts/μg total RNA)
1	10.0	2.9
2	9.3	2.0
3	9.4	2.2
4	12	2.2
5	12	2.1
6	12	2.0
Mean ± SD	10.0± 1.8	2.2± 0.3

B. Expression of α-Cardiac and α-Skeletal Actin mRNA in Regenerating Muscle

Alpha-skeletal actin mRNA was detected in solei muscles of all experimental groups and at all time points evaluated, however, not in the same quantities (Fig. 31). Denervated muscles, whether or not the hyperthyroid state was present, had less α-skeletal actin mRNA than innervated regenerates. The greatest amounts of α-skeletal actin mRNA were found in hyperthyroid regenerates at times later than 7 d.

Messenger RNA for α-cardiac actin was detected in very small amounts in 4 d innervated and denervated regenerates and 7 d hyperthyroid/denervated regenerates. When the values for α-cardiac actin and α-skeletal actin mRNA were added to determine total α-actin mRNA, the contribution of α-cardiac actin was found to be statistically insignificant using a two-tailed t-test ($p<0.01$) (Table 13). Therefore, all values for quantity of MHC (cts/μg total RNA) were normalized to α-skeletal actin mRNA only.

Table 13: Alpha-Skeletal Actin mRNA and α-Cardiac Actin mRNA Content in Early Stages of Regenerating Muscle

Group	α-Skeletal Actin mRNA[1]	α-Cardiac Actin mRNA[1]	Total α-Actin mRNA[1]	Significance[2]
4 d NTX	66.0	1.2	67.0	$p< 0.01$
4 d NTX/DEN	9.0	0.5	9.4	$p<0.01$
7 d NTD	37.0	1.4	38.0	$p<0.01$

1 Each value represents the content of α-skeletal or-cardiac actin in 10^4 cts/μg total RNA.

2 The values for total α-actin (i.e. the sum of the values of α-cardiac and α-skeletal actin) were compared to those of α-skeletal actin alone using a two-tailed t-test ($p<0.01$).

C. Expression of MHC mRNA in Regenerating Muscle

RNA slot blots were hybridized with probes designed to detect mRNA for β, IIB, IIA and IIX MHC. The IIX MHC probe did not bind to any of the regenerate samples. The amount in counts/μg total RNA for each of the MHC isoforms in each of the experimental groups before normalization (a-d) and after normalization (e-h) are presented in Fig. 32. Examples of results after hybridizing the slot blot with probes designed to detect β (Fig. 33) and IIA (Fig. 34) MHC mRNA, correspond to the non-normalized data in Fig. 32 (a-d).

1. Innervated Regenerating Soleus

β MHC mRNA was detected at all time points in the innervated regenerates (Fig. 32e and 33). IIA and IIB MHC mRNAs were first detected at 7 d and continued to be present to at least 21 d, although in smaller amounts than β MHC mRNA.

2. Denervated Regenerating Soleus

In denervated regenerating solei there was a greater content of the fast MHC mRNAs (especially IIA) than in innervated regenerates (Fig. 32f). The amounts of IIB MHC mRNA in denervated regenerates increased with time and by 21 d was greater than that in innervated regenerates, although IIA MHC mRNA was still present in a greater quantity. No β MHC

mRNA was detected in denervated regenerating soleus muscles (Fig. 32g and 33).

3. Hyperthyroid Regenerating Soleus

The hyperthyroid regenerating soleus revealed a different pattern of MHC mRNA accumulation than was seen in the other regenerates. At 7 d there was more mRNA for β and IIA MHC than was found for innervated or denervated regenerates (Fig. 32f, 33 and 34). The amount of β MHC mRNA continued to increase up to 21 d, at which point it was the major form of mRNA present. On the other hand, the content of IIA MHC mRNA quickly declined after 7 d and remained at a level slightly greater than was detected in innervated regenerates.

4. Hyperthyroid/Denervated Regenerating Soleus

The hyperthyroid condition did not cause an increased amount of β MHC mRNA in denervated muscles as it had in innervated regenerates; there were no differences between quantities of β MHC in denervated and hyperthyroid/denervated regenerates at 7 d or 14 d (Fig. 33). The amounts of IIB and especially IIA MHC mRNA were greater than those found in innervated regenerating soleus at 7 d and 14 d (Fig. 32h). The accumulation of IIA MHC mRNA was slower than in hyperthyroid regenerates since the levels attained by 7 d in innervated hyperthyroid regenerates were not reached until 14 d in denervated hyperthyroid regenerates (Fig. 34). The

increased amount of IIB MHC mRNA in the hyperthyroid/denervated condition was similar to that found in the denervated regenerating soleus, but less than that found in hyperthyroid regenerates.

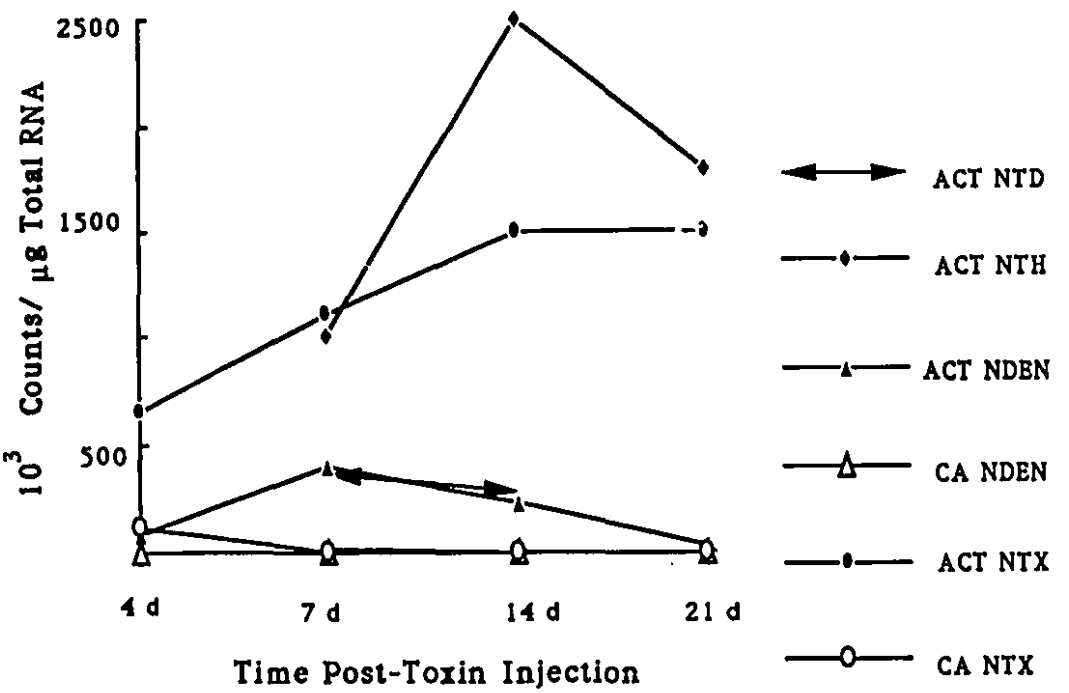
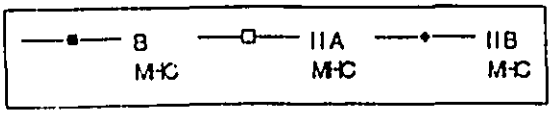


Figure 25. Content of α-cardiac (CA) and α-skeletal (ACT) actin mRNA in regenerating rat soleus which were either innervated (NTX), denervated (NDEN), hyperthyroid (NTH) or hyperthyroid/denervated (NTD). There were no results for α-cardiac actin and hyperthyroid regenerates, and the value for hyperthyroid/denervated regenerates overlaps that of innervated regenerates at 7 d, after which it decreases to zero.

Figure 32. Content of various isoforms of MHC mRNA in regenerating rat soleus by slot blot hybridization using probes designed to detect β, IIA, or IIB MHC mRNAs. Amount of mRNA expressed as 10^3 counts/μg (cts/μg) for absolute data (a-d) or data normalized to α-skeletal actin, (e-h) are shown on the y-axis. The number of days post-toxin injection is shown on the x-axis. Results are shown for innervated (a, e), hyperthyroid (b, f), denervated (c, g), and hyperthyroid/denervated (d, h) regenerating rat soleus. The different MHC mRNAs are represented by the following symbols.



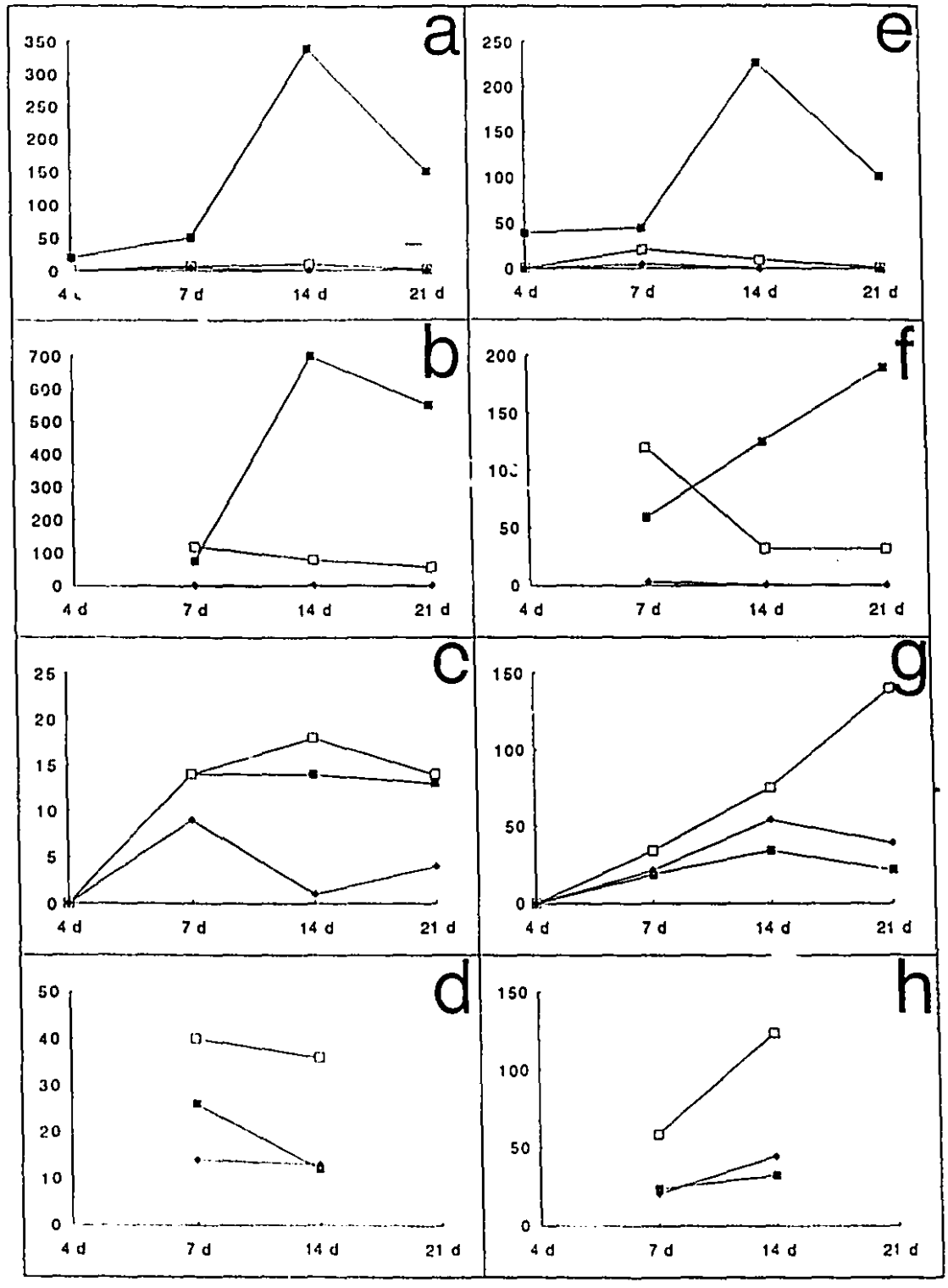

a
350
300
250
200
150
100
50
0
4 d
7 d
14 d
21 d
e
250
200
150
100
50
0
4 d
7 d
14 d
21 d
b
700
600
500
400
300
200
100
0
4 d
7 d
14 d
21 d
f
200
150
50
0
4 d
7 d
14 d
21 d
c
25
20
15
10
5
0
4 d
7 d
14 d
21 d
g
150
100
50
0
4 d
7 d
14 d
21 d
d
50
40
30
20
10
0
4 d
7 d
14 d
21 d
h
150
100
50
0
4 d
7 d
14 d
21 d

Figure 33. Content of β MHC mRNA in regenerating rat soleus. The β MHC probe was hybridized to a slot blot filter containing two-fold serial dilutions of RNA from innervated (NTX), denervated (NDEN), hyperthyroid (NTH), and hyperthyroid/ denervated (NTD) regenerating soleus. Three time points are shown: 7 d, 14 d, and 21 d post-toxin injection.

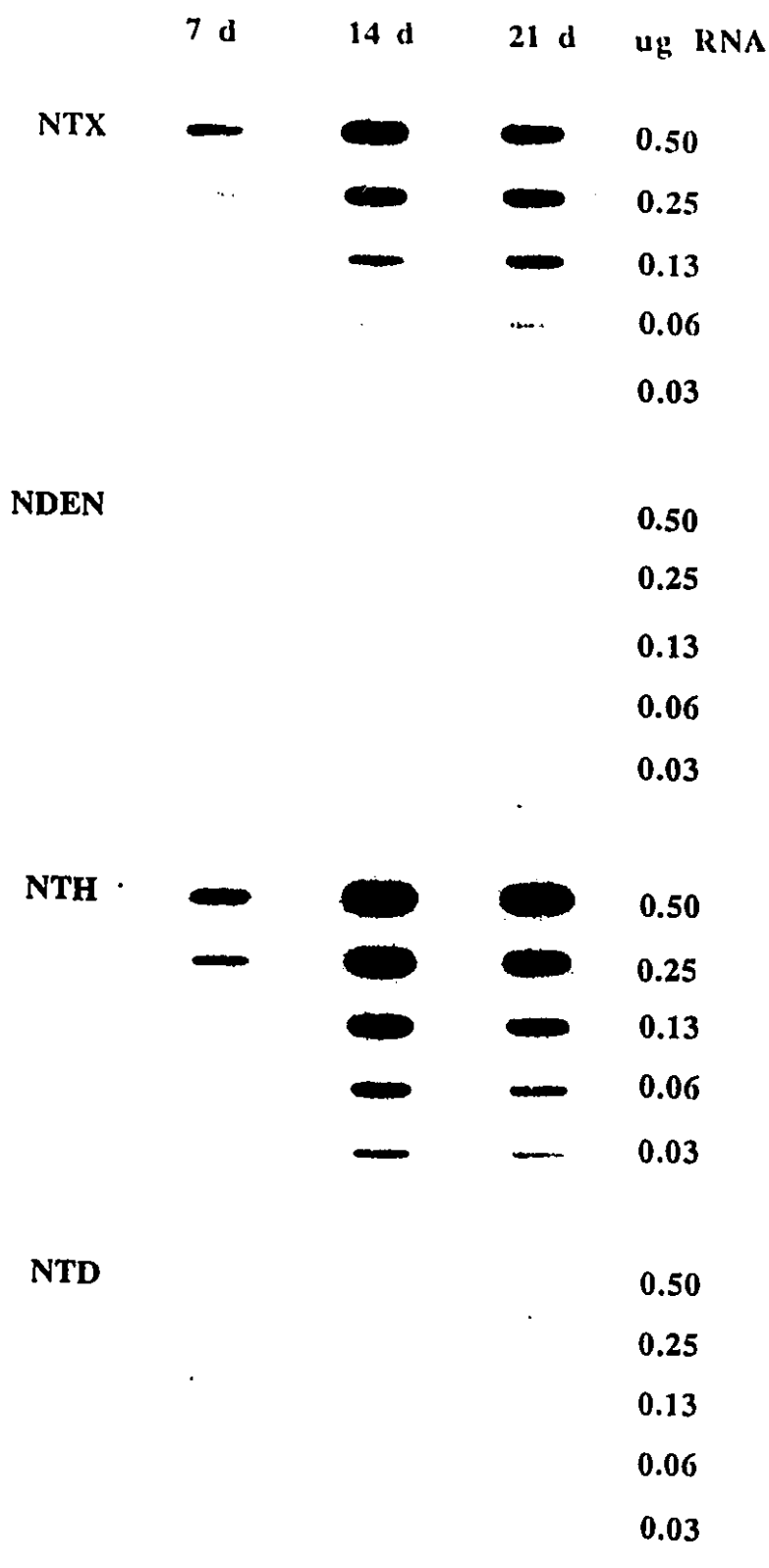
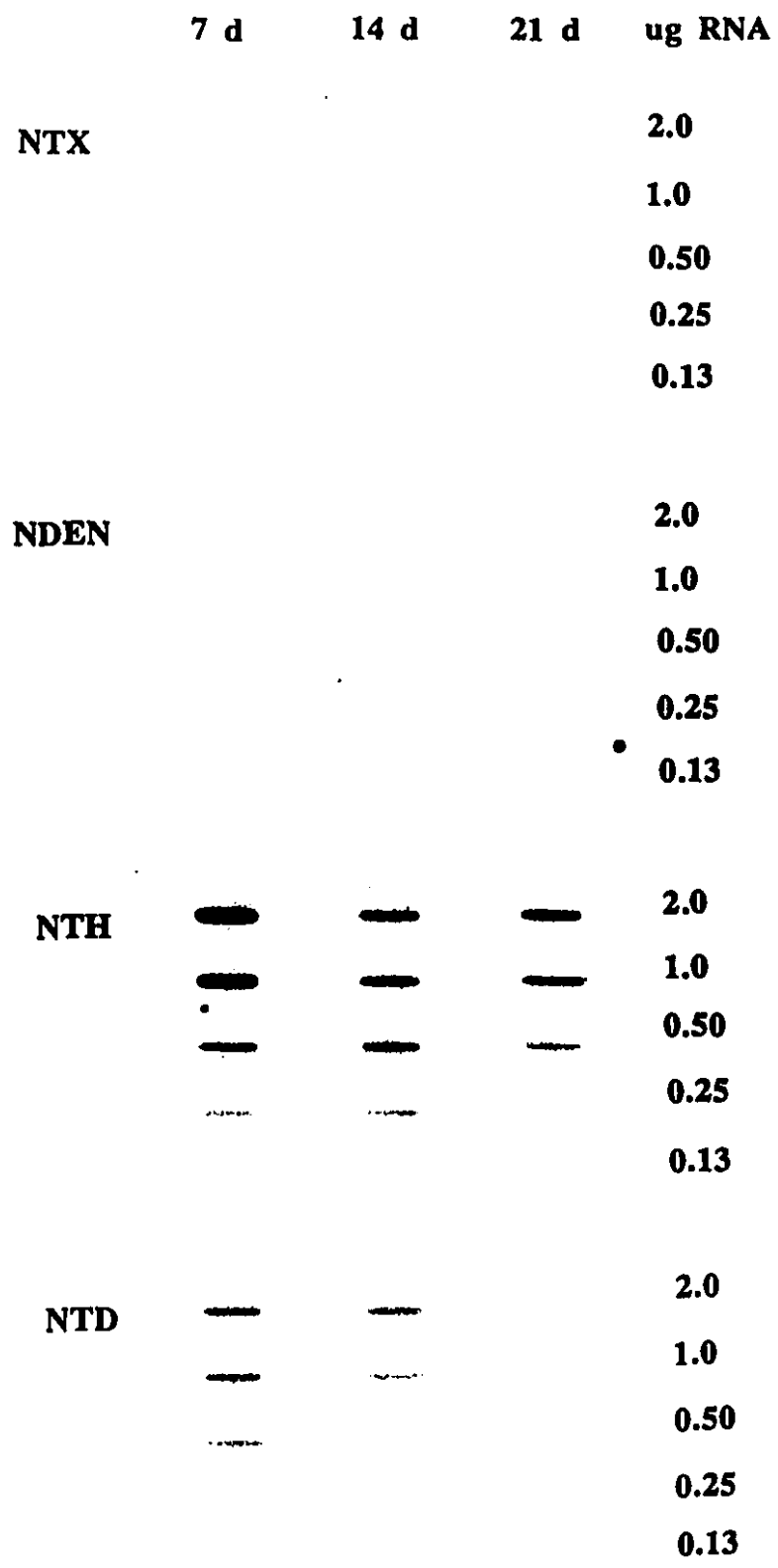

7 d
14 d
21 d
ug RNA
NTX
0.50
0.25
0.13
0.06
0.03
NDEN
0.50
0.25
0.13
0.06
0.03
NTH
0.50
0.25
0.13
0.06
0.03
NTD
0.50
0.25
0.13
0.06
0.03

Figure 34. Content of IIA MHC mRNA in regenerating rat soleus. The IIA MHC probe was hybridized to a slot blot filter containing two-fold serial dilutions of RNA from innervated (NTX), denervated (NDEN), hyperthyroid (NTH), and hyperthyroid/ denervated (NTD) regenerating soleus. Three time points are shown: 7 d, 14 d, and 21 d post-toxin injection.


7 d
14 d
21 d
ug RNA
NTX
2.0
1.0
0.50
0.25
0.13
NDEN
2.0
1.0
0.50
0.25
0.13
NTH
2.0
1.0
0.50
0.25
0.13
NTD
2.0
1.0
0.50
0.25
0.13

VI. COMPARISON OF mRNA CONTENT AND PROTEIN ACCUMULATION

The content of MHC mRNA, as determined using slot blots, and the isoform of MHC protein accumulated, as determined by immunohistochemistry, were compared for different muscles (soleus, extensor digitorum longus, tibialis anterior, plantaris, gastrocnemius, diaphragm, masseter and heart) taken from 30 d old rats.

Slot blots of RNA were hybridized with probes for α-cardiac and α-skeletal actin mRNA and β, IIA and IIB MHC mRNA. Since only α-skeletal actin mRNA (i.e. no α-cardiac actin mRNA), was detected in the various skeletal muscles, all PhosphoImager values (cts/μg total RNA) for MHC mRNA were normalized to α-skeletal actin.

In the normal soleus, the amounts of mRNA (cts/μg total RNA) and percent (%) of fibres staining for that isoform were for β MHC- 820 cts/μg total RNA and 87%, and for IIA MHC- 9.2 cts/μg total RNA and 13%. These latter values contrast with the EDL, which gave 9.9 cts/μg total RNA but had 56% of fibres staining for that isoform. The IIX MHC probe was also hybridized to this blot but it was detected at background levels (Table 14).

Table 14: Correlation of mRNA and Protein for MHC Isoforms

Muscle[1]	MHC Isoform					
	β mRNA[2] (cts/μg)	β protein (%)[3]	IIA mRNA[2] (cts/μg)	IIA protein (%)[3]	IIB mRNA[2] (cts/μg)	IIB protein (%)[3]
SOL	820	87	9.2	13	0	0
EDL	8.5	2	9.9	42	98.0	56
Mass[4]	170	0	40.0	74	9.8	7
Diaph[5]	0	14 or 30	12.0	32 or 50	0	40 or 53
Gast[6]	52	30/0	9.0	62/16	41.0	84/65

[1] All values of mRNA and protein content are from adult rat muscles. SOL= soleus, EDL= extensor digitorum longus, Mass= masseter, Diaph= diaphragm, Gast= gastrocnemius

[2] The mRNA values represent the content of a MHC mRNA isoform in counts/μg total RNA.

[3] The amount of protein represents the percent (%) of muscle fibres expressing the particular MHC isoform (Armstrong and Phelps, 1984; Pette and Staron, 1990; LaFramboise *et al.*, 1991; Parker-Thornburg *et al.*, 1992).

[4] The amount of protein represents the percent of all MHCs which are of that isoform (Bar and Pette, 1988).

[5] The range of values for proteins are from two different studies: in each case, the lower value was obtained with muscles of 30 d old rats (LaFramboise *et al.*, 1991) and the higher values from adult rats (Pette and Staron, 1990), except for IIX/IID, where it was found in 40-50% of the muscle fibres in both studies.

[6] The protein values are for the MHC content in the red/white regions of the gastrocnemius (Phelps and Armstrong, 1984).

VII. SUMMARY OF RESULTS

In summary, the most important findings of this study are:

(i) embryonic/neonatal MHC persists longer in the denervated state, indicating that innervation is required to suppress its expression.

(ii) thyroid hormone accelerates the loss of the embryonic/neonatal isoforms of MHC.

(iii) IIB and/or IIX are the major MHCs accumulated in denervated regenerates.

(iv) β MHC mRNA is initially expressed regardless of the state of innervation; however, innervation is required for continued accumulation of β MHC mRNA and protein.

(v) thyroid hormone does not act to suppress β MHC accumulation in regenerating muscle as it does in developing slow-twitch muscle (Table 2).

(vi) the hyperthyroid state causes a transition from slow (β) to fast (IIA) MHC in innervated regenerates and from IIX to IIB MHC isoforms in denervated regenerates.

(vii) Expression of IIA MHC is influenced by both thyroid hormone and innervation; the hyperthyroid state enhances whereas innervation suppresses accumulation of IIA MHC mRNA.

(viii) the regulation of MHC expression is generally at the level of transcription; however, accumulation of IIA MHC protein may also be controlled by translational or post-translational mechanisms.

DISCUSSION

I. CHOICE OF MODEL

The present project was undertaken to analyse the effects of thyroid hormone and innervation on MHC expression in regenerating muscle. The MHCs are of interest because there are several different isoforms, which are expressed in a stage- and tissue-specific manner, and which determine some of the physiological characteristics of myofibres. It is of interest to study regenerating muscle because it occurs as a result of trauma, or as a response to many of the acquired or inherited neuromuscular disorders. Indeed, in the case of some diseases such as Duchenne muscular dystrophy, severe disability results because of a failure of regeneration to occur.

A. Regenerating Muscle

Regenerating muscle was obtained by using snake venom from *Notechis scutatus scutatus*, which contains the toxin notexin, to first produce degeneration of existing mature myofibres. The notexin model of regeneration was chosen since (i) the basal lamina is left intact so it can provide a scaffold within which the regenerating muscle fibre can form; (ii) virtually all of the muscle fibres undergo degeneration, thus there is little risk that surviving mature muscle fibres will complicate interpretation of the results; (iii) the

neurovascular supply is not damaged; and (iv) the muscle cells regenerate in a homogenous manner such that all regenerating fibres will be at approximately the same stage of development at the same time (Carlson, 1973).

B. Denervated Muscle

The role of innervation on MHC expression was studied in hindlimb muscles which were denervated via sciatic neurectomy. This procedure involved the removal of a 10-15 mm section of the sciatic nerve just below the sciatic notch, resulting in the degeneration of the nerve stump distal to the denervation site. Reinnervation does not occur following such nerve resection because the regenerating nerve is unable to find its target, the muscle. Successful denervation was determined by the loss of the flexor response in the hindlimb.

C. Hyperthyroid State

The hyperthyroid condition was produced by daily intraperitoneal injections of L-T3 according to the protocols used by Russell *et al.* (1988) and Kirschbaum *et al.* (1990). T3 was used because it has higher biological activity than T4. The L-T3 form was used in preference to the D-T3 form because of the stereospecific transport system which favors the movement of L-T3 from the cytoplasm into the nucleus of the cell (Krenning and Docteur, 1986).

D. Analysis of mRNA

Messenger RNA accumulation in a given muscle sample was evaluated using PCR and slot blot hybridization. The PCR was used to determine the presence of a particular mRNA and slot blot hybridization was used to quantitate it. The PCR is more sensitive than slot blot hybridization to detect mRNA; therefore, it is useful for detecting transcripts present in small amounts. It is possible to use PCR for quantifying different transcripts using techniques such as competitive PCR which entails the addition to the sample of competitor DNA which shares sequence homology with the amplified target but either has an intron added on or a portion of the sequence deleted (Siebert and Larrick, 1992; Diviacco, Norio, Zentilin, Menzo, Clementi, Biamonti, Riva, Falaschi and Giacca, 1992). This technique is beneficial because variables that affect amplification will affect both targets equally. Comparing the amount of competitor DNA yielding equal amounts of products gives the initial amount of target gene.

Although this technique was available to use, slot blot hybridization was chosen to quantify the transcripts for different isoforms of MHC because: (i) it was possible to produce probes specific for one of the isoforms of MHC mRNA, (ii) the transcripts of interest were abundant and (iii) the availability of the PhosphoImager facilitated analysis and quantification of the data.

It was important to verify that the time course of MHC mRNA accumulation during regeneration was similar in muscles of different animals since the analysis of mRNA content was done on pooled RNA samples. In an earlier study, which analysed rat solei muscles regenerating after notexin injection, the time course of morphological changes (myoblast formation and fusion, hypertrophy of myofibres) and the expression of different MHC isoforms were found to be temporally consistent between animals (Whalen *et al.*, 1990). However, it had not been determined whether this was true at the mRNA level, and this was essential to know in order to validate the pooling of all solei of a given experimental group for analysis of types of mRNA present. Therefore, RNA was prepared from six individual solei (each from a different animal) taken 21 d following notexin-induced degeneration. No differences were found between amounts and types of mRNA in individual samples (as determined by two-factor analysis of variance), thus it was considered valid to pool muscles to determine changes in mRNA levels for a given treatment group.

E. Analysis of Protein Expression

The particular isoform of MHC protein present in a muscle fibre will determine some of the physiological characteristics of that fibre. In this study, the presence of different isoforms of MHC protein were detected using

monoclonal antibodies. These were used in preference to polyclonal antibodies because they are directed to a single epitope on the antigen molecule, which ensures specificity of staining. As such, there is little background staining, which could hamper detection of the signal.

II. MHC AND ACTIN EXPRESSION IN REGENERATING MUSCLE: DOES REGENERATION RECAPITULATE DEVELOPMENT?

In the innervated regenerating soleus the embryonic and neonatal MHC isoforms were detected as early as 4 d, along with the mRNAs for β and IIX MHC. The β MHC mRNA and protein gradually replaced the more immature isoforms starting at the time of reinnervation (approximately 7 d), and by 21 d its protein was present in almost 100% of the muscle fibres. At this time, it was also the predominant MHC mRNA present. No IIA or IIB MHC protein were found, although very low levels of mRNAs for these isoforms were detected.

By comparing the results of the present study on regenerating muscle to those found previously in developing muscle, it can be seen that regeneration generally recapitulates development with respect to expression of MHCs

and α-actin, although there are certain qualitative and temporal differences.

Skeletal muscle fibre development in the soleus occurs along two different lineages, each of which have a specific sequential expression of the different isoforms of MHC. One of these lineages arises from the primary myoblasts, which give rise to primary myotubes, which express embryonic and β MHCs and eventually form muscle fibres containing β MHC. The second lineage is derived from the secondary myoblasts, which arise later and form myotubes which express embryonic MHC, followed by neonatal MHC, which is then replaced by β or IIA MHC in myofibres of soleus (Whalen *et al.*, 1981; Butler-Browne and Whalen, 1984; Narasuwa *et al.*, 1987). The adult rat soleus has 85% β MHC-containing fibres and 15% with IIA MHC.

In this study on regenerating soleus muscle, embryonic and neonatal isoforms of MHC protein were first detected followed by the β isoform. Although this follows the same sequence as in developing soleus, the transition occurred sooner. Most notable, however, was that all fibres in regenerated soleus stained for β MHC, and none for IIA MHC.

Using PCR analysis, it was determined that IIX MHC mRNA was present throughout the course of regeneration in the innervated regenerating soleus. An antibody specific to IIX MHC protein is unavailable. However, using a combination of several different antibodies to the isoforms of MHC and subtractive analysis, Schiaffino *et al.* (1989) have shown that

IIX MHC protein is absent in adult soleus. If the pattern of IIX MHC expression in regenerating muscle follows that of developing muscle, one would not expect to see IIX MHC protein in (at least) the later stages of regeneration. There are several possibilities which may account for the probable absence of IIX MHC protein despite the presence of its mRNA in regenerating muscle: (i) IIX MHC mRNA was detected by PCR but not slot blot hybridization, which is less sensitive, thus there may not be enough mRNA to produce detectable amounts of protein, (ii) IIX MHC protein may be produced, but is so unstable that it does not accumulate or (iii) it is also possible that the mRNA is never translated. A block in translation of IIX MHC mRNA is possible as it is known to occur for other contractile mRNAs, including that of MLC3f in normal soleus (Kirschbaum *et al.*, 1990).

Expression of α-actin differs in developing and regenerating soleus muscle. In developing mouse muscle, α-cardiac actin mRNA is more abundant than is α-skeletal actin mRNA in the early pre-natal period (Sassoon *et al.*, 1988). Alpha-skeletal actin mRNA, which gradually increases during the pre-natal period, is the predominant form at birth (Cox and Buckingham, 1992). In contrast, regenerating rat soleus muscle had much less α-cardiac actin mRNA than α-skeletal actin mRNA, even in the early stages of myoblast formation, and it disappeared faster than during development.

There are four basic differences which may account for changes in MHC expression in regenerating and developing

muscles: i) source of stem cells (satellite cells versus mesenchymal cells), ii) state of innervation (earlier versus later innervation), iii) hormonal environment (adult versus fetal), and iv) structural environment (pre-existing basal lamina scaffold versus no organized structural support).

III. EFFECT OF THYROID HORMONE ON MHC EXPRESSION IN REGENERATING MUSCLE

Since it has been shown that levels of thyroid hormone influence expression of MHC isoforms during myogenesis, it was of interest to determine whether thyroid hormone also influences MHC expression during regeneration, and if so, to determine whether its effect is similar to that during development.

In the present study, it was shown that thyroid hormone does affect MHC accumulation in regenerating muscle, but not in a manner identical to that seen in developing muscle.

In regenerating rat soleus, excess thyroid hormone accelerated the disappearance of the immature forms of MHC as compared to innervated regenerating soleus, similar to the results of Sesodia *et al.* (1993) in regenerating rat soleus. In addition, it resulted in fewer fibres with β and more fibres with IIA MHC protein. This is similar to the effect of the hyperthyroid state on developing rat soleus muscle, in which

fast myosin predominated (d'Albis, Chanoine, Janmot, Mira and Couteaux, 1990). Despite high thyroid hormone levels, β is the major isoform of MHC present (for both protein and mRNA) in hyperthyroid regenerates, suggesting a greater negative effect of thyroid hormone on β MHC expression in developing than in regenerating muscle.

The accumulation of IIA MHC protein is associated with an initial rise in its corresponding mRNA (at 7 d) followed by a decline in IIA MHC mRNA to levels slightly greater than those in innervated regenerates. The persistence of the IIA MHC protein despite low levels of its mRNA may be accounted for by increased efficiency of translation of the mRNA and/or decreased degradation of the protein.

Physiological serum levels of thyroid hormone change throughout development. Newborn rat and mouse are basically hypothyroid at birth after which their thyroid levels rise to peak values at approximately 16 d post-natally, followed by a slight decline to the steady-state condition in the adult (d'Albis, Lenfant-Guyot, Janmot, Chanoine, Weinman and Gallien, 1987). To determine whether the accelerated appearance of adult isoforms in regenerating adult muscle was due to the presence of adult levels of thyroid hormone, d'Albis, Couteaux, Janmot and Mira (1989) induced regeneration in muscles of newborn rats, i.e., muscles regenerated in a thyroid hormone environment mimicking that of developing muscle. The sequence and timing of expression of fast MHCs (IIA and IIB) in these muscles was

similar to that seen in development but β myosin appeared later, than during development. Thus, the fast MHC's are similarly regulated by thyroid hormone in normal developing and developing regenerating muscle, but β MHC expression depends on another factor, such as innervation.

In summary, the hyperthyroid state appears to cause accumulation of fast MHC's in both developing and regenerating muscle, and it results in down-regulation of β MHC gene expression in developing but not regenerating muscle.

IV. EFFECT OF DENERVATION ON MHC EXPRESSION IN REGENERATING MUSCLE

Since it is known that the state of innervation affects expression of the isoforms of MHC in both developing and adult muscle, it was of interest to determine how it would affect MHC expression in regenerating muscles of adult animals.

The present findings show that denervation of regenerating solei muscles resulted in a different pattern of accumulation of the different isoforms of MHC, than that seen in innervated regenerates.

Neonatal MHC mRNA and protein persisted longer in denervated than innervated regenerates, indicating that

innervation plays a role in suppressing the expression of these early isoforms.

At the earliest time point analysed, β MHC mRNA, but not protein, was detected in both innervated and denervated muscles. Thereafter, the denervated regenerates no longer contained β MHC mRNA or protein, a situation which contrasts sharply with that in innervated regenerates where it became the predominant isoform. The innervation-dependent production of β MHC protein in regenerating rat soleus agrees with results of previous immunohistochemical studies (Whalen *et al.*, 1990). In addition, the present results indicate that the initial transcription of the β MHC gene occurs independent of innervation.

The expression of β MHC in developing muscle had been thought to be entirely dependent on innervation since neonatal denervation resulted in its complete absence (Rubenstein and Kelly, 1978). More recent evidence indicated that the initial appearance of β MHC mRNA and protein is independent of innervation, but that its continued expression requires normal innervation (Narasuwa *et al.* 1987; Condon *et al.*, 1990). In developing avian muscle, β MHC has been detected in primary myotubes prior to the time of innervation of the limb or even when the neural tube had been removed prior to myogenesis (Fredette and Landmesser, 1991). As development continued there was a loss of β MHC expression in certain non-innervated myofibres but not in others. This indicates a differential requirement for innervation for the

continued expression of β MHC in different muscle fibres (Fredette and Landmesser, 1991).

Although developing avian muscle clearly shows β MHC expression in the absence of innervation, the evidence is less clear for mammalian muscle. Condon *et al.*, (1990) concluded that the initial appearance of β MHC in developing rat muscle did not require innervation based on the fact that it appeared in muscles treated with β-bungarotoxin prior to the arrival of motor innervation to the muscles. This result was similar to that for aneural avian muscle (Fredette and Landmesser, 1991); however, in the mammalian study, the muscles were not truly denervated. The use of β-bungarotoxin is not equivalent to a non-innervated state, and although contractile activity would not occur, the nerve could still influence the muscle with myotrophic substances. In addition, it is possible that there were myotoxic effects of β-bungarotoxin. It will only be possible to clarify whether β MHC appears independent of innervation in developing muscle by denervating mammalian hindlimbs *in utero* prior to myoblast formation.

Levels of IIA MHC mRNA were higher in denervated than innervated regenerates, but despite this, some muscle fibres were only lightly stained for IIA MHC protein. This suggests that innervation had a suppressive effect on IIA MHC mRNA accumulation and despite the greater content of IIA MHC mRNA, translation occurred at a very low level, or the protein did not accumulate.

The major MHC protein in denervated regenerating soleus was IIB and/or IIX (it was not possible to identify the actual isoform since none of the antibodies available could differentiate between IIB and IIX MHC).

Analysis of expression of the IIA MHC gene in developing mouse hindlimb muscles, denervated at birth, or in denervated adult muscle indicated that the IIA MHC protein exhibited a dependence on innervation for its production and in the absence of innervation IIB and/or IIX isoforms accumulated in its place (Russell, Cambon and Whalen, 1993). If this is the case, then denervation of muscles, be they developing, adult or regenerating, leads to expression of the IIB and/or IIX MHC isoform.

In the present study, low levels of the mRNA for IIB MHC and possibly its corresponding protein are detected in denervated regenerates, indicating that fast isoforms of MHC (IIB and IIX as discussed above) accumulate in the absence of innervation in regenerating soleus. This suggests that the "slow" nerve suppresses the accumulation of fast isoforms. In developing fast-twitch muscle, IIB MHC mRNA and protein were detected regardless of the state of innervation (Butler-Browne *et al.*, 1982; Russell *et al.*, 1988; Russell *et al.*, 1993). However, in adult denervated fast-twitch muscle the content of IIB MHC decreased, to be replaced by IIA or IIX (it is difficult to distinguish between these two using SDS PAGE), indicating a possible change in nerve-dependence for expression of IIB MHC expression as the muscle fibres mature

(Jakubiec-Puka, Kordowska, Catani and Carraro, 1990). It is difficult to compare results on regenerating soleus muscle to those on developing muscle because an antibody specific for IIB MHC protein was not available.

In summary, the present results indicate that the effect of innervation on MHC genes is similar in regenerating and developing muscle in that: (i) the initial transcription of β MHC gene occurs independent of innervation, but its continued transcription and the production of the β MHC protein requires normal innervation, (ii) IIA MHC protein is present in low amounts in denervated muscle and in regenerating muscle, innervation has a suppressive effect on IIA MHC mRNA accumulation and (iiii) IIB and/or IIX MHC protein accumulate in denervated solei. These results suggest an active role for innervation in allowing β MHC accumulation, and in the absence of which fast MHC accumulates.

V. INTERACTION OF EFFECTS OF THYROID HORMONE AND INNERVATION ON MHC EXPRESSION IN REGENERATING MUSCLE

It was important to analyse the interaction between hormonal and neuronal influences in consideration of the findings of Nwoye *et al.* (1982) which showed that the hyperthyroid condition resulted in accumulation of fast

myosin in adult slow-twitch muscle, regardless of the state of innervation, but that the hypothyroid condition resulted in an increased content of slow myosin in innervated soleus and fast myosin in denervated soleus. These results indicate that the influence of denervation on myosin accumulation can dominate over that of thyroid hormone.

The hyperthyroid state in innervated regenerates resulted in an accelerated disappearance of the immature isoforms of MHC as compared to the euthyroid state. By contrast, in denervated regenerates the immature isoforms of MHC persist in the eu-or hyper-thyroid state. This suggests a greater importance for innervation than thyroid hormone in suppressing the expression of embryonic and neonatal MHC and allowing the accumulation of the adult isoform. However, when the muscle is innervated, thyroid hormone can accelerate the transition from immature to adult isoforms. The embryonic MHC is similarly regulated by innervation and thyroid hormone in developing muscle (d'Albis *et al.*, 1990).

Some expression of β MHC is innervation-independent since a small amount of β MHC mRNA was detected in denervated regenerates, only at the early time points. However, the expression of β MHC is much less sensitive in denervated than innervated soleus to the stimulating effect of thyroid hormone. Although regenerating myofibres in hyperthyroid/denervated muscles contain a small amount of β MHC mRNA (detected by PCR), which persists (unlike denervated regenerates where it disappears), no protein

accumulates, indicating that innervation plays a greater role than thyroid hormone in influencing β MHC accumulation.

Expression of the IIA MHC gene in regenerating muscle is influenced by both innervation and thyroid hormone. It appears that innervation of regenerating muscle provides or allows for a very low level of IIA MHC gene expression which is detectable only at the level of mRNA. In the denervated state, levels of IIA MHC mRNA are much greater, although the corresponding protein is detected faintly. In the hyperthyroid state, IIA MHC mRNA levels in regenerating muscle are initially quite high, but coincident with reinnervation they decrease to a low level which is only slightly higher than that in innervated regenerates. In the hyperthyroid condition, IIA MHC protein is produced, unlike in innervated regenerates. When denervated muscles regenerate in a hyperthyroid environment, IIA MHC mRNA levels are high but little protein accumulates, similar to what was seen in denervated regenerates. These results indicate that innervation has an inhibitory influence on the production of IIA MHC mRNA, which can override the positive influence thyroid hormone has on its accumulation. However, even with high IIA MHC mRNA levels, the protein is only weakly translated, suggesting the existence of translational and/or post-translational control mechanisms. It is possible that the translational efficiency of the IIA gene is not very high or that the IIA protein is susceptible to degradation. Thyroid hormone, in the presence

of innervation, may counteract these control mechanisms, resulting in significant IIA MHC protein production.

There is evidence of a transition in gene expression from IIX to IIB MHC mRNA in hyperthyroid/denervated regenerates as the mRNA for the former is detected at 7 d but not 14 d at which time IIB MHC mRNA is detected. Thus, addition of thyroid hormone causes a transition to the faster isoforms of MHC mRNA. In hyperthyroid/denervated regenerates, the immature isoforms of MHC are present at 14 d post-toxin injection, thus preventing the possible detection of any of the adult fast MHC protein isoforms (via subtraction of results from staining with different combinations of antibodies).

The interaction of innervation and thyroid hormone on MHC expression has also been examined in innervated and denervated muscles which were artificially electrically stimulated. Chronic low frequency stimulation (CLFS) of innervated fast-twitch muscle in a euthyroid environment induced the expression of IIX and IIA MHC at the expense of IIB, but no β MHC was detected (Kirschbaum *et al.*, 1990). In denervated slow-twitch muscle, which was stimulated at high frequency, there was less β MHC and more of the fast isoforms of MHC (IIA and IIX), although none of the fastest (IIB) was detected (Ausoni *et al.*, 1990).

The hypothyroid state induced expression of the β MHC gene and this was even greater if coupled with CLFS (Kirschbaum *et al.*, 1990). The addition of thyroid hormone resulted in an increased content of IIB MHC, but when coupled

with CLFS this increase was partially counteracted. Thus, thyroid hormone appears to act antithetically to innervation. Innervation provides for a limited range of MHC transitions, but the addition or deletion of thyroid hormone permits a full range of MHC transitions. Kirschbaum *et al.* (1990) hypothesized that there is a graded suppressive effect of thyroid hormone on MHC isoform expression in the order IIX<IIA<I (β) and an antagonistic effect of CLFS decreasing in the same order. Furthermore, the control of MHC expression by innervation and thyroid hormone is primarily at the level of transcription because similar changes were seen for mRNA and protein.

In summary, the results of this study suggest that each of the MHC isoforms is independently modulated by thyroid hormone and innervation and that, generally, changes at the protein level follow changes at the mRNA level. An exception to this involves the control of IIA MHC expression for which there may be further translational and/or post-translational control mechanisms operating to modify protein accumulation. There are several possible mechanisms through which MHC gene expression could be controlled.

VI. MECHANISMS OF REGULATION OF MHC GENE EXPRESSION

The results of this study indicate that in regenerating muscle the accumulation of a given MHC protein is more or less proportional to the amount of the corresponding mRNA. The regulation of gene expression may occur at several different points, between transcription and the eventual degradation of the gene product. The influence of a given regulatory factor may vary at different points in the process of gene expression or at different developmental stages of the tissue. The results of this study indicate that production of an isoform of MHC i.e. protein in regenerating muscle largely follows that of its corresponding mRNA. This suggests that expression of MHC genes depends predominantly on regulation of transcription or stability of mRNA. However, there is some evidence that under certain conditions there may also be translational and post-translational regulation.

A. Transcriptional Regulatory Mechanisms

Gene transcription is controlled by cis- and trans-acting factors. Positive and negative cis-regulatory elements have been identified for embryonic, β and IIB MHC genes. These are acted upon by different trans-acting factors (e.g. thyroid hormone), which can cause concurrent activation and

repression of different genes, resulting in coordinated gene expression throughout development (Bouvagnet *et al.*, 1987; Cribbs *et al.*, 1989; Shimizu *et al.*, 1992; Takeda *et al.*, 1992).

The present study provides clear evidence for the action of innervation in suppressing or activating MHC gene expression (e.g. IIA and β isoforms respectively), although it was not determined if the neural influence is at the level of transcription or on stability of mRNA. Innervation had already been shown to play a role in modulating expression of genes for other contractile proteins. For example, a 181 bp sequence, which confers electrical activity-dependent gene expression, was identified in the 5' flanking region of the DNA for δ subunit of AChR (Dutton, Simon and Burden, 1993). The mechanism of interaction between electrical activity and this cis-acting element is unknown.

The gene for cardiac troponin-T (cTn-T) is expressed in early embryonic muscle, but coinciding with the time of innervation, its expression is repressed (Long and Ordahl, 1988). Furthermore, addition of nerve cells or nerve extract to cultures of cTn-T expressing-myoblasts causes the cTn-T protein to disappear (Toyota and Shimada, 1983). This indicates that a neurogenic factor may be responsible for the disappearance of cTn-T.

Thyroid hormone can directly or indirectly bind to specific cis-acting sequences (thyroid response elements -TRE) of a gene and cause up- or down-regulation of gene transcription (Nwoye *et al.*, 1982; Gambke *et al.*, 1982; Russell *et al.*,

1988). TRE have been located in the α-cardiac and β MHC genes, and binding of thyroid hormone to them leads to transcriptional up-regulation of α-cardiac MHC and down-regulation of β MHC in cardiac muscle (Rottman *et al.*, 1990). A decrease in β MHC content is observed in hyperthyroid developing and adult skeletal muscle (Nwoye et al., 1982; Izumo et al., 1986). The small effect of thyroid hormone on β MHC expression indicates that the influence of thyroid hormone on MHC expression in regenerating skeletal muscle differs from that in developing and adult skeletal and cardiac muscle.

At present, it is not known whether the influence of thyroid hormone on expression of the MHC genes in regenerating muscle changes over time. This is possible since it is known to occur with other genes. For example, expression of the gene for keratin in head skin of *Xenopus laevis* is initially independent of thyroid hormone, but during metamorphosis keratin gene expression increases in the presence of thyroid hormone (Mathisen and Miller, 1987). During myogenesis, the expression of certain isoforms of MHC is influenced by thyroid hormone. Hyper- and hypo-thyroid conditions can still induce MHC isoform transitions in adult muscle fibres, which otherwise have stable expression of MHC, suggesting that the expression of MHC is continuously sensitive to thyroid hormone levels.

Myogenic regulatory factors, such as MyoD, myogenin, myf 5 and MRF4 (herculin in the mouse) are able to induce

myogenesis in some fibroblastic cell lines (Davis *et al.*, 1987). Since such muscle regulatory factors have different timing of expression, they may play a role in modifying transcription during deter-mination and differentiation of muscle cells. To date, no correlation between the expression of any of these factors and that of MHC genes has been determined (Miller *et al.*, 1990; Lyons *et al.*, 1990). However, there is evidence that myogenic factors can activate the synthesis of an unidentified protein, which is required for an active transcriptional complex, affecting the IIB MHC promoter (Takeda, North, Miyagoe and Whalen, 1993).

In regenerating mouse muscle, small amounts of MyoD and myogenin are found in some myoblasts, but both are detected in all newly fused myotubes (Fuchtbauer and Westphal, 1992). MyoD and myogenin are still detectable after 15 d of regeneration, by which time the myofibres have matured. Thus, there is a correlation between the timing of the appearance of MyoD and myogenin and the events of myoblast fusion and muscle differentiation in regenerating muscle, similar to that which occurs in developing muscle. The mechanisms of action of the muscle regulatory factors are not known.

B. Post-Transcriptional Regulatory Mechanisms

Increased amounts of mRNA and protein are not necessarily due to increased gene expression, but can also be

due, partially or completely, to greater stability of the mRNA. This mechanism may be operating with respect to the MHC's. It has previously been shown, using pulse radio-labelling techniques, that a 26S RNA (the size of the mRNA for MHC) is present in large quantities in myoblasts, and when these fuse into myotubes it becomes much less plentiful, but its stability increases five fold. Concurrent with the decreased amount of 26 S RNA was the commencement of synthesis of several muscle specific proteins, including myosin (Buckingham, Caput, Cohen, Whalen and Gros, 1974).

Several factors which affect stability of mRNA have been identified. One of these is the length of the poly(A) tail, which seems to protect the mRNA from digestion by nucleases by binding to a cytoplasmic poly(A) binding protein (PABP) (Bernstein and Ross, 1989). The poly(A) tail is long immediately after transcription, but becomes shortened over time (Medford *et al.*, 1980), and resistance to degradation is lost if it becomes shorter than 30 bases. This is thought to be because the poly(A) tail is no longer large enough to bind to PABP.

Cis-acting sequences may also influence mRNA stability. Specific sequences have been identified which when activated may result in a less stable mRNA. For example, the AU-rich sequence in the 3'-untranslated region (3' UTR) of c-myc and c-fos genes can bind to PABP, and bring about release of the poly(A) tail (Shyu, Balasco and Greenberg, 1991). The free poly(A) tail, upon losing its "protector", becomes vulnerable to

degradation (Bernstein and Ross, 1989; Jackson and Standart, 1990).

C. Translational Regulatory Mechanisms

Translational efficiency is determined by examining the relative proportion of mRNA and its corresponding protein. Evidence that it can influence MHC accumulation is suggested by the results of the analysis of mRNA content and its corresponding protein in a given muscle.

The adult soleus has high levels of β MHC mRNA and 87% of the muscle fibres contain β MHC protein. This mRNA/protein ratio contrasts that found for other MHC isoforms. In the fast-contracting EDL, the fast MHC isoforms are more efficiently translated than fast or slow isoforms in the slow-contracting soleus, as indicated by lower mRNA/protein ratios. This does not rule out that rate of protein degradation may influence accumulation of MHC.

Innervated and hyperthyroid regenerating muscles have lower mRNA/protein ratios for β MHC mRNA than normal adult soleus, indicating that their efficiency of translation is greater. In contrast, denervated and hyperthyroid/ denervated regenerates had more IIA MHC mRNA but less protein than innervated regenerates, indicating that innervation may cause either decreased translational efficiency of the IIA MHC mRNA or increased degradation of the protein.

Although, in general, thyroid hormone appears to regulate MHC gene expression at the level of transcription, it seems to also affect expression of the IIA isoform by increasing the efficiency of translation or reducing the degradation of the protein. This was suggested by the finding that hyperthyroid regenerates accumulated IIA MHC protein, whereas innervated regenerates did not, despite the fact that they had similar amounts of IIA MHC mRNA.

The MLC3f gene is an example of another gene found to be under both transcriptional and translational control (Kirschbaum *et al.*, 1990). Normal soleus was found to contain mRNA but not the protein for MLC3f, suggesting translational control of its expression. However, chronic low frequency stimulation of fast- and slow-twitch muscle in hyper- or hypo-thyroid rats resulted in alterations in both MLC3f mRNA and protein, suggesting transcriptional regulation.

Although the mechanism of translational control of MHC gene expression is not known, two distinct possibilities exist: (i) there are specific sequences on the 5' side of the initiation site (AT/UG) which affect initiation of translation, and/or (ii) the length of the poly(A) tail of the mRNA may change and affect stability of the protein.

A sequence, CCA/GCCAUG, which is found 5' of the translation-initiation site of many eukaryotic mRNAs, serves as a consensus sequence for the initiation of translation (Kozak, 1984). Although this exact sequence was not observed 5' of the initiation sites in all the different mRNA

sequences examined, a purine (usually A) was commonly found three nucleotides upstream from AUG, and the pyrimidine C, was commonly found one, two, four, and five nucleotides upstream from a functional AUG site. Analysis of the 5' sequences of the β (rat), embryonic (rat), neonatal (human) and IIB (mouse) MHC genes shows a strong similarity to the above consensus sequence:

	A
Consensus sequence:	CC G CC AUG
β MHC	CC G TC AUG
Emb MHC	AC A CT AUG
Neo MHC	CA G CC AUG
IIB MHC	CA G CC AUG

The specific mechanism by which these sequences modulate translation is not known, but it is possible that proteins or other cis-acting sequences (e.g. 3' UTR) may interact with this site and alter initiation of translation.

The poly(A) tail, in addition to its role in influencing mRNA stability, is known to influence translation since the 60S and 40S ribosomal subunits bind to each other more efficiently in the presence of the poly(A)-PABP complex, which in turn improves the efficiency of translation (Sachs, 1990; Jackson and Standart, 1990).

The 3' UTR sequences, which are thought to decrease mRNA stability, may also affect translation. It is possible that the 3' UTR interacts with (i) the consensus sequence located 5' of the initiation site and/or (ii) the poly(A) tail to form a

translational control RNA (tcRNA) as suggested by Bester *et al.* (1975). In the latter situation, the 5' poly(U) tract of the tcRNA hybridizes with the 3' poly(A) tail of the adjacent mRNA, which circularizes the mRNA, making it resistant to translation. However, if the 3' UTR were hydrolysed, the tcRNA-mRNA complex would open up and translation would ensue.

The possibility that the rate of protein degradation plays a role in MHC accumulation cannot be ruled out. Analysis of MHC mRNA and protein expression in muscles stimulated with a chronic low frequency pattern has provided evidence for such post-translational regulation (Termin and Pette, 1992). Stimulated fast-twitch TA muscles exhibited changes in MHC mRNA (decreased IIB, increased IIA and IIX), but similar changes in the respective proteins were not observed until several days later. Termin and Pette (1992) suggested that the slow turn-over rate of IIB MHC protein was responsible for the delayed accumulation of the IIA and IIX MHC protein. Since newly synthesized MHCs could not have been incorporated into the sarcomere until the IIB MHC was degraded, they may be susceptible to degradation as free proteins.

In summary, the regulation of MHC expression in regenerating muscle seems to involve control mechanisms acting on both transcription and translation, and stability of mRNA and rate of protein degradation may also play a role.

VII. SATELLITE CELLS

Although it appears that differences exist in the mechanisms controlling MHC gene expression between developing and regenerating muscle, it is also possible that satellite cells may respond differently than embryonic muscle precursor cells to hormonal and neuronal cues. The results of the present study support this possibility since (i) developing soleus contains both β MHC and IIA MHC, whereas regenerating muscle contains predominately β and no IIA MHC and (ii) β MHC mRNA and protein accumulate in hyperthyroid regenerating muscles as in typical regenerating muscle, whereas the hyperthyroid state in developing or adult muscle results in fewer β MHC-containing muscle fibres than in regenerating fibres.

The presence of neonatal, IIX and β MHC mRNA very early in regeneration indicates that satellite cells in the soleus are intrinsically programmed to express fast and β isoforms. However, the type of MHC protein which ultimately accumulates is largely determined by external factors such as innervation and thyroid hormone. Feldman and Stockdale (1991) identified two distinct populations of avian satellite cells: (i) "fast" satellite cells which form fibres containing only fast MHC and (ii) "slow" satellite cells which form fibres containing mixed fast and slow MHC, or fast MHC only. This suggests that a difference in MHC expression in avian satellite

cells is the basis of MHC diversity seen in regenerating avian muscle.

Hughes and Blau (1992) analysed the importance of cell lineage *in vivo* using retrovirally-labelled clones of myoblasts, which were derived from adult leg muscle or cell lines, injected into rat muscle. They found that the myoblasts did not preferentially fuse with any one type of fibre but could fuse with either fast or slow muscle fibre types. Upon fusion, the MHC gene expression of the myoblasts was reprogrammed to that of the host muscle fibre. Thus, it would seem that external cues rather than cell lineage determine MHC accumulation in developing muscle. These results, which were obtained from *in vivo* experiments probably give a more accurate assessment of what is happening physiologically than results derived from *in vitro* experiments, where typical extrinsic influences are absent. The results of the present study also support the importance of extrinsic influences in determining the pattern of MHC expression in regenerating muscle.

One can not rule out the possibility that the detection of β MHC mRNA in 4 d regenerating muscle, which is prior to innervation, may have been due to the continued presence of original fibres still undergoing degeneration or not affected by the toxin. If this was the case, and regenerating myofibres did not in fact contain any β MHC mRNA at 4 d, satellite cells in the soleus would resemble secondary fetal myoblasts, in that they initially express embryonic and neonatal MHC and

do not express adult fast or slow MHC until influenced by innervation and thyroid hormone. Since all fibres in the muscles appeared centronucleated, and none stained for β MHC protein as would be expected if pre-existing fibres had survived, the present results indicate appearance of β MHC mRNA in the early regenerates.

It is possible that fetal myoblasts contain low levels of β MHC mRNA, which have gone undetected by techniques previously used. In the present study, use of PCR enabled the detection of very low amounts of mRNAs.

Muscle fibres in the regenerated soleus differ from the original myofibres with respect to MHC isoforms accumulated. While normal mature soleus contains both β and IIA MHC, innervated regenerates contain only β MHC. The loss of IIA MHC expression may be because myoblast-derived and satellite cell-derived fibres may have different mechanisms of control of MHC expression. Whatever the reason, it is not necessarily true for all species since d'Albis *et al.* (1988) found that myosin expression in regenerated mouse soleus muscle resembled that in normal control muscle.

Future experiments are required to clarify which mechanisms are actually responsible for the regulation of MHC expression, specifically, it will be necessary to differentiate between (i) regulation of transcription and stability of mRNA, and (ii) regulation of translation and protein turnover. Nuclear run off assays could be a means by which transcriptional regulation could be differentiated from mRNA

stability or other post-transcriptional regulatory mechanisms. This would involve isolation of nuclei from muscles at different stages of development or regeneration, and these would then be incubated with radioactively labelled UTP and an excess of cold NTPs, which would become incorporated into the newly transcribed mRNA. Since only isolated nuclei would be used, the interpretation of the results would not be influenced by the presence of cytoplasmic mRNA. One limitation of such an experiment might be technical difficulty in isolating the myonuclei owing to the presence of connective tissue, and the speed of isolation of the nuclei is important for their viability.

Pulse-chase experiments could be a means by which translational regulation could be differentiated from protein turnover. With this technique, all components necessary for maintenance or growth of muscle cells in culture are provided, including radioactively labelled amino acids (pulse). This is followed by incubation in medium containing excess unlabled amino acid (chase). By varying the length of time of the "pulse" before the "chase" of cold amino acids, different length chains of radio-labelled amino acids could be made. The kinetics of the relationship between the length of the amino acid chain and the length of time of the pulse-chase labelling could be determined to provide an indication of protein production and degradation.

CLAIM FOR ORIGINAL WORK

The results presented in this thesis represent the following original contributions to the knowledge of the role of innervation and thyroid homone in regulating the expression of different isoforms of MHC.

Firstly, this thesis confirms that innervation acts at the level of transcription, but the results demonstrate that for different isoforms it may promote (β MHC) or suppress (IIA MHC) their accumulation. Removal of innervation leads to an increase in IIA MHC mRNA compared to innervated regenerates. Conversely, innervation is required for the accumulation of β MHC mRNA. The inhibitory effect of innervation on IIA MHC mRNA accumulation may involve transcriptional regulation or stabilization of the IIA MHC mRNA.

Secondly, in the absence of innervation IIB and/or IIX MHC accumulates in regenerating muscle. It has previously been documented that a fast MHC isoform accumulates in the absence of innervation. The results presented here indicate that it is IIB and/or IIX, and not IIA MHC, which predominate in denervated regenerating soleus.

Thirdly, the results confirm that thyroid hormone acts at the level of transcription and it provides new evidence for a role of thyroid hormone in stabilizing MHC protein. In innervated regenerates no IIA MHC protein is detected, but in

the hyperthyroid condition IIA MHC protein accumulates without an associated increase in the corresponding mRNA. This suggests a possible role for thyroid hormone in influencing MHC protein accumulation possibly by reducing MHC protein degradation.

Fourthly, despite a large increase in IIA MHC mRNA in denervated regenerates, little IIA MHC protein accumulates, which suggests translational or post-translational regulation. A comparison of MHC mRNA:protein content i.e. translational efficiency, in adult muscle fibres indicates the importance of efficiency of translation in regulating the accumulation of isoforms of MHC.

Finally, this thesis provides evidence that satellite cells are not predetermined to accumulate fast or slow MHC, but rather the final accumulation of isoforms of MHC is determined by external factors such as the state of innervation and the thyroid hormone condition. More specifically, evidence is provided which indicates the presence of fast and slow MHC mRNA at the early stages of regeneration. Innervation is required to maintain the production of β MHC mRNA and for the accumulation of the corresponding protein.

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