Neural and Thyroidal Control of IIb Myosin Heavy Chain and its mRNA During Development

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Abstract

Myosin heavy chain (MHC) switching is a hallmark of developing skeletal muscle. Factors responsible for changes in MHC gene expression during development include endogenous, neural, and hormonal signals. After perturbing innervation and/or thyroid hormone levels, we have examined the neonatal to (adult) IIb MHC transition in rat hindlimb muscles. This switch takes place between 5 and 15 days *post partum (p.p.)* in innervated muscles of euthyroid rats. Denervation does not qualitatively affect the timing of appearance of IIb mRNA or protein, but the disappearance of neonatal MHC gene transcripts is delayed. Hypothyroidism delays the appearance of IIb MHC mRNA in the innervated limb. In the denervated hypothyroid limb, however, IIb MHC is synthesized at moderate levels. Hyperthyroidism causes an early increase in IIb MHC mRNA and protein accumulation in both innervated and denervated muscles. These results suggest that while thyroid hormones can alter the levels of the IIb myosin heavy chain - and can even accelerate its appearance during development, as in the hyperthyroid animals - they are probably not the cue for the shift from neonatal to adult myosin heavy chains. Rather, we suggest that the synthesis of IIb myosin heavy chain and its mRNA in the normal animal is the result of an interaction among neural and hormonal factors on all elements of the motor unit.

Key words: myosin heavy chain, mRNA, skeletal muscle, thyroid hormone, development, rat hindlimb, motor neuron, denervation, PTU.

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A pattern of myosin heavy chain (MHC) switching is a hallmark of developing avian and mammalian muscles. In the fast muscles of the rat hindlimb, there is a sequential appearance of an embryonic myosin heavy chain *in utero*, followed by a neonatal or perinatal heavy chain during the immediate pre- and postnatal period, and finally one or more adult fast heavy chain(s) beginning around 7 days *post partum (p.p.)* [39]. The embryonic and neonatal MHCs disappear in the same sequence: the former around two weeks *p.p.*, the latter at approximately 4 weeks of age [10]. Similar developmental sequences of appearance and disappearance of these myosin heavy chain gene transcripts have been described [30, 37], suggesting that MHC gene expression may be regulated at the level of transcription rather than translation.

The mechanisms which regulate this temporal sequence of appearance of myosin types remain unclear. Both neural and hormonal controls of muscle differentiation in the developing and adult animal have been demonstrated [reviewed in 9, 12, 19]. Neural control is clearly important from the classic experiments of cross innervation of fast and slow twitch muscles, a perturbation that leads to reciprocal transformation of the muscles' properties [2]. Similarly, imposition of the slow muscle pattern of stimulation on fast muscle leads to a switch from fast to slow myosin isoforms [32, 36]. Testosterone has a general anabolic effect on skeletal muscles, and, in special cases, directly influences the MHC content of the muscle [22].

Thyroid control of myosin isoforms is also apparent, since hypothyroidism leads to an increase in slow MHCs, while hyperthyroidism increases fast heavy chains [16, 17]. Whether the thyroid exerts its influence on myosin isozymes by acting directly on muscle fibers or indirectly by altering the properties of the motor neurons is a matter

of controversy. Transfection experiments with cardiac myocytes have shown that thyroid hormones do, in part, regulate contractile protein isoforms by directly acting on the muscle cell [13]. In normal, euthyroid development of fast muscles, denervation does not qualitatively affect the sequential synthesis of myosin isozymes [3, 10]. Consistent with these findings, daily injections of newborn rats with triiodothyronine (T3) leads to a precocious expression of fast MHC mRNA in both innervated and denervated rat hindlimb muscles [33].

We and others have shown that the synthesis of fast heavy chains coincides with the increase in serum thyroid hormones from 5-15 days *p.p.*, is blocked in hypothyroid animals, and occurs precociously in hyperthyroid animals [8, 10]. We do not know, however, whether these changes in thyroid status affect muscle fibers directly or through some neurally mediated mechanism. Moreover, we also do not know whether thyroid hormones alter fast myosin synthesis at the transcriptional or translational level. Translational controls of some MHC mRNAs have been suggested [15], including perinatal MHC [23]. However, the identification of different thyroid hormone receptor isoforms which differentially activate cardiac α-MHC transcription [18] suggests that synthesis of at least some MHCs is regulated at the transcriptional level.

In this paper, we have examined the normal transition from a neonatal to a fast (type IIb) isoform of myosin heavy chain. We have examined the control of this isoform switching by perturbing innervation and thyroid hormone concentration and have investigated the interaction of these two control mechanisms. The results suggest that the normal transition from neonatal to IIb MHC synthesis is an endogenously programmed event which is closely orchestrated by the changing hormonal and neuronal status of the animal.

Materials and Methods

Protein Purification

Purified myosins were prepared according to a modification of the method of Whalen et al [38]. Minced rat gastrocnemius muscles were extracted with 0.3 M KCl, 0.015 M sodium phosphate buffer, pH 6.5, 7 mM ß-mercaptoethanol, 5 mM ATP. After centrifugation to remove insoluble material, actomyosin was precipitated from the supernatant by dilution with 6 volumes of distilled H_2O. The precipitated actomyosin was redissolved in 0.5 M NaCl, 20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, and centrifuged at 100,000x g for 4 hrs. to remove actin. Crude myosin was again precipitated out of the supernatant by dilution with distilled H_2O and redissolved in column buffer (0.5 M NaCl, 20 mM sodium phosphate buffer, pH 6.5, 1 mM $MgCl_2$, 1 mM ATP). The myosin-rich supernatant was then chromatographed on a Sepharose 2B (Pharmacia P-L Biochemicals) column in the presence of this same buffer. If SDS gel electrophoresis showed actin contamination of this column-purified sample, the sample was rechromatographed in the same buffer. The purity of all samples was checked by electrophoresis on SDS gels.

Peptide Mapping

Peptide maps of purified myosins were performed following the method described in detail in Lyons *et al.* [22]. Peptides were analyzed on 12.5% acrylamide-SDS gels according to Laemmli [21] as modified by Milstone and McGuire [26].

Isolation of RNA

Total RNA was isolated from newborn rat muscles by the method of Cox [7]. Muscles frozen in liquid N_2 were dropped directly into Buffer A (8 M guanidine HCl, 10 mM sodium acetate, pH 5.0, 1 mM dithiothreitol) and homogenized with a Polytron homogenizer. After centrifugation to remove debris, DNA was sheared through an 18g needle and the RNA precipitated overnight in 50% ethanol at -20°C. The RNA pellet was collected by centrifugation and redissolved in Buffer A containing an additional 20 mM EDTA, pH 7.0. This solution was first extracted by addition of 0.5 volumes of phenol saturated with 10 mM EDTA, 0.5 volumes of 20 mM EDTA, pH 8.0, 0.5 volumes of chloroform:isoamyl alcohol (24:1). The supernatant was reextracted with the phenol and chloroform:isoamyl alcohol, then twice with 0.5 volumes of chloroform:isoamyl alcohol. RNA was precipitated overnight by the addition of 0.1 volume of 3 M sodium acetate, pH 5.0, and 2.5 volumes of ethanol. After a final wash in 80% ethanol, the RNA was dissolved in DEPC-treated H_2O. Samples were stored at -70°C. Yields of total RNA were usually 200-300 µg/g of tissue (wet weight). RNAs were electrophoresed on denaturing gels to verify that no degradation had occurred and to verify the concentration measured by A_{260}.

Northern blots

Northern blots were prepared essentially by the method of Rave *et al* [31] after electrophoresis of total RNA on formaldehyde - 1% agarose gels. Ten micrograms total RNA was loaded per well. Separated RNAs were transferred to GeneScreen (Dupont/NEN) overnight. GeneScreen was then baked at 90°C for 2-4 hrs prior to hybridization.

Oligonucleotide Hybridization

Nylon membranes containing total RNAs were hybridized with oligonucleotide probes specific to the 3 untranslated region of the neonatal and IIb myosin heavy chain mRNAs. The nucleotide sequences for these probes are identical to those published in Gustafson *et al* [14], adapted from the sequences published by Nadal-Ginard and his colleagues [24, 28, 30]. The following sequences were used as probes:

IIb mRNA: GTGTGATTTCTTCTGTCACC
Neonatal: GCGGCCTCCTCAAGATGCGT

These probes were end-labelled with γ-32P-ATP according to Maxam and Gilbert [25]. The membranes were prehybridized with the following solution: 6x NET (NET = 0.15 M NaCl, 15 mM Tris-HCl, pH 8.0, 1 mM EDTA), 10x Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, 10% dextran sulfate. Prehybridization and hybridization temperatures were 61°C for the neonatal probe and 53°C for the IIb probe. Labelled oligonucleotide probe was added to the prehybridization solution and hybridization was allowed to proceed for 2 hours. Northern blots were then washed thoroughly and autoradiographed with two Lightning Plus (DuPont) screens at -70°C overnight.

Dot blots

Four or ten micrograms of formalin-denatured total RNA/well was blotted onto GeneScreen using a Schleicher & Schuell Minifold II apparatus. Dot blots for quantitative studies were baked and hybridized in the same manner as Northerns [35]. Each dot blot for quantitation was repeated a minimum of three times.

Thyroid hormone perturbations

Hypothyroidism was induced by adding 0.05% propylthiouracil (PTU) to the drinking water of pregnant rats from 3 days gestation onwards and to the water of their offspring from birth through 60 days *post partum*. Mothers and pups were also maintained on a low iodine diet. Hyperthyroidism was induced by injecting rat pups intraperitoneally with 25 µg of thyroxine (Sigma) on alternate days after birth. Serum thyroid levels were monitored as described [10]. Distal hindlimbs were denervated at birth by removal of a 2 mm segment of the sciatic nerve in the proximal thigh.

Results

For this study, we used two oligonucleotide probes specific to the 3' untranslated regions of the neonatal and IIb myosin heavy chain mRNAs. RNAs were isolated from the fast gastrocnemius muscle at a series of stages after birth, and the presence of neonatal and IIb mRNAs were established by Northern blots. Quantitative figures were derived by dot blots, followed by scintillation counting. As shown in Figure 1A, neonatal myosin heavy chain mRNA is present in innervated muscle at both 1 (lane a) and 10 (lane b) days *p.p.*. At 20 and 30 days (lanes c, d) *p.p.*, however, no neonatal mRNA is detectable with our probe on Northern blots. Quantitative measurements (Fig. 2) show that neonatal myosin heavy chain mRNA is present in high

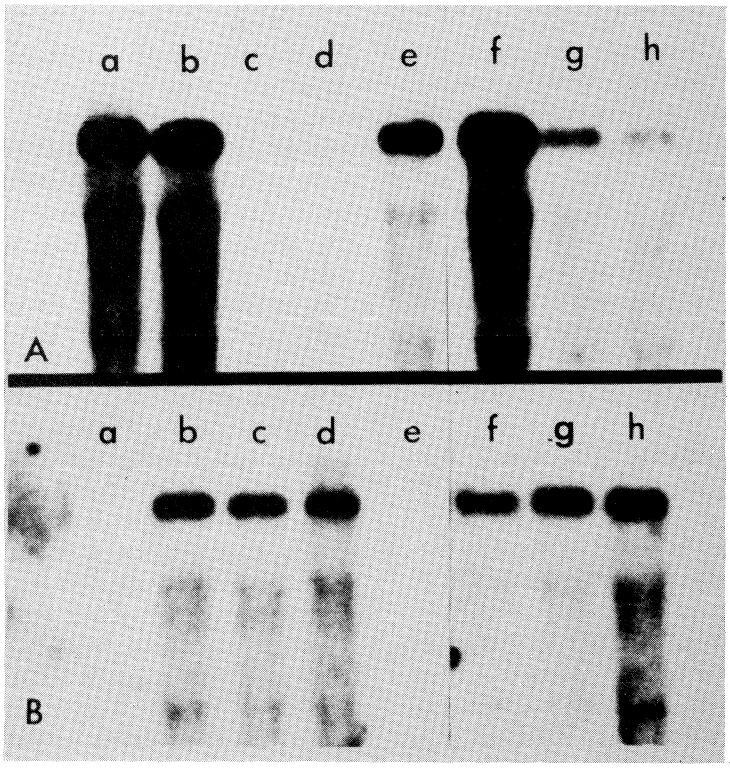

Figure 1. A) Neonatal and B) IIb myosin heavy chain mRNAs in developing innervated and denervated muscles. In both A and B, RNA samples were from the innervated gastrocnemius at day 1 (lane a), day 10 (lane b), day 20 (lane c) and day 30 (lane d) p.p. RNA samples were isolated from the gastrocnemius denervated at birth and examined on day 1 (lane e), day 10 (lane f), day 20 (lane g) and day 30 (lane h) p.p. Mobility of 28S rRNA indicated by arrowheads.

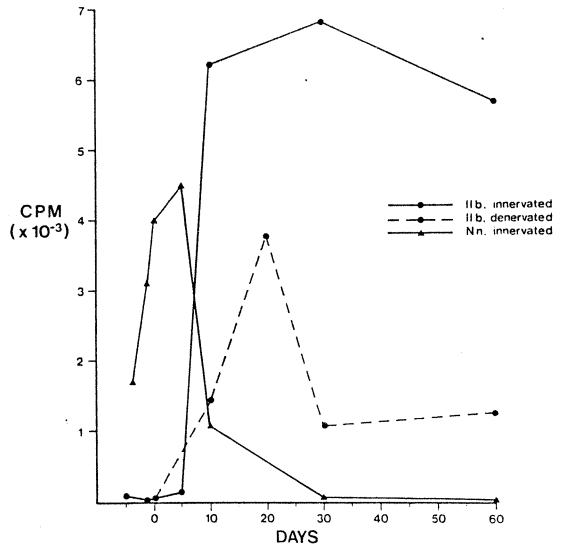

Figure 2. Quantitative changes in neonatal and IIb myosin heavy chain mRNAs during development. Total RNA from developing gastrocnemius muscle was analyzed by dot blots followed by hybridization with labelled oligonucleotide probe. After autoradiography, individual spots from the GeneScreen were cut out and radioactivity was determined by scintillation counting in Aquasol.

concentrations prior to birth, shows peak values during the first week *post partum*, and decreases rapidly thereafter. These results are consistent with those of Periasamy et al. [30] who detected neonatal mRNA as late as 28 days *post partum* using S1 nuclease protection assays.

IIb myosin heavy chain mRNA in the gastrocnemius shows a complementary pattern (Figs. 1B and 2) to that of neonatal MHC. The mRNA for IIb MHC is not apparent prior to 1 day *p.p.* (lane a), but is present in significant amounts at 10 days (lane b). This correlates with the appearance of fast myosin heavy chains [39]. IIb mRNA levels are stable at least through 60 days *p.p.* (Fig. 1B, lanes c, d; Fig. 2).

When the hindlimb is denervated at birth, IIb mRNA is still not seen at birth (Fig. 1B, lane e) but is apparent by 10 days *p.p.* (lane f) and increases through 20 days (lane g), reaching levels approximately one half of those in the innervated muscle at comparable stages (see Fig. 2). By 30 days, however, there is a three-fold decrease, relative to the innervated muscle, in IIb mRNA (lane h). Although lowered total levels of myosin heavy chain mRNA may reflect, in part, atrophy of the leg muscles with denervation, the quantitative studies measured IIb mRNA as a fraction of total RNA. Hence, innervation is at least partly responsible for maintaining increased levels of this mRNA during development, even though it is not likely to be responsible for initiating its synthesis.

Protein analyses of denervated fast muscles have shown that 25 day muscle still contains predominantly adult fast myosin heavy chain (Fig. 3, lane g). The disappearance of neonatal mRNA does not occur as rapidly in denervated muscle as in innervated muscle. There appear to be large amounts of neonatal mRNA at 1 and 10 days (Fig. 1A, lanes e, f) and small, but decreasing amounts at 20 and 30 days (lanes g and h). Hence, denervation at birth prevents neither the initiation of IIb myosin heavy chain mRNA nor its initial increase; it impedes but does not completely block the disappearance of neonatal mRNA.

The neural independence of IIb mRNAs correlates with the previously demonstrated neural independence of fast myosin heavy chain protein synthesis. Figure 3A is a set of peptide maps of isolated myosin heavy chains from the fast gastrocnemius muscle. While the 5 day innervated muscle contains a myosin peptide map characteristic of neonatal myosin heavy chain (lane a), at 25 days there is clearly a shift to another peptide pattern, characteristic of adult fast myosin heavy chain (lane f). Even after denervation at birth, the adult pattern is evident at 25 days (lane g). Thus, in the euthyroid animal, the synthesis of fast IIb myosin heavy chain and its mRNA appear to be independent of innervation.

The transition from neonatal to adult fast myosin heavy chains is, however, dependent on thyroid hormones. In the hypothyroid, innervated muscle at 10 days (lane b) and 25 days (lane d) the pattern remains unchanged and adult fast heavy chains do not appear. If the gastrocnemius of the hypothyroid animal is denervated at birth, however, a different situation occurs. Lane c shows the peptide map of myosin heavy chains in the 10 day hypothyroid, denervated muscle. Only the neonatal pattern exists. In the 25 day hypothyroid denervated muscle, however, a significant switch to adult fast myosin can be seen by comparing the peptide map (lane e) with the euthyroid innervated muscle's peptide map at 25 days (lane f).

Hyperthyroidism causes a precocious appearance of IIb mRNA and protein, and this precocity is independent of innervation. Figure 3B shows another series of peptide maps of gastrocnemius myosin heavy chains under various conditions. Lane a is the gastrocnemius at 5 days *p.p.*, a time at which the muscle contains predominantly the neonatal myosin heavy chain. In the hyperthyroid, innervated animal, at both 10 and 25 days *p.p.* (lanes b and d, respectively), adult, IIb myosin heavy chain can be seen (compare to lanes f and g). This precocious appearance of IIb myosin heavy chain in hyperthyroid animals occurs even when the muscle is denervated at birth (lanes c, e).

Like the protein, the IIb mRNA is dependent on thyroid hormones. Animals were made hypothyroid by feeding them PTU as described in the Materials and Methods. RNAs were isolated and probed on Northern blots using the synthetic oligonucleotides. In Figure 4, lanes a, b, and c show the hybridization of the IIb oligonucleotide probe with RNAs from hypothyroid EDL muscles at 10, 20 and 30 days respectively. In these innervated, hypothyroid

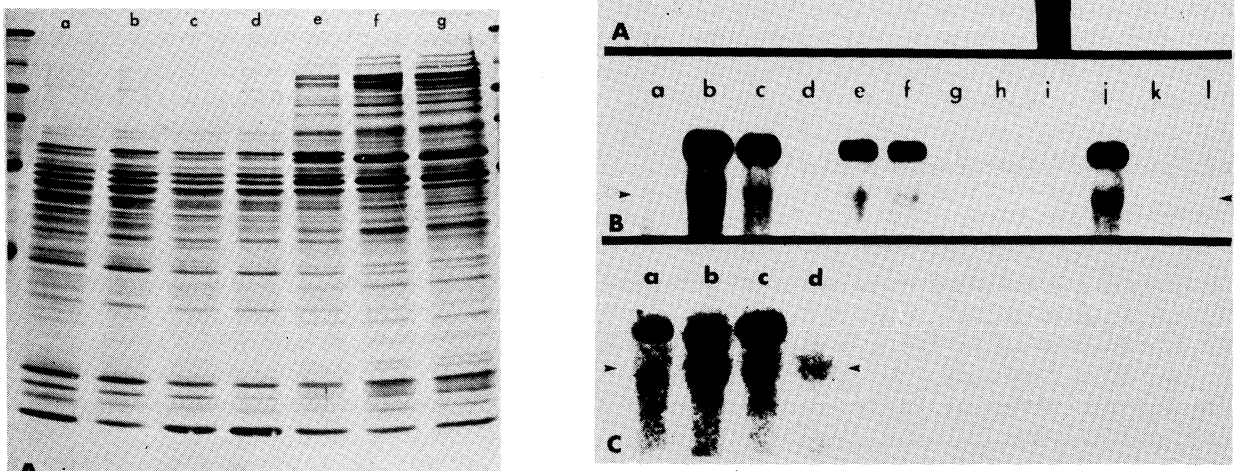

Figure 4. A) IIb and B) neonatal myosin heavy chain mRNAs after thyroid and neural perturbations. In both A and B, RNAs were isolated from hypothyroid, innervated muscles at 10 (lane a), 20 (lane b) and 30 (lane c) days p.p.; from hypothyroid muscles, denervated at birth, examined at 10 (lane d), 20 (lane e), and 30 (lane f) days p.p.; from hyperthyroid, denervated muscles at 10 (lane g), 20 (lane h), and 30 (lane i) days p.p.; and from hyperthyroid muscles, denervated at birth, examined at 10 (lane j), 20 (lane k), and 30 (lane 1) days p.p. In C, RNAs were isolated from 5 day hyperthyroid denervated (lane a), 5 day hyperthyroid innervated (lane b), 10 day euthyroid innervated (lane c), and 5 day euthyroid innervated (lane d) gastrocnemius muscles. Mobility of 28S rRNA indicated by arrowheads.

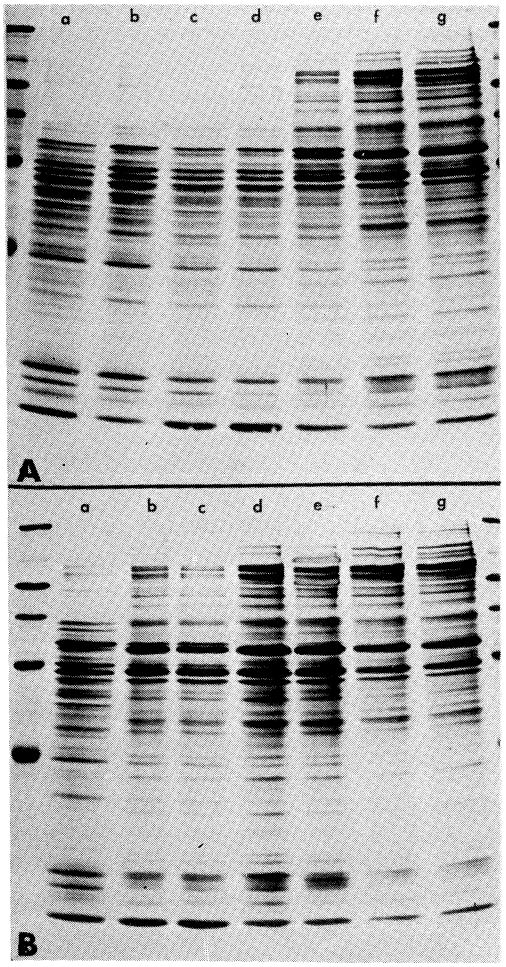

Figure 3. A) Peptide maps of myosin heavy chains of developing innervated and denervated euthyroid and hypothyroid gastrocnemius muscles. Myosins were isolated from: lane a, euthyroid muscle, 5 days p.p.; lane b, hypothyroid muscle, 10 days p.p.; lane c, hypothyroid muscle, 10 days p.p., denervated at birth; lane d, hypothyroid muscle, 25 days p.p.; lane e, hypothyroid muscle, 25 days p.p., denervated at birth; lane f, euthyroid muscle, 25 days p.p.; lane g, euthyroid muscle, 25 days p.p., denervated at birth. B) Peptide maps of myosin heavy chains from developing innervated and denervated hyperthyroid muscles. Myosins were isolated from: lane a, euthryoid muscle, 5 days p.p.; lane b, hyperthyroid muscle, 10 days p.p.; lane c, hyperthyroid muscle, 10 days p.p., denervated at birth; lane d, hyperthyroid muscle, 25 days p.p.; lane e, hyperthyroid muscle, 25 days p.p., denervated at birth; lane f, euthyroid muscle, 25 days p.p.; lane g, euthyroid muscle, 25 days p.p., denervated at birth.

muscles, IIb mRNA does not appear. In the hypothyroid denervated muscles, however, IIb mRNA - not detectable at 10 days (lane d) - is clearly present at 20 and 30 days (lanes e, f). Thus, although hypothyroidism blocks the appearance of IIb myosin heavy chain and its mRNA, denervation somehow removes this suppression.

This same type of neurally independent control in the hyperthyroid muscle can be seen when transcription of IIb myosin heavy chain mRNA is examined. Figure 4A shows the Northern blot of various EDL muscles after probing with the synthetic oligonucleotide for IIb mRNA. IIb mRNA can be seen in the 10, 20, and 30 day hyperthyroid EDL, whether the muscle is innervated (lanes g, h, i) or denervated (lanes j, k, l).

In the hypothyroid animal, changes in neonatal mRNA do not exactly complement changes in the IIb mRNAs. At 10 days *p.p. (Fig.* 4B, lane a) very little neonatal mRNA is detected on Northern blots from innervated, hypothyroid animals. This occurs because hypothyroidism interferes with the rapidity of the embryonic/neonatal transition. Hypothyroidism causes a prolonged presence of em-

bryonic myosin, as demonstrated by our monoclonal antibody 2B6 [11] At 20 and 30 days *p.p.* (lanes b, c), however, neonatal mRNA is readily detected. In the hypothyroid muscle denervated at birth, there is a less obvious increase in neonatal mRNA at 20 and 30 days (Fig. 4B, lanes d-f). This correlates with the increased IIb mRNA at this stage. Hyperthyroidism, started at birth, shows neonatal mRNA at 10 days *p.p.*, but not at 20 or 30 days (lanes g, h, i). This is independent of innervation (lanes j, k, l).

Discussion

The period between 5 and 15 days *post partum (p.p.)* in the rat hindlimb is marked by several developmental changes: (a) there is a replacement of neonatal by adult fast, predominantly IIb, myosin heavy chains (MHC); (b) there is a change from a hypothyroid state to a physiologically hyperthyroid state; and (c) there is a maturation of motor neurons and the neuromuscular junction. Perturbations in thyroid hormone status and innervation can profoundly affect normal development of the neuromuscular system.

The sequential synthesis of several myosin heavy chain isoforms during development is the product of sequential transcription of distinct myosin heavy chain mRNAs [24,28,30,37]. Using S1 nuclease protection assays with several different cDNA probes, Nadal-Ginard and his coworkers have demonstrated different patterns of expression of myosin heavy chain mRNAs during development [24,28,30]. The embryonic myosin heavy chain mRNA is present in high quantities at 20 days *in utero*, but decreases greatly by 2 days *p.p.* It is barely detectable at 14 days. This correlates with our demonstration by radioimmunoassay, using a specific monoclonal antibody, that the embryonic myosin heavy chain peaks prior to birth and is reduced to background levels by 20 days *p.p.* [11]. The neonatal myosin heavy chain mRNA, which peaks around 7 days *p.p.*, decreases until it is barely detectable at 28 days and not detectable at 3 months [30]. Finally, we have now shown that the fast IIb myosin heavy chain mRNA is detectable between 5 and 10 days *p.p.* and rapidly rises between 10 and 15 days *p.p.* This is similar to the increase in fast myosin heavy chain seen around day 10 [39].

Profound changes in thyroid hormone levels also occur during the first two weeks *p.p.* in the rat. Serum T4 levels have shown that the rat is essentially hypothyroid at birth [10]. There is a significant rise in serum T4 levels between 5 and 15 days to a level higher than that seen in the normal, euthyroid adult. These T4 levels decrease to mature levels by 35 days *p.p.* It should be noted that while serum T4 levels are practically undetectable at birth, tissue levels of T4 can be quite high even during the prenatal period [27]. In any case, we have previously demonstrated that the rise in serum T4 levels coincides with the transition from neonatal to adult fast myosin heavy chains and that this transition is dependent on adequate levels of thyroid hormones [10]. In the hypothyroid animal the transition is prevented, while in the hyperthyroid animal it occurs precociously. Whalen *et al.* have demonstrated that this effect of thyroid hormones is not mediated via growth hormone [40].

The transition from predominantly neonatal to predominantly adult fast myosin heavy chain is not qualitatively disturbed by changes in innervation in the euthyroid animal. The motor neuron and the neuromuscular junction in the newborn rat are physiologically immature compared to their adult counterparts. For example, the frequency of miniature end-plate potentials increases from one per 40 seconds at birth to one per second at three weeks. Similarly, the extrajunctional sensitivity to acetylcholine decreases by two orders of magnitude during this period. Kawa and Obata [20] have shown that these changes, as well as the decrease in polyneuronal innervation, are thyroid sensitive. Also Navarette and Vrbova [29] have shown that adult patterns of neuromuscular activity do not become established until several weeks after birth.

We have now shown several additional features of thyroidal and neural control of myosin heavy chain isoform transitions. First, hypothyroidism blocks the transition from neonatal to adult IIb myosin at the transcriptional level. In the absence of thyroid hormones, no IIb myosin heavy chain mRNA can be detected by our probes. Second, denervation, regardless of the thyroid status of the animal, does not block the synthesis of IIb myosin heavy chain mRNA, although decreased amounts of this mRNA are found in the denervated limb. Third, hyperthyroidism, regardless of the innervation status of the limb, causes a precocious synthesis of IIb myosin heavy chain mRNA. This is consistent with the results of Russell *et al.* [33]. Finally - and surprisingly - while the hypothyroid, innervated gastrocnemius fails to synthesize either IIb myosin heavy chain or its mRNA, the hypothyroid, denervated gastrocnemius synthesizes both.

The influence of thyroid hormones on contractile protein gene expression in developing skeletal muscle is not a general effect.

Although MHC transitions are clearly affected, myosin light chain developmental switches appear to be unaffected [5,10]. The cardiac and skeletal α-actin genes appear to be affected independently by thyroid hormone [1]. The skeletal α-actin promoter contains a thyroid hormone response element (TRE) [1, 6] which suggests that T3-induced increases in α-actin mRNA in animals [6, 41] may be mediated by a direct transcriptional mechanism. A TRE has not yet been identified in the promoter of the IIb MHC gene [34], however, suggesting that thyroid hormones may not have a direct effect on this MHC gene expression. TREs have been identified in the promoter regions of the cardiac MHC genes [18] which may explain the apparently different mechanisms of action of thyroid hormones in cardiac and skeletal muscle [4].

Our results suggest that it is unlikely that thyroid hormones themselves must interact with the muscle fiber to effect the transition from neonatal to adult fast myosin synthesis. Moreover, since the denervated, hypothyroid

animals do make adult IIb myosin heavy chain, thyroid hormones themselves must not be essential to the transition. This is supported by transfection experiments using the cardiac α-myosin heavy chain and cultured myocytes which have shown that some portions of the 5' untranslated region are important in thyroid regulation of cardiac myosin isoforms. Yet removal of most of the 5' region of the transfected gene did not completely abolish thyroid sensitivity [13].

Thus, we conclude that while thyroid hormones can alter the levels of the IIb myosin heavy chain - and can even accelerate its appearance during development, as in the hyperthyroid animals - they are probably not the cue for the shift from neonatal to adult myosin heavy chains. Rather, we suggest that the synthesis of IIb myosin heavy chain and its mRNA in the normal animal is the result of an interaction among neural and hormonal factors on all elements of the motor unit. One possible explanation for our results is that the immature motor neuron, in some way, blocks the synthesis of IIb myosin heavy chain mRNA. This inhibition can be removed in one of two ways. Either the motor neuron can be allowed to mature by the normal rise in T4 levels (this can occur precociously in hyperthyroid animals) or the motor neuron can itself be removed by denervation. In either case, the results suggest that the normal transitions from embryonic to neonatal to adult fast myosin heavy chains are endogenously programmed during development, but are closely orchestrated by the changing hormonal and neuronal status of the animal.

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References

[1] Alonso S, Lyons G, Garner I, Kelly AM, Proust E, Buckingham M: Expression of α-actin genes in developing skeletal muscles of hypothyroid rats. *BAM*; in press.

[2] Buller A, Eccles J, Eccles R: Interaction between motoneurones and muscles in respect of the characteristic speed of their responses. *J Physiol* 1960; 150: 417-439.

[3] Butler-Browne G, Bugaisky L, Cuenoud S, Schwarz K, Whalen RG: Denervation of newborn rat muscles does not block the appearance of adult fast myosin heavy chain. *Nature* 1982; 299: 830-833.

[4] Butler-Browne G, Prulière G, Cambon N, Whalen RG: Influence of the *Dwarf* mouse mutation on skeletal and cardiac myosin isoforms. *J Biol Chem* 1987; 262: 15188-15193.

[5] Butler-Browne G, Barbet JP, Thornell L: Myosin heavy and light chain expression during human skeletal muscle development and precocious muscle maturation induced by thyroid hormone. *Anat Embryol* 1990; 181: 513-522.

[6] Collie ESR, Muscat GEO: The human skeletal α-actin promoter is regulated by thyroid hormone: Identification of a thyroid hormone response element. *Cell Growth Diff* 1992; 3: 31-42.

[7] Cox RA: The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol* 1968; 12B: 120-129.

[8] d'Albis A, Chanoine C, Janmot C, Mira JC, Couteaux R: Muscle-specific response to thyroid hormone of myosin isoform transitions during rat postnatal development. *Eur J Biochem* 1990; 193: 155-161.

[9] Florini JR, Ewton DZ, Magri KA: Hormones, growth factors and myogenic differentiation. *Ann Rev Physiol* 1991; 53: 201-206.

[10] Gambke B, Lyons G, Haselgrove J, Kelly A, Rubinstein NA: Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. *FEBS Lett* 1983; 156: 335-340.

[11] Gambke B, Rubinstein NA: A monoclonal antibody to the embryonic myosin heavy chain of the rat. *J Biol Chem* 1984; 259: 12092-12100.

[12] Gunning P, Hardeman E: Multiple mechanisms regulate muscle fiber diversity. *FASEB J* 1991; 5: 3064-3070.

[13] Gustafson TA, Markham BE, Bahl JJ, Morkin E: Thyroid hormone-regulates expression of a transfected α-myosin heavy chain fusion gene in fetal heart cells. *Proc. Natl. Acad. Sci. USA* 1987; 84: 3122-3126.

[14] Gustafson TA, Markham EE, Morkin E: Analysis of thyroid hormone effects on myosin heavy chain gene expression in cardiac and soleus muscles using a novel dot blot assay. *Biochem Biophys Res Commun* 1985; 130: 1161-1167.

[15] Havarnis AS, Heywood SM: Cytoplasmic utilization of liposome-encapsulated myosin heavy chain messenger ribonucleoprotein particles during muscle cell differentiation. *Proc Natl Acad Sci USA* 1981; 78: 6898-6902.

[16] Ianuzzo C, Patel P, Chen V, O'Brien P (1980) A possible thyroidal trophic influence on fast and slow skeletal myosin. in: Pette D (ed) The *Plasticity of Muscle.* 1980 Walter de Gruyter, Berlin pp. 593-605.

[17] Izumo S, Nadal-Ginard B, Mahdavi V: All members of the MHC multigene family respond to thyroid hormone in a highly tissue specific manner. *Science* 1986; 231: 597-600.

[18] Izumo S, Mahdavi V: Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC transcription. *Nature* 1988 334: 539-543.

[19] Jansen JKS, Fladby T: The perinatal reorganization of the innervation of skeletal muscle in mammals. *Prog Neurobiol* 1990; 34: 39-90.

[20] Kawa K, Obata K: Altered developmental changes of neuromuscular junction in hypo- and hyperthyroid rats. *J Physiol* 1982; 329: 143-161.

[21] Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage. *Nature* 1970; 227: 680-685.

[22] Lyons GE, Kelly AM, Rubinstein NA: Testosterone-induced changes in contractile protein isoforms of the sexually-dimorphic temporalis muscle of the guinea pig. *J Biol Chem* 1986; 261: 13278-13284.

[23] Lyons GE, Ontell M, Cox R, Sassoon D, Buckingham M: The expression of myosin genes in developing skeletal muscle in the mouse embryo. J *Cell Biol* 1990; 111: 1465-1476.

[24] Mahdavi V, Chambers AP, Nadal-Ginard B: Cardiac α- and β-myosin heavy chain genes are organized in tandem. *Proc. Natl. Acad. Sci. USA* 1984; 81: 2626-2630.

[25] Maxam AM, Gilbert W: Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* 1980; 65: 499-543.

[26] Milstone LM, McGuire J: Different polypeptides form the intermediate filaments in bovine hoof and esophageal epithelium and in aortic endothelium. *J Cell Biol* 1981; 88: 312-316.

[27] Morreale de Escobar G, Pastor R, Obregon MJ, Escobar de Rey F: Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues before and after the onset of fetal thyroid function. *Endocrinology* 1985; 117: 1890-1900.

[28] Nadal-Ginard B. Medford RM, Nguyen HT, Periasamy M, Wydro RM, Horning D, Gubits RM, Garfinkel LI, Wieczorek DF, Mahdavi V: Structure and regulation of a mammalian sarcomeric myosin heavy chain gene, in Pearson ML and Epstein HF (eds): *Muscle Development: Molecular and Cellular Control.* 1982 Cold Spring Harbor: Cold Spring Harbor Laboratory, pp 143-168.

[29] Navarette R, Vrbova G: Changes of activity patterns in slow and fast muscles during postnatal development. *Dev Brain Res* 1983; 8: 11-19.

[30] Periasamv M, Wieczorek D, Nadal-Ginard B: Characterization of a developmentally regulated perinatal myosin heavy chain gene expressed in skeletal muscle. *J Biol Chem* 1984; 259: 13573-13578.

[31] Rave N, Crkvenjakov R, Boedtker H: Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. *Nuc Acids Res* 1979; 6: 3559-3567.

[32] Rubinstein NA, Mabuchi R, Pepe FA, Salmons S, Gergely J, Sreter F: Use of type specific antimyosins to demonstrate the transformation of individual fibers in chronically stimulated rabbit fast muscles. *J Cell Biol* 1978; 79: 252-261

[33] Russell SD, Cambon N, Nadal-Ginard B, Whalen RG: Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb muscle. J *Biol Chem* 1988; 263: 6370-6374.

[34] Takeda S, North DL, Lakich MM, Russell SD, Whalen RG: A possible regulatory role for conserved promoter motifs in an adult-specific muscle myosin gene from mouse. J *Biol Chem* 1992; 267: 16957-16967.

[35] Thomas P: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci* USA 1980; 77: 5201-5205.

[36] Weeds A, Trentham D, Kean C, Buller A: Myosin in cross-reinnervated cat muscles. *Nature* 1974; 247 : 135-139.

[37] Weydert A, Daubas P, Caravatti M, Minty A, Bugaisky G, Cohen A, Robert B, Buckingham M: Sequential accumulation of mRNAs encoding different myosin heavy chain isoforms during skeletal muscle development *in vivo* detected with a recombinant plasmid identified as coding for an adult fast myosin heavy chain from mouse skeletal muscle. *J Biol Chem* 1983; 258: 13867-13874.

[38] Whalen RG, Butler-Brcwne GS, Gros F: Identification of a novel form of myosin light chain present in embryonic muscle tissue and cultured muscle cells. *J Mol Biol* 1978; 126: 415-431.

[39] Whalen R, Sell S, Butler-Browne G, Schwartz K, Bouveret P, Pinset-Harstrom I: Three myosin heavy chain isozymes appear sequentially in rat muscle. *Nature* 1981; 292: 805-809.

[40] Whalen RG, Toutant M, Butler-Browne G, Watkins S: Hereditary pituitary dwarfism in mice affects skeletal and cardiac myosin isozyme transitions differently. *J Cell Biol* 1985; 101: 603-609.

[41] Winegrad S, Wisnewsky C, Schwartz K: Effect of thyroid hormone on the accumulation of mRNA for skeletal and cardiac α-actin in hearts from normal and hypophysectomized rats. *Proc Natl Acad Sci USA* 1990; 87: 2456-2460.