Slow Myosin in Developing Rat Skeletal Muscle

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Abstract. Through S1 nuclease mapping using a specific cDNA probe, we demonstrate that the slow myosin heavy-chain (MHC) gene, characteristic of adult soleus, is expressed in bulk hind limb muscle obtained from the 18-d rat fetus. We support these results by use of a monoclonal antibody (mAb) which is highly specific to the adult slow MHC. Immunoblots of MHC peptide maps show the same peptides, uniquely recognized by this antibody in adult soleus, are also identified in 18-d fetal limb muscle. Thus synthesis of slow myosin is an early event in skeletal myogenesis and is expressed concurrently with embryonic myosin.

By immunofluorescence we demonstrate that in the 16-d fetus all primary myotubes in future fast and future slow muscles homogeneously express slow as well as embryonic myosin. Fiber heterogeneity arises owing to a developmentally regulated inhibition of slow MHC accumulation as muscles are progressively assembled from successive orders of cells. Assembly involves addition of new, superficial areas of the anterior tibial muscle (AT) and extensor digitorum longus muscle (EDL) in which primary cells initially stain weakly or are unstained with the slow mAb. In the developing AT and EDL, expression of slow myosin is unstable and is progressively restricted as these muscles specialize more and more towards the fast phenotype. Slow fibers persisting in deep portions of the adult EDL and AT are interpreted as vestiges of the original muscle primordium.

A comparable inhibition of slow MHC accumulation occurs in the developing soleus but involves secondary, not primary, cells. Our results show that the fate of secondary cells is flexible and is spatially determined. By RIA we show that the relative proportions of slow MHC are fivefold greater in the soleus than in the EDL or AT at birth. After neonatal denervation, concentrations of slow MHC in the soleus rapidly decline, and we hypothesize that, in this muscle, the nerve protects and amplifies initial programs of slow MHC synthesis. Conversely, the content of slow MHC rises in the neonatally denervated EDL. This suggests that as the nerve amplifies fast MHC accumulation in the developing EDL, accumulation of slow MHC is inhibited in an antagonistic fashion.

Studies with phenylthiouracil-induced hypothyroidism indicate that inhibition of slow MHC accumulation in the EDL and AT is not initially under thyroid regulation. At later stages, the development of thyroid function plays a role in inhibiting slow MHC accumulation in the differentiating EDL and AT. The effects of the nerve and of thyroid hormone on these developing fast muscles therefore appear synergistic. In the adult AT and EDL, hypothyroidism causes a significant rise in proportions of slow MHC, which selectively accumulates in type IIA and not IIB fibers. This pattern of accumulation is not a simple recapitulation of early programs of slow MHC expression.

Myosin heavy chains (MHCs) are encoded by a multigene family which is developmentally regulated (30–32, 36, 37, 40, 49). For example, during differentiation of future fast muscles in the rat, fibers sequentially express heavy-chain genes for embryonic, neonatal, and adult fast myosin (31). Less is known about expression of slow MHC genes during development. In previous studies we have drawn attention to the relationship between expression of MHCs and the pattern of muscle histogenesis (19, 20, 29, 43). Whereas all muscle fibers initially synthesize the embryonic MHC, early developing or primary muscle cells (18) react, in addition, with our polyclonal antibody against the heavy chain of adult slow myosin. Many of these primary fibers correspond to adult slow, type I, cells in distribution.

Abbreviations used in this paper: AT, anterior tibial muscle; EDL, extensor digitorum longus muscle; MHC, myosin heavy chain; PTU, phenylthiouracil.
Most later developing or secondary generation cells fail to react with this antibody and apparently mature into fast, type II, fibers. We have shown that these distinctions in staining between primary and secondary cells are expressed in the rat as early as in 18-d gestation. This indicates that the initial commitment of fibers to their specialized genetic program must take place at an even earlier stage of development. Consequently, we have proposed that the generation of diversity is a slowly progressive amplification and modification of distinctions in programming that are set early in myogenesis (29).

In previous studies we have questioned whether the matching of neurons to fibers initiates diversity (29), or whether there are intrinsic differences between the distinct generations of fibers (19). Some more recent studies in the chick have graphically demonstrated that early diversity precedes innervation and is not the consequence of neuromuscular synapsing (3, 7, 33, 34, 41). Initial fiber specialization in the chick is thus controlled by factors other than by neuromuscular contact, which suggests that, once a nerve penetrates a muscle, it must then search for its appropriate myotube target which already is partially determined. Elegant studies showing normal patterns of fiber diversification in chick limbs that had received inappropriate innervation as a result of transplantation support this conclusion (25). However, all of these results leave open the question of why polyneural innervation and is not the consequence of neuromuscular innervation occurs in developing muscle and why reinervation appears nonselective in the adult.

Our interpretation of muscle specialization requires that either the definitive adult slow MHC or a closely related species is expressed in a subset of fibers in utero. To support this idea we have presented biochemical evidence for the presence of small amounts of slow skeletal MHCs in prenatal rats, both by pyrophosphate gel electrophoresis and by peptide maps of purified myosin (29). Dhoot (8) has recently presented evidence that correlates well with these results. However, the identity of the slow isozyme remains unclear, because the mobility of early slow isozymes on nondenaturing gels does not appear identical to the mobility of slow isozymes of adult muscle. Despite these results, the question of slow myosin expression and its differential distribution in early developing muscle remains controversial. Gauthier et al. (11) claim that all fibers in the fetal rat hind limb react with antibodies purported to be specific to either fast or slow MHCs. The possibility that this represents cross-reactivity with embryonic or neonatal myosin is discussed by Gauthier et al. (11), but in contrast to our results, these authors emphasize that peptide analysis of myosin from embryonic rat muscle reveals an absence of slow myosin. This conclusion appears to correlate with the classic biochemical analysis of developing rat skeletal muscle myosins by Whalen et al. (46), in which slow myosin isozymes were not detected until ~14 d postpartum. These studies therefore suggest that the initiation of diversity coincides with development of posture and imply that tonic neural activity initiates adult slow myosin synthesis.

Because of these ambiguities, we have performed SI nuclease mapping, which allows the detection of closely related gene products with high sensitivity and specificity (2). We have used a cDNA probe specific to the slow MHC gene (27, 30, 31) to determine when this gene is expressed in fetal and neonatal rat limb muscles. We have also used a monoclonal antibody, NOQ7.5.4D, which is highly specific to the slow MHC, in order to probe the accumulation and control of slow myosin in developing muscle. We show that the same slow β-MHC gene that is expressed in the adult soleus and fetal ventricle (27) is also expressed in all fetal rat limb muscles. In view of this finding, we have followed the differential expression of slow MHC in primary and secondary cells of the soleus, extensor digitorum longus muscle (EDL), and anterior tibial muscle (AT) from 16 d of gestation to 6 mo of age. We have also explored the potential of the nerve and of thyroid hormone to regulate slow myosin synthesis during development.

**Materials and Methods**

**RNA Isolation**

Total cytoplasmic RNA was isolated from various muscles by the hot phenol procedure (44) and stored at −20°C in ethanol.

**SI Nuclease Mapping Analysis**

The cDNA clone, pCMHCS, was digested with restriction endonuclease Pst I, 3'-end-labeled with tritiated cytidine triphosphate (3) and size-separated by 6% acrylamide gel electro- phoresis as described (27). The desired fragment was isolated and strand separated as described (27). The probe was then hybridized in DNA excess to 20 μg of total cellular RNA in 25 μl of 80% formamide, 10 mM Pipes (pH 6.4), 1 mM EDTA, and 0.05% SDS for 16 h at 42°C. SI nuclease digestion was performed with 150 U of enzyme (New England Nuclear, Boston, MA) for 1 h at 25°C, and the digestion products were separated on a 5% polyacrylamide, 8.3 M urea, sequencing gel as described (27). The autoradiogram was performed at −80°C with an intensifying screen (Dupont Co., Wilmington, DE).

**Protein Purification**

Actomyosins or column purified myosins were prepared according to previously described methods (43, 47). Samples for gel electrophoresis were redissolved in Laemmli sample buffer (24). For RIA, samples were dissolved in pyrophosphate buffer (20 mM Na3PO4, 1 mM EDTA, pH 8.8) containing 50% glycerol. Protein concentrations were determined by the method of Lowry et al. (28) using bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, MO) as the standard.

**Thyroid Hormone Perturbation**

Hypothyroidism was induced by adding 0.05% phenylthiouracil (PTU) to the drinking water of pregnant rats from 10 d of gestation onwards and to the drinking water of their offspring from birth to 24 wk of age as described previously (12). This regime inhibits the rise in T3 and T4 values characteristic of the adolescent rat (5, 12).

**Monoclonal Antibody (mAb) Preparation**

Myosins prepared from adult human muscle and from neonatal rat limb muscles were used as immunogens. BALB/c mice were immunized as described previously (13). Isolated splenocytes were fused with an NSO myeloma cell line. Hybridoma cells were screened for antimyosin immunoglobulin secretion with an indirect, solid-phase RIA (1) or by ELISA. Positive wells were cloned by both agar plate colony and limiting dilution.

**Solid-Phase RIA**

A solid-phase RIA was used for screening cell line supernatants for specific immunoglobulins and for assaying slow MHC concentrations of various muscles during development, as described by Gambke and Rubinstein (13). 125I-labeled rabbit anti-mouse F(ab) for the assay was provided by Dr. M. Cancro, University of Pennsylvania. Bound label was assayed by spectrometry (Gamma Trac 1290, Tracor Analytic, Elkgrove, IL).
SDS PAGE and Immunoblots

Actomyosins and column-purified myosins were analyzed by electrophoresis on SDS PAGE using the buffer system of Laemmli (24). Gels were 1.0 mm thick, 15% acrylamide, and 0.05% in N,N'-methylene-bis-acrylamide. Gels were stained in 0.1% Coomassie Blue in methanol and acetic acid and destained according to previously described methods (13). Proteins on unstained gels were electrophoretically transferred to nitrocellulose paper at 0.4 A for 1 h using a Transphor TE-42 electrophoresis cell ( Hoefer Scientific Instruments, San Francisco, CA) with 0.05 M Tris, 0.2 M glycine, and 30% methanol as the transfer buffer. After the transfer, the nitrocellulose paper was incubated in wash buffer, 0.05 M Tris, 0.15 M NaCl, 0.1% (w/v) bovine serum albumin, 0.05% (v/v) Nonidet P-40 overnight.

Antibody binding to proteins on the nitrocellulose paper using mAb NOQ7.5.4D supernatant was at a 1:40 dilution with wash buffer containing 5 mg/ml normal rabbit serum. At this antibody dilution and both higher and lower dilutions, NOQ7.5.4D was found to be highly specific for slow MHC.

Immunofluorescence

Indirect immunofluorescence was performed on 8-μm-thick frozen sections of whole limbs of rats from 16 d of gestation to 15 d postpartum. At later stages, individual muscles were sectioned. Sections were first incubated in a 1:30 dilution of NOQ7.5.4D supernatant in standard salt solution for 1 h at 37°C, then washed three times in standard salt, and incubated for 1 h at 37°C with fluorescein-labeled goat anti-mouse IgG (heavy and light chain) antibody (Cappel Laboratories, Cochranville, PA) diluted 1:20 with standard salt. Sections were washed three times with standard salt and mounted with standard salt containing 25% glycerol. Controls for immunofluorescence included sections stained with negative hybridoma culture supernatants and with fluorescein-labeled goat anti-mouse IgG alone.

Animals

All muscles were obtained from Sprague-Dawley rats. For RIA, material obtained from rats younger than 30 d of age was pooled from at least five animals. Measurements were performed using muscles from two separate groups of animals for each stage and each treatment. For animals older than 30 d, muscles were obtained from at least two females at each stage and for each treatment. At 24 wk, material was from two "retired breeder" female rats. Denervation of the distal hind limb was obtained by removing a 4-mm segment of the sciatic nerve in the proximal thigh from rats at birth when isolated on non-denaturing gels.

Figure 1. S1 nuclease-mapping analysis showing slow MHC gene expression in developing skeletal and cardiac muscle. The cDNA probe was hybridized to 20 μg of total cellular RNA from fetal heart (lane B), 20-d fetal soleus (lane C), adult soleus (lane D), and 18- (lane E) and 20- (lane F) d fetal bulk limb muscle. Lane G is from hind limb muscle at 7 d postpartum and lane H from a mixed adult skeletal muscle. The probe used was the 3'-end-labeled, single-stranded Pst I fragment of pCMHC5, a cDNA clone specific to the rat slow β-MHC gene. This 347-nucleotide (nt)-long probe (lane A) contains 180 nt of coding sequence at the carboxyl end of the heavy chain and the entire 3'-untranslated sequence of the slow β-MHC gene. It also contains 43 nt of oligo-dT and -dG tails. When hybridized to an homologous mRNA, this probe yields a 304-nt-long fragment whereas the α-MHC mRNA produces a 180-nt-long, partially protected fragment (15, 27). The other MHC mRNA species are not detected with this probe.

Results

Slow MHC Expression

We have searched for the presence of slow MHC mRNAs in the early embryo by S1 nuclease mapping using as a probe, a 3'-end Pst I fragment of pCMHC5, a cDNA clone specific for the rat slow MHC gene (27, 30). With this probe it has been demonstrated that the cardiac β-MHC and skeletal slow MHC are the products of the same gene (27, 30).

When this probe is hybridized to RNAs from the adult soleus or from fetal heart, a fully protected, 304-nucleotide-long fragment is generated (Fig. 1, B and D). The fetal heart also generates a 180-nucleotide-long fragment that corresponds to the α-MHC mRNA (not shown). The fetal soleus at 20 d of gestation contains a high level of slow MHC mRNA (Fig. 1 C). The full protection of the probe was also detected with RNAs from 18- and 20-d fetal bulk limb muscle (Fig. 1, E and F) and from muscle at 7 d postpartum (Fig. 1 G). Thus even these future fast muscles, which are predominantly expressing embryonic and neonatal myosin (31, 40), also express slow MHC mRNA, before birth. The slow MHC gene is being transcribed as early as at 18 d of gestation, the earliest time point examined, and it is more actively expressed in the future slow, soleus muscle than in the future fast muscles in utero. It is likely that the gene is expressed at even earlier times as is shown below.

Immunohistochemical Analysis: Antibody Specificity

An IgG mAb, NOQ7.5.4D, specifically recognizes slow, and not fast, MHC of adult muscle (Fig. 2). This antibody does not react with the heavy chain of rat muscle at 8 d in culture (entirely embryonic myosin), nor does it react with embryonic or neonatal myosin isoforms from the rat masseter at birth when isolated on nondenaturing gels.

Figure 2. Specificity of mAb NOQ7.5.4D to slow MHC in adult muscle. MHC from adult rat fast muscles, the EDL (b), tensor fascia lata (c), tongue (e), and AT (f) and from the slow-twitch soleus (d) was isolated and displayed on 12.5% SDS polyacrylamide gels. 5 μg of protein was loaded per lane. The Coomassie Blue-stained gel is on the left and the immunoblot of an identical gel reacted with the slow monoclonal antibody is on the right. Lane a contains C protein. Only MHC from the soleus (d) reacts with the antibody.
To investigate the presence of slow MHC accumulation in developing muscle, we have column-purified myosin obtained from the adult soleus, the soleus and masseter muscles of the rat at birth, and from 18-d fetal bulk muscle. Samples were subjected to limited proteolysis by chymotrypsin and the products were analyzed on 15% SDS polyacrylamide gels. The specific peptides recognized by the slow mAb were distinguished on immunoblots (Fig. 3 A and B).

Peptides binding the antibody in 18-d fetal bulk muscle and in the neonatal and adult soleus muscles have precisely the same electrophoretic mobility. The neonatal masseter is predominantly composed of fibers containing embryonic and neonatal myosin. This muscle does, however, include a small population of slow fibers. Blots made from peptide maps of purified neonatal masseter myosin, demonstrate only faint staining of peptides identical to those in the soleus (Fig. 3 B).

These results correlate with the cDNA analysis by demonstrating the same slow MHC peptides uniquely recognized in adult soleus are also present and uniquely recognized by the antibody in fetal and neonatal muscle.

Immunocytochemistry

Frozen sections were obtained from the AT, EDL, and soleus muscle at successive stages of development. Serial sections were stained by an indirect technique with NOQ7.5.4D and with 2G3, an IgG mAb that binds with embryonic, neonatal, and adult fast MHCs but not with slow MHCs.

In the 16-d fetus, the AT and EDL as well as the soleus muscle are entirely composed of primary generation myotubes (43), all of which uniformly stain with both antibodies (Fig. 4 a). Hence, all primary generation myotubes present in both the future fast AT and EDL and in the future, slow soleus muscle initially express slow MHC as well as embryonic MHCs. Similar, homogeneous patterns of staining in the 16-d gastrocnemius, diaphragm, intercostals, pectoralis, and rectus abdominis at 16 d in utero suggest that accumulation of slow MHC consistently accompanies expression of embryonic MHC in early muscle primordia.

By 18 d of gestation, the sizes of the AT and EDL are greatly increased. The AT, which at 16 d lay apart from, and had a simple straight border facing the EDL (Fig. 4 a), now abuts and forms a superficial crest over the EDL (Fig. 4 b). In contrast to 16 d, there is heterogeneity of antibody staining among myotubes of the EDL and AT at 18 d of gestation (Fig. 4 b), indicating that in these two muscles, fibers are now demonstrably committed to distinct pathways of specialization.

In the AT and to a lesser extent in the EDL at 18 d in utero, two domains are apparent after staining with NOQ7.5.4D. In deep, axial regions, primary myotubes lie close to one another and stain intensely with this slow mAb. By contrast, the myotubes are more dispersed and have variable but generally weaker affinity for NOQ7.5.4D in the superficial portions of the AT and EDL (Fig. 4 b). An exception is the peripheral border of the AT, which is delineated by a continuous row of myotubes, the majority of which stain well with NOQ7.5.4D (Fig. 4 b). The reason for this circumferential border of myotubes is unclear but may relate to positional growth and the addition of muscle bundles to the expanding AT.

In the AT at this stage, the deep regional distribution of primary myotubes staining well with NOQ7.5.4D corresponds to the distribution of slow, type 1 cells in the red portion of the adult AT (see, for example, Fig. 5 b). Consequently, we interpret the superficial and deep domains of the fetal AT as precursors of the red and white portions of the AT in the adult.

The size of the AT and EDL is further increased by 21 d of gestation and differential antibody staining between superficial and deep regions is more marked than previously (Fig. 5 a). Primary myotubes in the deep areas of both muscles continue to stain well with NOQ7.5.4D, but affinity for this antibody is markedly reduced among primary cells in the superficial areas. In addition, secondary cells, which are now abundant throughout both muscles, fail to stain with NOQ7.5.4D. These findings suggest that as histogenesis proceeds, accumulation of slow myosin is progressively inhibited among the fibers that are determined to be phenotypically fast.

Examination of the EDL and AT after birth reinforces this conclusion. At 10 d, the superficial areas of the EDL and AT are now without fibers that stain with NOQ7.5.4D. All fibers in these areas stain well with 2G3, indicating progressive
specialization towards the fast phenotype (Fig. 5 b). Our results therefore illustrate that the equivalent staining of primary and secondary fibers in the superficial, fast regions of the AT is the result of convergence of phenotypes with differing developmental histories. By contrast, primary fibers in the deep regions of the AT and EDL at 10 d stain well with NOQ7.5.4D, whereas staining with 2G3 is now weak (Fig. 5 b). Hence, while this select population increasingly specializes as slow type I cells, accumulation of embryonic and neonatal myosin is progressively inhibited.

The numbers of fibers staining with the slow antibody continue to decline in the EDL and AT until maturity. For example, in the EDL the original 300 slow-staining fibers present at 21 d of gestation is progressively reduced to ~100 slow fibers in the adult (Table 1). Thus the switch towards fast phenotypes is a long-term modulation and with age the EDL and AT progressively become more and more specialized as fast muscles.

Differentiation of the soleus follows a modification of the program in the EDL and AT. Growth of this unipennate mus-
Figure 5. Immunofluorescence of transversely sectioned AT and EDL at 21 d of gestation and 10 d postpartum, prepared as in Fig. 4. (a) At 21 d of gestation the contrast in staining with NOQ7.5.4.4 D between superficial and deep domains of the AT and EDL is more marked than at 18 d. Bar, 75 μm. (b) At 10 d postpartum, staining with NOQ7.5.4D is eliminated from fibers in superficial regions of the AT and, to a lesser extent, of the EDL. Fibers in axial regions of both muscles now exclusively react with the slow antibody and no longer react with 2G3. We interpret these slow fibers as vestiges of the primordium present at 16 d. Bar, 180 μm.
Table I. Numbers of Fibers Reacting with the Slow MHC Antibody in the EDL and AT from 21 d of Gestation to Maturity

| Muscle | 21-day fetus | 10 | 15 | 25 | 30 | 45 | Adult |
|--------|--------------|----|----|----|----|----|-------|
| EDL    | 289          | 238| 282| 196| 202| 192| 94±16 |
| AT     | 628          | 320| 311| 289| 233|- - - |

Fiber counts were made from montages reconstructed from transverse sections through the middle of the two muscles at each stage of development. In both muscles the numbers of slow staining fibers progressively declines with age.

The muscle is entirely by interstitial addition of fibers and regional variations of diversity do not occur. At 18 d of gestation the soleus is composed of 300–400 primary myotubes at comparable stages of development. These cells homogeneously stain well with NOQ7.5.4D but poorly with 2G3 (Fig. 6).

At 21 d in utero the soleus contains an average of 1,633 ± 208 fibers (n = 3). Of these, 1,100 ± 100 primary and secondary cells stain with NOQ7.5.4D. Although a majority of secondary cells stain with NOQ7.5.4D at this stage, the intensity of staining is not equivalent to that of the larger primary cells (Fig. 7 a). Throughout the muscle, all fibers have equivalent weak staining with 2G3.

Secondary-generation cells continue to be added to the soleus for 1–2 d postpartum but, in contrast to intrauterine development, these newly added cells stain well with 2G3 and do not stain with NOQ7.5.4D. As a result, heterogeneity of staining with 2G3 emerges in this muscle (Fig. 7 b). At 2 d the soleus contains 2,600 ± 282 fibers (n = 3). Of these, 1,700 ± 141 fibers stain with NOQ7.5.4D. The remainder stain exclusively with 2G3. At 10 d the total fiber population remains at 2,600. Of this population, 1,500 fibers stain well with NOQ7.5.4D and do not stain with 2G3 (Fig. 7 c). These are type I cells. Conversely, 1,100 fibers exclusively stain with 2G3 and appear to be precursor type IIa fibers.

These results indicate that, as secondary fibers are added to the soleus late in gestation, a majority accumulate slow MHC. Some of these secondary cells must differentiate into slow fibers, for we are otherwise unable to account for the total population of type I cells in this muscle. Secondary fibers added after birth do not accumulate slow MHC and become fast IIa fibers. Thus, as in the developing EDL and AT, there is a progressive shift from slow to fast pathways of specialization as the soleus progressively differentiates. This conclusion is consistent with our previous studies and with recent studies by Dhoor (8).

Radioimmunoassay

Normal Development. We have used NOQ7.5.4D to measure the relative proportions of slow MHC in the EDL, AT, and soleus while these muscles progress through postnatal maturation. 1 µg of purified myosin was immobilized on polyvinyl chloride microtiter plates and slow myosin quantitated by solid-phase RIA with NOQ7.5.4D and a radiolabeled rabbit anti-mouse antibody. Slow myosin is reported as counts per minute per 1-µg sample.

Relative proportions of slow myosin in the AT drop from 600 cpm at birth to 100 cpm at 30–40 d (Fig. 8). In the EDL, proportions of slow myosin rise from birth until 15 d and then decline. Slow myosin in the gastrocnemius (not shown) follows a course similar to that in the EDL. These results correlate with the perinatal rise in slow MHC transcripts illustrated in Fig. 1 and apparently reflect a progressive increase in slow MHC accumulation as type I fibers progressively specialize postpartum.

The relative proportions of slow MHC in the soleus at...
Figure 7. Soleus at 21 d of gestation (a and b), 2 d postpartum (c and d), and 10 d postpartum (e and f). The transverse sections are stained with NOQ7.5.4D (a, c, e) and with 2G3 (b, d, f). At 21 d of gestation, most secondary-generation cells stain with both 2G3 and NOQ7.5.4D (arrows, a and b). At 2 d, staining of some secondary cells with NOQ7.5.4D persists (black arrows, c and d). Heterogeneity of staining with 2G3 is now present and a population of secondary-generation fibers which uniquely stain with 2G3 is evident (white arrows, c and d). This population is a prominent feature of the muscle by 10 d. Corresponding sections of a differentiating slow fiber at 10 d (arrows, e and f). Bar, 40 μm.

Figure 8. RIA of the relative proportions of slow MHC in the AT, EDL, and soleus during normal development and after neonatal denervation. The assay was made with NOQ7.5.4D. At each stage measurements were made from more than one animal, and for each sample measurements were made in triplicate. Results are expressed as the mean ± 1 SD. Whereas proportions of slow MHC in the soleus decline after denervation, they rise in the denervated EDL.
birth are significantly higher than in the EDL and AT and emphasize that specialization commenced at an earlier stage (Fig. 8). Proportions of slow MHC rise by 30% to 15 d and then continue to rise slowly until 24 wk. The initial rise may be due to hypertrophy and elimination of embryonic myosin among a defined population of slow fibers. After 30 d we correlate the more slowly progressive rise with transformation of secondary-generation, fast type IIA into slow fibers, a process specifically known to occur in the maturing soleus (6, 23).

**Denervation.** To investigate influences of motor innervation on slow MHC expression during terminal differentiation, we denervated the hind limb of rats at birth by removing a 4-mm segment of the sciatic nerve in the proximal thigh. Relative proportions of slow MHC in the denervated EDL and soleus were measured at 5, 10, 15, and 30 d (Fig. 8). Proportions of slow MHC in the soleus declined from 3,500 cpm at 5 d to 850 cpm at 30 d, indicating that expression of slow myosin in this developing muscle is neurally dependent. This result was anticipated from previous studies (12, 42). However, we did not anticipate the change in the EDL after denervation. Proportions of slow MHC in this denervated muscle rose from 600 cpm at 5 d to 2000 cpm at 30 d. Consequently, at 30 d, the content of slow MHC in the denervated EDL exceeded that of the denervated soleus by a twofold margin (Fig. 8).

**Hypothyroidism.** Because expression of the slow β-MHC gene is reported to be inhibited perinatally in cardiac muscle as the result of the development of thyroid function (5, 26), we questioned whether it is similarly regulated in differentiating skeletal muscle. Hypothyroidism was induced by continuously supplying 0.05% PTU to the drinking water of pregnant rats from 10 d of gestation until 6 mo of age. This method has proved effective in inhibiting the elevation of thyroid levels in young rats (12).

There is remarkable plasticity in expression of slow MHC in the ventricle of euthyroid animals as the rat matures (Fig. 9). Slow β-MHC declines from 3,000 cpm at birth to almost undetectable levels at 30 d and then recovers, rising back to over 3,000 cpm by 24 wk. Differences between the hypothyroid and euthyroid ventricle emerge between birth and 5 d and our results support the conclusion that the decline in slow MHC accumulation in the ventricle of euthyroid animals is under thyroid control.

Fig. 9 also outlines the relative proportions of slow MHC in the soleus, EDL, and AT muscles of hypothyroid animals from birth to 24 wk. Throughout this period, hypothyroidism does not affect accumulation of slow MHC in the soleus. This is consistent with recent studies by Izumo et al. (17). We find no significant difference in slow myosin concentrations in the EDL and AT from euthyroid and hypothyroid animals between birth and 10 d of age. After 15 d, differences become increasingly evident as levels progressively rise in thyroid-deficient animals and, by 24 wk, levels are elevated.
Figure 10. Montage made from transverse sections of AT from the deep, axial region (lower) to the peripheral region (upper). Muscle is from euthyroid (a) and hypothyroid (b) rats at 6 mo of age. The hypothyroid rat was continuously exposed to PTU from 10 d in utero. The hypothyroid AT is small and contains a remarkable increase in the numbers of slow fibers in axial regions. However, there is no reexpression of slow MHC in the periphery of the muscle. Bar, 400 μm.

two- to fourfold. Thus the thyroid appears to assume control over slow MHC synthesis in the EDL and AT during adolescence.

Immunohistochemically, we find that the numbers and distribution of fibers staining with NOQ7.5.4D in the hypothyroid AT and EDL at 10 d postpartum is identical to that of euthyroid animals. Thus, thyroid hormone does not appear to be involved in the initial inhibition of slow MHC accumulation in these muscles.

Immunocytochemical studies also show that the rise in slow MHC concentration in the hypothyroid AT between adolescence and maturity is due to an absolute increase in numbers of slow fibers. However, these are restricted to the deep, red portions of the muscle and do not involve superficial white regions (Fig. 10). Increased accumulation of slow myosin in the hypothyroid AT is thus limited to a spatially determined subset of fibers, probably type IIa fibers, and does not represent a simple recapitulation of early programs of slow myosin synthesis.

Discussion

We show by S1 nuclease mapping, using a specific cDNA probe, that the slow β-MHC gene characteristic of the adult soleus and fetal ventricle, is expressed in bulk hind limb muscle obtained from the 18-d rat fetus (Fig. 1). At this stage, the level of expression is low but it progressively increases during the perinatal period. We support these results by immunohistochemical analysis of developing limb muscles using a monoclonal antibody, NOQ7.5.4D, which is highly specific to adult slow MHC (Fig. 2). Immunoblots of MHC peptide maps show that the same peptides, uniquely recognized by this antibody in the adult soleus are also present in 18-d bulk fetal limb muscles (Fig. 3). Immunocytochemically we find slow myosin is homogeneously expressed by all primary generation cells in primordia of the EDL, AT, and soleus of the 16-d fetus (Fig. 4). This appears to be a general characteristic of early muscle histogenesis for we find similar, uniform patterns of slow MHC staining in the primordia of the diaphragm, intercostals, pectoralis, triceps, and rectus abdominis at 16 d in utero. Synthesis of slow MHC is therefore an early event in myogenesis of both future fast and future slow skeletal muscles and is expressed concurrently with embryonic myosin in one population of cells. Inasmuch as the early development of primary generation cells is considered to be autonomous (7, 15, 33), we conclude that the initial activation of the slow MHC gene is independent of innervation. This evaluation is based on the characterization of NOQ7.5.4D with known MHCs. Our results are consistent with studies showing staining with a mAb to slow MHC in rat muscle at 14 d gestation (8) and with the report of slow myosin expression in explanted cultures of rat muscle (9).

Unlike the chick in which fiber heterogeneity is reported to be present from the inception of muscle histogenesis (34), fiber heterogeneity in the rat EDL, AT, and soleus progressively evolves from the homogeneous primordium. This evolution, which involves modulation of the slow MHC gene, appears to be directed by at least two developmental mechanisms. One mechanism involves regional patterns of fiber specialization and is of importance in the EDL and AT but not the soleus. By 18 d of gestation, staining with

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NOQ7.5.4D shows that the AT and, to a lesser extent, the EDL are divided into two domains that anticipate specialization into red, the oxidative, and white, the glycolytic, regions in the adult (Fig. 4 b). This partition is due to amplification of slow MHC accumulation in select populations of primary myotubes lying in the axial portions of the EDL and AT, and to progressive inhibition of slow MHC accumulation in primary and secondary generation cells throughout the superficial portions of these muscles (Figs. 4 and 5). The primary cells, in which slow MHC is amplified, differentiate as slow type I fibers (Figs. 4 and 5) as we have described previously (43). Fibers, in which slow MHC is inhibited, become fast, type II fibers and are particularly abundant in the superficial portions of the AT and EDL. These sharply defined, regional differences therefore suggest that after 16 d in utero the pattern of MHC accumulation is somehow influenced by myotube position in the developing limb.

The second developmental process involves the temporal pattern of skeletal muscle histogenesis from successive generations of cells (17). Regardless of regional differences, the largest accumulations of slow MHC in the EDL and AT occur in primary-generation cells. Slow myosin persists longest in these cells as inhibition advances (Figs. 4 and 5). Occasional secondary-generation cells also react with NOQ7.5.4D (cf. Dhoot [8]). In these cells, staining is always weak and slow MHC accumulates only transiently as it is replaced, probably by neonatal MHC, by 21 d of gestation. Secondary-generation cells formed in the EDL and AT after 18 d of gestation do not express slow MHC. Thus programs of specialization appear to shift from slow to fast phenotypes as histogenesis proceeds and a continuous spectrum of fiber heterogeneity emerges very early in histogenesis, because of this temporal progression.

This analysis illustrates a correlation between an unfolding pattern of diversity and the temporal progression of muscle histogenesis. However, we find that slow myosin continues to be eliminated from the EDL and AT from birth to maturity. As a result there is a progressive, long-term transformation of primary slow fibers toward fast pathways of specialization. For example, the ~300 slow-staining fibers present in the 21-d fetal EDL are depleted by >60% as the animal matures (Table I). This result is consistent with findings in the mouse (48) and the chicken (22) and illustrates the plasticity of myosin expression as specialization advances in fast muscles. Thus, as the hind limb of the adolescent rat is drawn under the animal to assume an adult posture, expression of slow myosin appears to become less and less of a requirement for the tasks these fast muscles must perform, and our results suggest that slow, type I fibers in the adult EDL and AT may be viewed as relics of early development (see Figs. 4 and 5).

A comparable, developmentally regulated inhibition of slow MHC accumulation occurs in the developing soleus, but initiates later than in the EDL or AT, and exclusively involves secondary-generation cells. In accordance with other studies (8, 43), we find all primary fibers in the 18-d fetal soleus are slow in type (Fig. 6). Some secondary cells also synthesize slow MHC (Fig. 7, a and b) and must mature as slow fibers in that the total complement of type I fibers can not otherwise be accounted for in this muscle. Other secondary cells, apparently those developing late in histogenesis, express slow myosin either transiently or not at all and go on to mature as fast fibers in the young rat (Fig. 7 and references 4, 8, 43). As has been shown by Kugelberg (23), many of the fast fibers present in the soleus of adolescent rats transform into slow, type I fibers at later stages of development. The slow soleus and fast EDL and AT, therefore, become more and more specialized along their divergent pathways as maturity advances in the rat.

Collectively our results demonstrate that primary and secondary fibers are not intrinsically committed to be either slow or fast in type. Rather, their fate is flexible and determined by their position and time of formation in the developing limb.

When rat motoneurons are sufficiently mature to regulate pathways of muscle fiber specialization is unknown. It seems unlikely that the differences in antibody staining present in the EDL and AT at 18 d of gestation can result from differences in frequency of neural impulse traffic, for primary and secondary myotubes are interconnected by gap junctions at this stage (21) and presumptive fast and slow motoneurons are activated phasically even at birth (35). In the chick the results of Butler et al. (3), Laing and Lamb (25), Fremont et al. (10), Phillips and Bennett (41), and Miller et al. (34) collectively demonstrate that the initial differentiation of primary myotubes into distinct fiber types is independent of the motor nerve supply. Instead, their results suggest that the identity of the target muscle cell plays a significant role in determining the type of synaptic connection that is formed. In view of this, the present finding showing that the muscle target progressively changes patterns of gene expression during the perinatal period therefore raises the possibility that this modulation may influence neural competition and the transient appearance of polyneural innervation.

We have explored the effects of neonatal sciatic neuratomy upon slow MHC accumulation in the developing EDL and soleus. These muscles respond to denervation in a complex and tissue specific manner. The slow MHC content of the denervated EDL rises significantly between 10 and 30 d (Fig. 8). This appears to be due to pronounced hypertrophy of slow fibers in deep portions of the muscle (45; Kelly, A. M., unpublished observations) together with hypoplasia of developing fast fibers. These alterations are similar to the changes seen in Werdnig-Hoffman's disease in humans and suggest that, as the nerve promotes fast MHC accumulation, it inhibits accumulation of slow MHC in an antithetic fashion. This idea correlates with studies by Khaskiye et al. (22), suggesting that in the developing fast-twitch posterior latissimus dorsi of the chick, activity promotes a decline in the slow fiber population.

We find the soleus responds to denervation in the opposite way as levels of slow myosin decline rapidly between 5 and 30 d (Fig. 8). This result is consistent with our previous studies illustrating the restriction of slow and the emergence of fast myosin isozymes in the soleus after denervation at birth (12, 42), and our finding supports the idea that the postural activity imposed by slow motoneurons on the developing soleus protects and amplifies the intrinsic program of slow myosin expression. These results also point out that slow type I fibers in the soleus and EDL are not equivalent.

Thyroid hormone is known to regulate slow β-MHC accumulation in cardiac and skeletal muscle of adult animals (5, 16, 27, 38, 39). During the perinatal period, the rat ventricular myocardium undergoes a developmentally regulated
switch from slow β to α patterns of MHC accumulation (5, 26). Because these exchanges of heavy chain in cardiac muscle appear contemporaneous to the slow to fast exchanges in skeletal muscle, we speculated that similar controls operate in both tissues particularly with respect to expression of the slow β-MHC gene. In the myocardium, the transition is influenced by the development of thyroid function (5), for thyroxine acts either in an instructive or permissive role, promoting α cardiac MHC synthesis. In differentiating fast muscles of the rat we have previously shown that thyroid hormone regulates the developmental program of MHC switching as the animal matures from an essentially hypothyroid state at birth to a physiologically hyperthyroid state by 15 to 20 d postpartum (12).

To test the possibility that slow β-MHC expression responds to the maturation of thyroid function in both skeletal and cardiac muscle in a unified way, we made animals hypothyroid by supplying 0.05 % PTU in the drinking water of pregnant rats from 10 d of gestation and throughout life postpartum. RIA of the ventricular myocardium shows that between birth and 5 d, hypothyroidism begins to inhibit the exchange of slow β-MHC (Fig. 9). However, the effects of hypothyroidism on slow MHC accumulation in the EDL and AT, do not emerge until 15–30 d postpartum (Fig. 9). This is later than in the ventricle and is consistent with the view that the myocardium is significantly more sensitive to thyroid control than skeletal muscle. Based on the present methods of creating hypothyroidism, our results therefore suggest that the development of thyroid function is not involved in the initial inhibition of slow MHC accumulation in either the EDL or AT. However, with the development of thyroid function, thyroid hormone assumes a role in inhibiting slow MHC accumulation in the EDL and AT (Fig. 9). Present results suggest this role may be synergistic with inhibitory neural controls. This thyroidal effect probably also contributes to the decline in numbers of type I fibers in the maturing EDL and AT (Table I). By contrast, slow MHC accumulation in the soleus appears comparatively insensitive to thyroidal control. This may be influenced by neural activity.

Our previous studies (17), together with recent studies by Gustafson et al. (14), show that the MHC multigene family responds to thyroid hormone in a highly tissue-specific manner. For example, as the adult rat converts from a hypo- to a hyperthyroid state, the content of type Ila MHC mRNA is selectively increased in the soleus but decreased in the EDL. It seems possible that similar effects are imposed as thyroid function develops after birth in the rat. This may regulate rates of fiber differentiation in a complex, tissue-specific manner, during development of the soleus, the EDL, and the AT.

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