Type 2X-Myosin Heavy Chain Is Coded by a Muscle Fiber Type-specific and Developmentally Regulated Gene

Costanza DeNardi, Simonetta Ausoni, Paolo Moretti, Luisa Gorza, Mark Velleca,* Margaret Buckingham,‡ and Stefano Schiaffino

Department of Biomedical Sciences and CNR Unit for Muscle Biology and Physiopathology, University of Padova, 35121 Padova, Italy; *Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri; and ‡Department of Molecular Biology, Institut Pasteur, Paris, France

Abstract. We have previously reported the identification of a distinct myosin heavy chain (MyHC) isoform in a major subpopulation of rat skeletal muscle fibers, referred to as 2X fibers (Schiaffino, S., L. Gorza, S. Sartore, L. Saggin, M. Vianello, K. Gundersen, and T. Lømo. 1989. *J. Muscle Res. Cell Motil.* 10:197–205). However, it was not known whether 2X-MyHC is the product of posttranslational modification of other MyHCs or is coded by a distinct mRNA. We report here the isolation and characterization of cDNAs coding a MyHC isoform that is expressed in type 2X skeletal muscle fibers. 2X-MyHC transcripts differ from other MyHC transcripts in their restriction map and 3' end sequence and are thus derived from a distinct gene. In situ hybridization analyses show that 2X-MyHC transcripts are expressed at high levels in the diaphragm and fast hindlimb muscles and can be coexpressed either with 2B- or 2A-MyHC transcripts in a number of fibers. At the single fiber level the distribution of each MyHC mRNA closely matches that of the corresponding protein, determined by specific antibodies on serial sections. In hindlimb muscles 2X-, 2A-, and 2B-MyHC transcripts are first detected by postnatal day 2–5 and display from the earliest stages a distinct pattern of distribution in different muscles and different fibers. The emergence of type 2 MyHC isoforms thus defines a distinct neonatal phase of fiber type differentiation during muscle development. The functional significance of MyHC isoforms is discussed with particular reference to the velocity of shortening of skeletal muscle fibers.

MYOSIN heavy chain (MyHC)[1] isoforms with distinctive fiber type–specific and developmental-stage-specific distribution have been identified in skeletal muscle. Type 1 (slow-twitch) and type 2 (fast-twitch) fibers are known to contain different MyHCs, which are responsible for their different myosin ATPase activity and speed of shortening. Three subpopulations of type 2 skeletal muscle fibers, referred to as type 2A, 2B, and 2X, have been described in rat skeletal muscle using anti-MyHC mAbs (Schiaffino et al., 1986, 1989a; Gorza, 1990) and three type 2 MyHC isoforms have been identified by electrophoretic and immunoblotting analysis (Bär and Pette, 1988; Schiaffino et al., 1989; Termin et al., 1989a; LaFramboise et al., 1990). Type 2A- and 2B-MyHCs, as well as the β/slow MyHC present in type 1 fibers, are coded by distinct genes (see Mahdavi et al., 1987). However, it is not known whether 2X-MyHC derives from posttranslational modification of other MyHCs or is coded by a distinct mRNA. Type 2X fibers have a relatively rich mitochondrial content (Schiaffino et al., 1986, 1989a; Gorza, 1990) and belong to motor units characterized by a resistance to fatigue intermediate between that of type 2A and 2B motor units and a contraction and relaxation time similar to that of the other type 2 units (Larsson et al., 1991b). The maximum velocity of shortening of muscles containing predominantly 2X-MyHC is intermediate between that of muscles containing predominantly 2B-MyHC and that of muscles that consist essentially of β/slow MyHC (Schiaffino et al., 1988). Physiological studies on skinned single fibers show that the maximum shortening velocity of the three type 2 muscle fiber subpopulations is determined by their MyHC as well as myosin light chain (MyLC) composition (Bottinelli et al., 1991; Bottinelli et al., 1994).

In this study we report that 2X-MyHC is coded by a distinct gene that is expressed in type 2X muscle fibers and is coexpressed in a number of fibers either with 2A- or with 2B-MyHC. We also show that the 2X-MyHC gene is developmentally regulated and its expression can be modulated by thyroid hormone and electrical stimulation.

---

1. *Abbreviations used in this paper:* EDL, extensor digitorum longus; MyHC, myosin heavy chain; MyLC, myosin light chain; UTR, untranslated region.

Materials and Methods

Isolation of cDNA Clones

A λgt11 cDNA library from adult rat diaphragm was prepared essentially as described (Gubler and Hoffman, 1983; Gubler, 1988). In brief, poly(A)$^+$ RNA was prepared from diaphragms of 2-mo-old Charles River CD rats. First strand synthesis was primed with an oligodT/NotI primer/adapter (Promega, Madison, WI) and carried out with Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD). After second strand synthesis, cDNAs were made blunt with T4 and Klenow polymerase, EcoRI methylated, and linkers were added. After digestion with NotI and EcoRI, cDNAs were size-selected with a Sephacryl S400 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Fractions >0.5 kb were cloned into the EcoRI and NotI sites of λgt11 SfiI-NotI (Promega). From 150 ng cDNA and 1 μg vector, 3.5×10^7 independent recombinants were obtained. Library amplified from 2.5×10^7 primary plaques was screened using two mAbs, RT-D9, which is specific for 2B- and 2X-MyHC, and SC-71, which is specific for 2A-MyHC (Schiaffino et al., 1989a). Both antibodies recognize epitopes present in the rod portion of the MyHC molecule (Schiaffino et al., 1989a). Duplicate filters of the same plates were screened with a 1.2-kb-long MyHC cDNA, previously isolated from a mouse skeletal muscle cDNA library screened with mAb RT-D9 and found to correspond to the 3′ portion of 2B-MyHC mRNA (Weydert et al., 1983). This clone includes regions of high homology between different MyHC mRNAs (Nadal-Ginard et al., 1982). Clones reactive both with the anti-MyHC antibodies and the cDNA probe, were selected for further study after screening with an oligo-d(T) probe. Positive clones were recovered from the agar plates and purified to homogeneity by repeated plating and rescreening with the antibodies.

Subcloning and Sequence Analysis

The cDNA inserts from selected clones were purified from agarose gels and subcloned into Bluescript plus vector (Stratagene, Milano, Italy). Partial sequencing of double-stranded plasmid DNA was performed with the dideoxy chain termination method (Sanger et al., 1977) using T7 polymerase (Pharmacia, Milano, Italy).

Probes

Four MyHC probes, derived from the 3′ untranslated region (UTR) of the mRNAs, were used in this study. (a) 2X-MyHC probe: 5′ATCGATCCAAAGCAGGAAAGTGACCAAAGAGATGAGCAAAATGTGAAGAT-CTTTGTCACTCCATTTTGTACTTACGACTTTGGGAGATAAAAA-ATTTATCTGCCAAAAAAAAAAAAAAAAAAAAA3′.This DNA fragment was excised from the 3′-most portion of a 2X-MyHC cDNA clone using ClaI, that cuts at the level of the termination codon, and was subcloned into pBK (Stratagene). These sequence data are available from EMBL/GenBank/DDSB under accession number X72591. (b) 2B-MyHC probe: 5′GAGGTTCACACCAAAGTCATAAGCGAAGAATAGCTCAATTC-CTTCTGTTGAAAGGTGACAGAAGAAATCACACAAATGTGACG-TTCTTTGTCACTGTCCTGTATATCAAGGAAATAAAAGCTGCAG-ATAATTTTGCAAAAAAAAAAAAA3′.This sequence was obtained by PCR amplification of the 3′-UTR sequence of 2B-MyHC cDNA synthetized by reverse transcriptase from rat diaphragm RNA. Primers used for PCR were oligo-dT and an oligonucleotide, 5′GAGGTTCACACCAAAGTCATA-AG3′, which corresponds to a sequence in the 3′-most coding region of the 2B-MyHC mRNA (see Fig. 2). The amplification product was subcloned into pBS (Stratagene). These sequence data are available from EMBL/GenBank/DDSB under accession number X72590. (c) 2A-MyHC probe: 5′AGCTCTGATGCTGTAGAATGACCGAAGAAAGGCACAA-AATGTGAAGCCTTTGGTCATGCCCCCATGTGATTCTATTTAAT-CCTATTGTAAGGAAATAAAGAGCCCAAGTTCTTGCAAGCAAA-AAAAAAAAAAAAAAAA3′.This DNA fragment was excised from the 3′-most portion of the 2A-MyHC cDNA clone using Fnu4HI that cuts 3 bp after the termination codon, and was subcloned into pBK. These sequence data are available from EMBL/GenBank/DDSB under accession number X72589. (d) β/slow-MyHC probe: 5′CAAGGGCCTGAATGAAGAGT-AGATCTTGCTCTACCCAACCCTAAGGATGCCTGTGAAGCCCTG-AGACC3′.This is a StyI–BstnI fragment comprising 19 nt of the coding region plus 50 nt of the 3′-UTR sequences of the β-MyHC gene subcloned into pBS (Schiaffino et al., 1989b). These sequence data are available from EMBL/GenBank/DDSB under accession number K01463.

Northern Blotting

Total RNA was prepared from different rat tissues as described (Chomczynski and Sacchi, 1987). Samples were electrophoresed on 1% agarose, 3% formaldehyde gels, transferred to Hybond N+ nylon membranes (Amersham, Milano, Italy) and hybridized to ^{32}P-labeled probes. The 2X- and 2B-MyHC–specific probes used for hybridization were isolated from the vectors and labeled by the random priming method (Feinberg and Vogelstein, 1983). Filters were washed in 0.1 × SSC at 60°C.

In Situ Hybridization and Immunocytochemistry

Vectors containing cDNAs specific to 2A-, 2X-, 2B-, and β-slow MyHC mRNAs were linearized with appropriate restriction enzymes. Sense or anti-sense RNA probes labeled with ^{35}S-UTP were transcribed with T3 or T7 polymerase according to manufacturer's conditions. Probes were reduced to 50-70 nucleotides in length by alkali hydrolysis to allow better penetration into the section and used at a final concentration of 25,000–50,000 cpm/μl.

In situ hybridization with cRNA probes and immunocytochemistry with anti-MyHC antibodies were performed on serial cryosections of rat skeletal muscles. We found that sections could be stored at −20°C for up to 2 mo without significant variation in reactivity with cRNA probes and antibodies. Cryosections were fixed for 30 min with 4% paraformaldehyde and subsequently processed for in situ hybridization following the protocol described by Sassoon et al. (1988). Unfixed serial cryosections were processed for immunocytochemistry as described (Schiaffino et al., 1989a). The following antibodies were used in this study: BA-D5, reactive with β/slow-MyHC, SC-75, reactive with all type 2 MyHCs, SC-71, reactive with 2A-MyHC, BF-F3 and BF-G6, reactive with 2B-MyHC, and BF-35, reactive with all MyHCs except 2X-MyHC (Schiaffino et al., 1989a; Bottinelli et al., 1991).

In situ hybridization studies were also performed on soleus muscles from adult rats treated for one week with daily intraperitoneal injections of thyroid hormone (T$_3$, 5 μg per 100 g) (Izumo et al., 1986; Schiaffino et al., 1990) and on soleus muscles denervated and stimulated with a high frequency impulse pattern (25 pulses at 150 Hz every 15 min) as previously described (Ausoni et al., 1990).
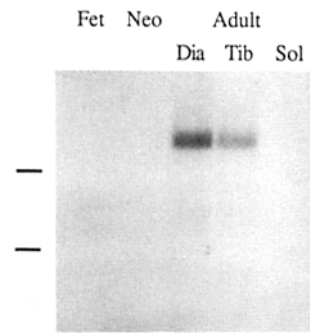
Results

Isolation of 2X-, 2B-, and 2A-MyHC cDNA Clones

Several positive clones were identified by double screening of a rat diaphragm expression library with monoclonal antibody RT-D9, specific for 2B- and 2X-MyHC, and with a mouse 2B-MyHC cDNA clone. Seven clones, reactive both with the antibody and the cDNA probe, were selected for further study after screening with an oligo-d(T) probe. Another cDNA was identified by double screening of the same expression library with monoclonal antibody SC-71, specific for 2A-MyHC, and with the mouse 2B-MyHC probe. Partial sequencing revealed that all selected clones correspond to MyHC genes, based on the high degree of homology of their 5′ ends with the rat embryonic MyHC gene (Strehler et al., 1986). Three groups of clones were identified on the basis of restriction endonuclease maps and 3′ end sequences (Figs. 1 and 2). These three groups will hereafter be referred to as 2A-, 2X-, and 2B-MyHC cDNAs, based on the fiber-type distribution of the corresponding mRNAs (see below). The 2A-MyHC cDNA, identified by its reactivity with SC-71, is ∼1.8 kb long and has a 3′ end sequence identical to one of the MyHC cDNAs expressed in adult fast muscle described by Nadal-Ginard et al. (1982, see Fig. 4 d of that article). Three of the seven clones identified by their reactivity with RT-D9 (all ∼1.7 kb long) have a 3′ end sequence identical to another MyHC cDNA expressed in adult fast muscle (see

0.5 kb
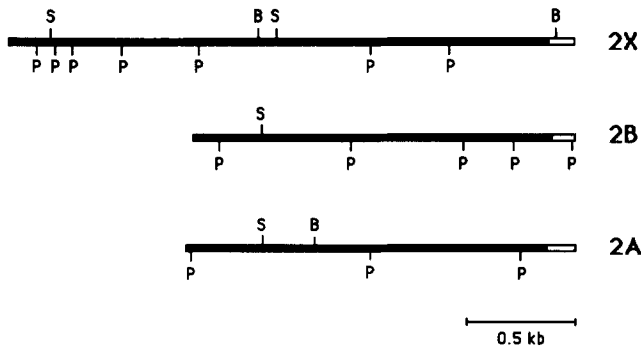
Figure 1. Restriction maps of 2X-, 2B-, and 2A-MyHC cDNA clones. The open area at the 3' end of each clone corresponds to the noncoding and poly(A) regions. Restriction enzyme abbreviations: *B*, BglII; *P*, PstI; *S*, SacI.

Fet Neo Adult
Dia Tib Sol

Figure 3. Expression of 2X-MyHC mRNA in rat muscles analyzed by Northern blotting. Total cellular RNA was isolated from hindlimb muscles from 18-d-old rat fetuses (*Fet*), 1-d-old neonates (*Neo*), and three muscles from adult rats: diaphragm (*Dia*), tibialis anterior (*Tib*), and soleus (*Sol*). Samples containing 10 μg of RNA were electrophoresed on agarose-formaldehyde gels, blotted onto nylon filters and hybridized with a ^{32}P-labeled 2X-MyHC probe. The probe corresponds to the 3'UTR of 2X-MyHC cDNA.

Fig. 4 *c* of Nadal-Ginard et al., 1982) and were found to correspond to 2B-MyHC mRNA. The other four clones reactive with RT-D9 (~3.8, 2.5, 2.4, and 2.3 kb long) were found to represent the same 2X-MyHC transcript: the restriction map of these cDNAs differs from that of the 2A- and 2B-MyHC clones, therefore 2X-MyHC transcripts must derive from a different gene (Fig. 1). The 3'UTR of the 2X-MyHC transcript differs from that of the 2A- and 2B-MyHC cDNAs (Fig. 2), as well as from that of five other rat sarcomeric MyHC genes whose 3'UTR sequence is known, i.e., embryonic-MyHC, neonatal MyHC, α-MyHC, β/slow MyHC, and extraocular-MyHC (Nadal-Ginard et al., 1982; Wieczorek et al., 1985). Two regions of high homology are present in the middle of the 3'UTR of 2A-, 2B-, and 2X-MyHC mRNAs. Similar regions are found in the 3'UTR of MyHC genes from different species (Weydert et al., 1983; Kavinsky et al., 1983; Saez and Leinwand, 1986; Maeda et al., 1987). The 3'-coding region of 2A-, 2B-, and 2X- MyHC transcripts shows a very high degree of homology. At the aminoacid level the sequence of the end of the 3'-coding segment of 2X-MyHC is identical to that of 2A- and 2B-MyHC, except for
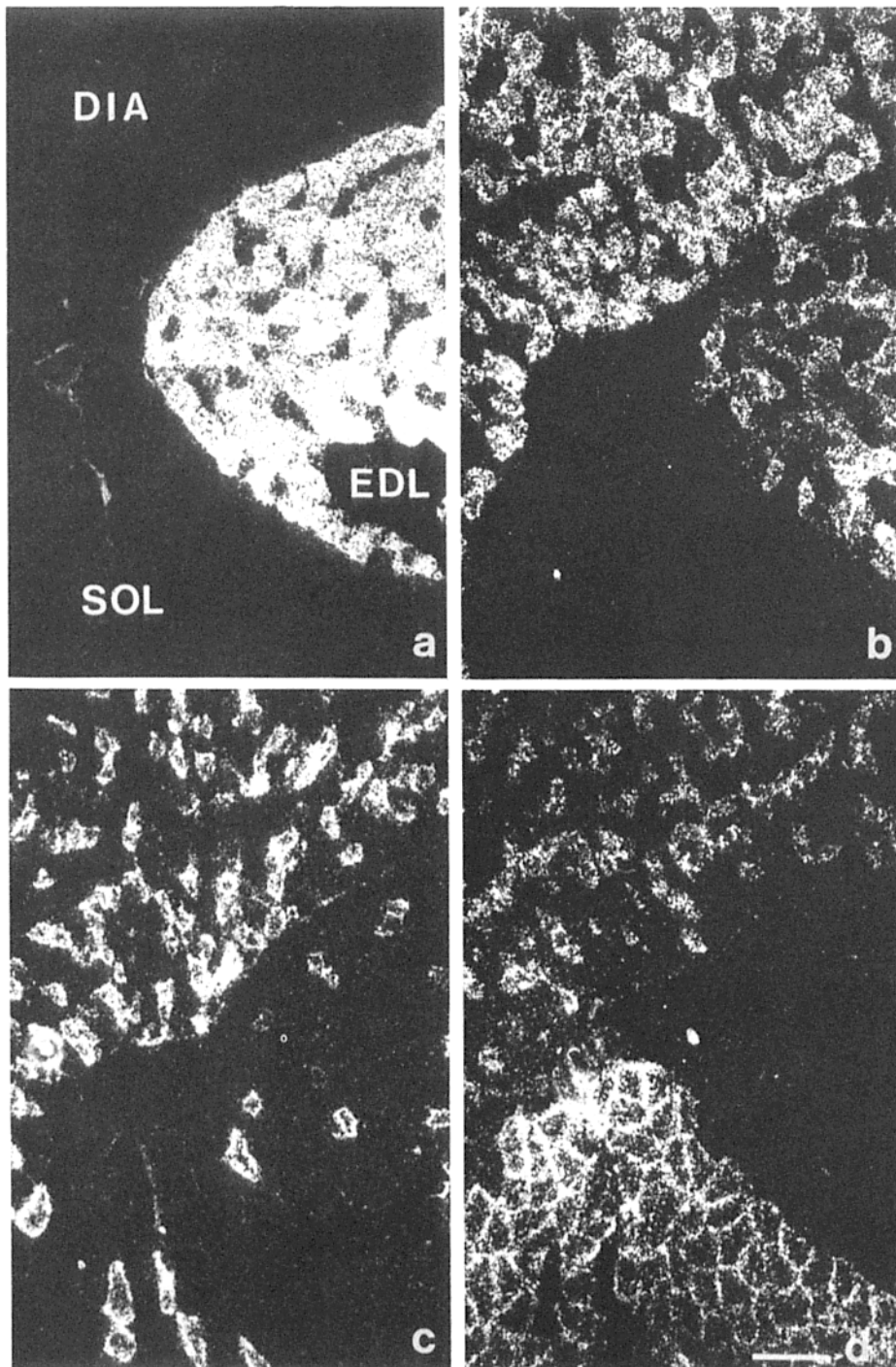
isoleucine instead of valine as the fifth residue from the carboxyl-terminal end (Fig. 2). This region is also very similar to the corresponding sequence of rat neonatal MyHC, whereas a greater divergence is found with the other known rat sarcomeric MyHC isoforms (see Nadal-Ginard et al., 1982 and Wieczorek et al., 1985).

The 3'-UTR of the 2X-MyHC cDNAs shows significant homology with that of a MyHC gene, called *MdMs* gene, recently described in the mouse (Parker-Thornburg et al., 1992) and with a MyHC cDNA clone (clone pSMHCA) previously isolated from an adult human skeletal muscle library (Saez and Leinwand, 1986). Neither of these sequences had been attributed to a specific MyHC or fiber type. The 3'-UTR of the rat 2X-MyHC mRNA shows 86.7% similarity with the 3'-UTR sequence of the human MyHC cDNA, and 91.4% similarity with the first 104 nt of the 3'-UTR of the mouse gene. A common feature of both the rat and human sequences is also the presence of the atypical polyadenylation signal GATAAA 13 nucleotides upstream from the poly(A). The rat and human sequences also show an identical aminoacid sequence at the carboxy-terminal end, including isoleucine as the fifth residue from the end.

Figure 2. Nucleotide and amino acid sequences of the 3' ends of 2X-, 2B-, and 2A-MyHC clones. Note that the protein carboxyl termini are highly homologous. The 3'UTRs are more divergent but contain homologous stretches (*overlined*). Stop codons are marked by asterisks and polyadenylation signals are underlined. These sequence data of 2X-, 2B-, and 2A-MyHC clones are available from EMBL/GenBank/DDSB under accession numbers X72591, X72590 and X72589, respectively.

```
         V   K   S   R   E   V   H   T   K   I   I   S   E   E   *
2X-MyHC  GTG AAG AGC CGC GAG GTT CAC ACC AAA ATC ATA AGC GAA GAG TGA  TCGATCCAAAGCAGGAAAGTGACCAAAGAGATG

         V   K   S   R   E   V   H   T   K   V   I   S   E   E   *
2B-MyHC  GTG AAG AGC CGA GAG GTT CAC ACC AAA GTC ATA AGC GAA GAA TAG  CTCAATTCCTTCTGTTGAAAGGTGACAGAAGAA

         V   K   S   R   E   V   H   T   K   V   I   S   E   E   *
2A-MyHC  GTA AAG AGC CGC GAG GTT CAC ACT AAA GTC ATA AGT GAA GAG TAA  GGCAGCTCTGATGCTGTAGAATGACCGAAGAAA

2X-MyHC  AGCAAAATGTGAAGATCTTTGTCACTCCATTTTGTACTTACGACTTTGGGAGATAAAAAATTTATCTGCC(A)n

2B-MyHC  ATCACACAAATGTGACGTTCTTTGTCACTGTCCTGTATATCAAGGAAATAAAAGCTGCAGATAATTTTGC(A)n

2A-MyHC  GGCACAAAATGTGAAGCCTTTGGTCATGCCCCCATGTGATTCTATTTAATCCTATTGTAAGGAAATAAAGAGCCCAAGTTCTTGCAAGC(A)n
```

Figure 4. Expression of 2X-MyHC mRNA, compared to that of 2B-, 2A-, and β/slow-MyHC mRNAs, analyzed in different rat muscles by in situ hybridization. Serial transverse cryosections of a composite block, containing soleus (*SOL*), EDL and diaphragm (*DIA*) muscles from adult rat, were hybridized with ³⁵S-labeled probes complementary to the 3'UTR of 2B- (*a*), 2X- (*b*), 2A-(*c*) and β/slow- (*d*) MyHC mRNAs. Dark-field micrographs.

2X-MyHC Transcripts Are Selectively Expressed in Type 2X Fibers and Can Be Coexpressed in a Number of Fibers Either with 2B- or with 2A-MyHC Transcripts

Northern blot analysis showed that 2X-MyHC clones hybridize with a 31S (~7 kb) MyHC mRNA that is expressed at high levels in the diaphragm and at lower levels in the tibialis anterior muscle, but not in the soleus muscle nor in fetal and neonatal hindlimb muscles (Fig. 3). Serial sections of rat extensor digitorum longus (EDL), soleus and diaphragm muscles were hybridized with cRNA probes specific for the 3'UTRs of 2X-, 2A-, 2B-, and β/slow-MyHC transcripts. In situ hybridization analysis showed that 2X-MyHC mRNA is expressed at high levels in the diaphragm and in the fast EDL muscle, with no expression detected in the slow soleus muscle (Fig. 4 *b*). In contrast, 2B-MyHC transcripts are expressed in many fibers in EDL, are weakly expressed in rare fibers in the diaphragm and are absent in the soleus (Fig. 4 *a*). 2A-MyHC transcripts were detected in many fibers present in all three muscles (Fig. 4 *c*) and β/slow-MyHC transcripts are expressed in most fibers in the soleus and numer-
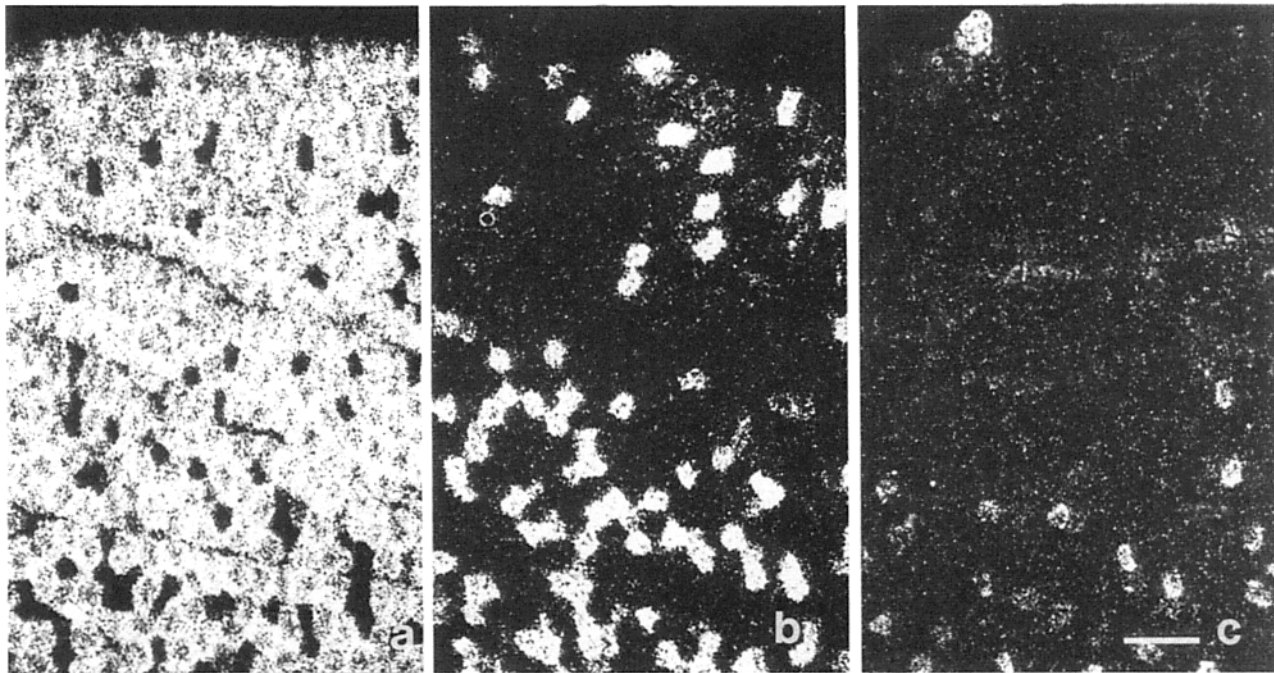
Figure 5. Regional variation in the distribution of 2B- (*a*), 2X- (*b*), and 2A- (*c*) MyHC mRNAs in the rat tibialis anterior muscle. The anterior surface of the muscle is at the top. Note that 2B-MyHC transcripts are abundant throughout and represent the major fiber population near the surface of the muscle, whereas 2A-MyHC transcripts are only seen in fibers present in deeper regions (the white spot at the top is an artifact). Fibers containing 2X-MyHC transcripts are distributed throughout, although with a tendency to be less numerous in the very superficial areas.

ous fibers in the diaphragm, whereas they are present only in rare fibers in EDL (Fig. 4 *d*). Variations in the distribution of the different MyHC transcripts were also seen within the same muscle. 2B-MyHC transcripts are more abundant in the superficial regions of tibialis anterior muscle (Fig. 5 *a*), whereas 2A-MyHC transcripts are more abundant in the deep regions (Fig. 5 *c*) and 2X-MyHC transcripts are present in both areas but are less numerous in the very superficial regions (Fig. 5 *b*). The corresponding sense probes gave no significant reaction in any muscle.

Serial sections were processed for in situ hybridization with cRNA probes and for immunocytochemistry with antibodies specific for 2A-, 2B-, and β/slow-MyHCs and with one antibody (BF-35) that reacts with all MyHCs except 2X-MyHC (Fig. 6). As previously described (Schiaffino et al., 1989*a*), type 2X fibers correspond to fibers unreactive with BF-35 (Fig. 6 *d*) as well as with antibodies specific for 2A-, 2B-, and β/slow-MyHCs (Fig. 6, *b*, *f*, and *h*), but reactive with an antibody (SC-75) reactive with all type 2 MyHCs (not shown). Analysis of serial sections of EDL and tibialis anterior muscles showed that the distribution of 2X-, 2B-, 2A-, and β/slow-MyHC transcripts closely matches that of the corresponding proteins. Many fibers contained a single type of MyHC transcript, however a significant proportion of fibers was found to coexpress either 2X- and 2B-MyHC mRNA or 2X- and 2A-MyHC mRNA (Figs. 6 and 7). The fibers with mixed mRNA composition appear to contain a mixture of the corresponding proteins, as suggested by the finding that they display an intermediate intensity of staining with both BF-35 and with the 2B-specific antibody, or with BF-35 and with the 2A-MyHC–specific antibody (see also

Schiaffino et al., 1989*a*, 1990). A number of fibers were also found to coexpress type 2A- and β/slow-MyHC transcripts and proteins. The fibers with mixed MyHC transcript composition, i.e., containing β/2A- or 2A/2X- or 2X/2B-MyHC mRNAs, account for almost one third of the whole fiber population in the rat EDL muscle (Table I). ∼55% of the fibers present in this muscle contain 2X-MyHC transcripts, either alone or together with 2A- or 2B-MyHC transcripts (Table I). Fibers with mixed MyHC composition are also present in soleus and diaphragm muscles. The rare fibers containing 2B-MyHC transcripts in the diaphragm were all found to contain also 2X-MyHC, thus no pure 2B fiber is present in adult rat diaphragm, based on MyHC transcript composition.

Expression of 2X-MyHC Transcripts Is Developmentally Regulated

Type 2X-MyHC transcripts, as well as 2B- and 2A-MyHC transcripts, were not detectable by Northern blotting and in situ hybridization in hindlimb muscles of 20-d-old fetal and 1-d-old neonatal rats (Fig. 3). The three type 2 MyHC transcripts were first detected by days 2–5 after birth in hindlimb muscles and display from their first appearance a differential pattern of expression (Figs. 8 and 9). 2A-MyHC transcripts were first detected by day 2 postnatal in a number of fibers in soleus, gastrocnemius and EDL, whereas 2X-MyHC transcripts were first detected by day 3 in EDL, and 2B-MyHC transcripts were first seen by day 4 in a number of fibers in the tibialis anterior (not shown). The distribution of MyHC transcripts in different leg muscles by day 5 is illustrated in
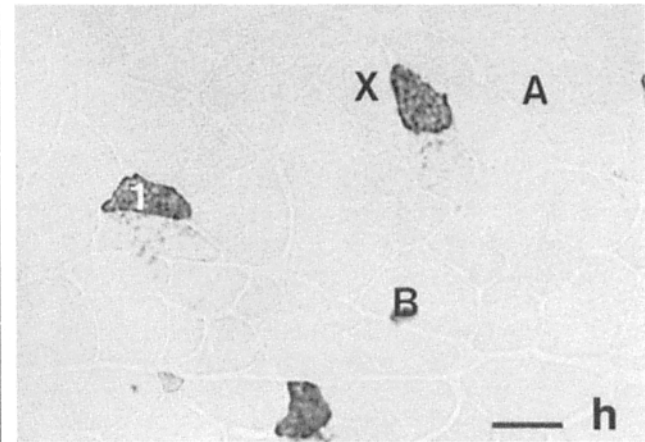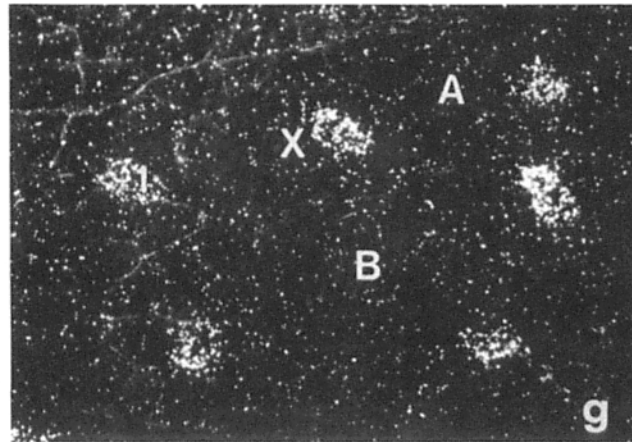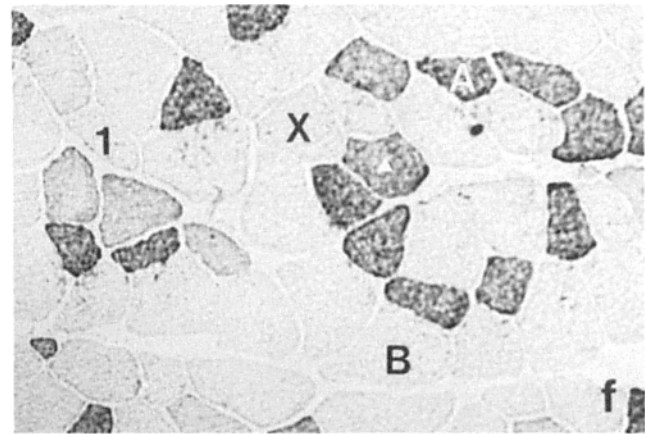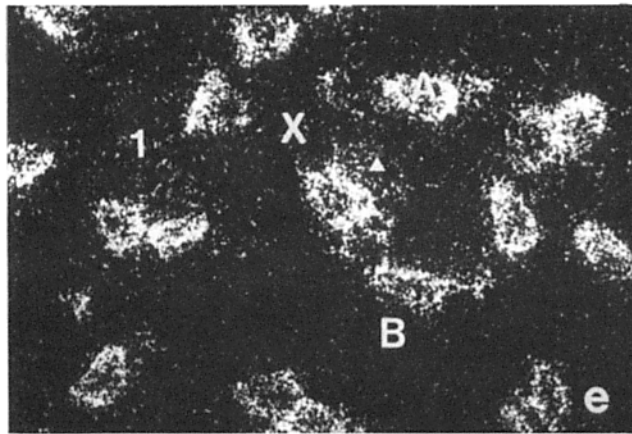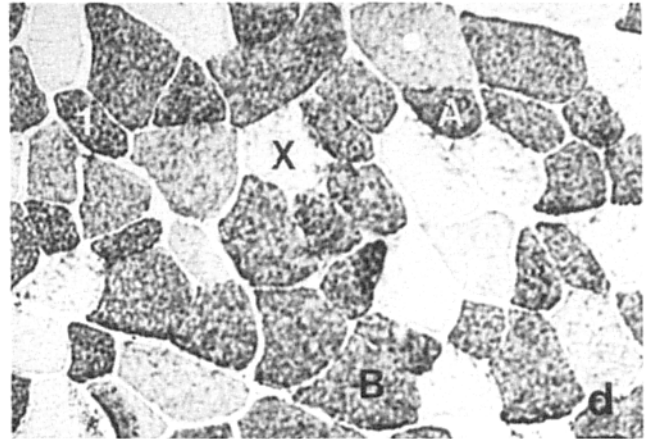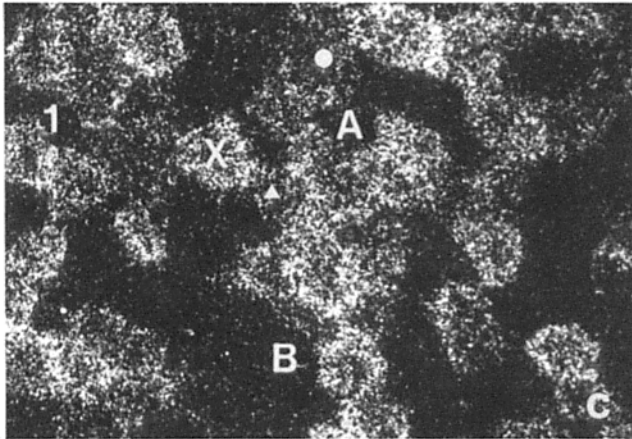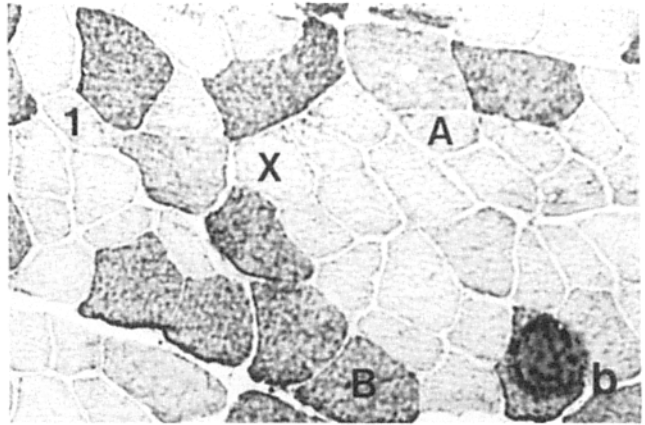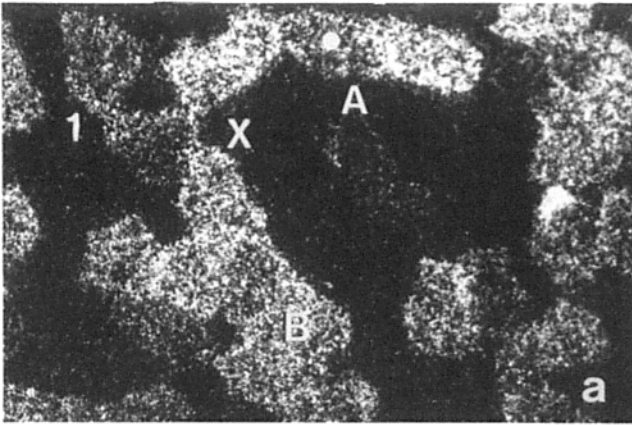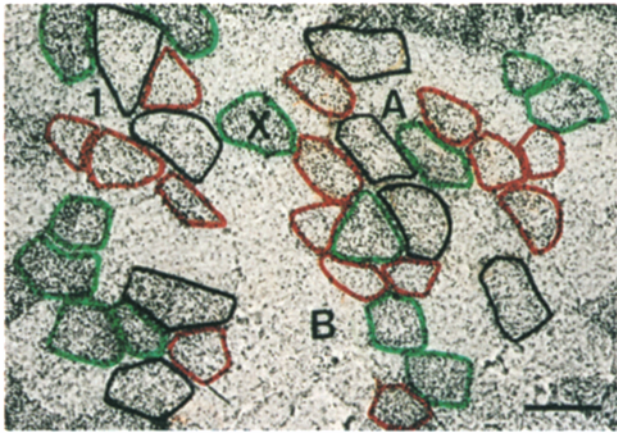
Figure 7. Expression of 2X-MyHC mRNA in the rat EDL muscle. Phase-contrast micrograph of the same field shown in Fig. 6 *c*. Fibers containing exclusively 2X-MyHC are outlined in green, those coexpressing 2X- and 2B-MyHC in black and those coexpressing 2X- and 2A-MyHC in red.

Fig. 8. In the soleus most fibers contain β/slow-MyHC transcripts and a significant proportion of fibers contain 2A-MyHC transcripts, whereas 2X- and 2B-MyHC transcripts were not detectable (Fig. 8, *a, c, e,* and *g*). 2X- and 2A-MyHC transcripts are relatively abundant in EDL muscle, whereas 2B-MyHC transcripts are more abundant in the tibialis anterior muscle, where they are mostly expressed in peripheral areas, except for a very superficial layer that contains fibers unreactive with the four probes (Fig. 8, *b, d, f,* and *h*). Since all fibers show embryonic and neonatal MyHC immunoreactivity at this stage of development (our unpublished observations) we conclude that these fibers have not yet begun to express the adult isoform. Comparative analysis of serial sections showed that many fibers at day 5 expressed exclusively one of the three type 2 MyHC transcripts, i.e., either 2X-MyHC transcripts (Fig. 9 *b, arrows*) or 2A-MyHC transcripts (e.g., in soleus), or 2B-MyHC transcripts (e.g., in tibialis anterior or peroneus digiti 4). In addition, a number of fibers were found to coexpress 2X- and 2B-MyHC (e.g., in tibialis anterior) or 2X- and 2A-MyHC (e.g., in EDL) or 2A- or β/slow-MyHC transcripts (e.g., in soleus). By day 7 the level of 2X-and 2A-MyHC transcripts, as determined by the intensity of the hybridization signal, was found to increase in the tibialis anterior and the level of 2B-MyHC transcripts has increased markedly in the EDL. By postnatal day 14 to 21 the pattern of distribution of the various transcripts is similar to that of adult muscle (not shown).

2X-MyHC Transcripts Are Induced in the Rat Soleus Muscle by Thyroid Hormone and High Frequency Electrical Stimulation

The expression of MyHC genes in rat skeletal muscle is known to be regulated by thyroid hormone (Izumo et al., 1986) and by different patterns of electrical stimulation

Table I. Distribution of MyHC Transcripts in Muscle Fibers of Adult Rat EDL Muscle

MyHC transcripts	Percent of fibers
β	2.2
β/2A	1.9
2A	11.2
2A/2X	9.9
2X	26.2
2X/2B	19.8
2B	28.8

Serial sections of rat EDL were processed for in situ hybridization with the four MyHC probes. The proportion of fibers that contained either one or two MyHC transcripts was determined on 313 fibers from three different fields.

(Gorza et al., 1988; Termin et al., 1989*b*; Ausoni et al., 1991). We have therefore examined the effect of hyperthyroidism and high frequency stimulation on the expression of 2X-MyHC transcripts in the rat soleus muscle. The soleus muscle in adult rats consists predominantly of type 1 fibers containing β/slow-MyHC and a minor, variable proportion of type 2A fibers; 2X- and 2B-MyHC are not detectable by immunoblotting and immunocytochemistry in this muscle (Schiaffino et al., 1989*a*; Ausoni et al., 1990). We have previously reported that thyroid hormone can induce the appearance of 2X-MyHC in a population of soleus muscle fibers that also express 2A-MyHC (Schiaffino et al., 1990). We have analyzed the same muscles by in situ hybridization. 2X-MyHC mRNA is not present in the normal soleus muscle (Fig. 10 *a*), but is readily detectable in a population of soleus muscle fibers after one week of treatment with thyroid hormone (Fig. 10 *b*).

High levels of 2X-MyHC can be induced in soleus muscle fibers after long-term high frequency electrical stimulation (Ausoni et al., 1990). Under these conditions the relative amount of 2X-MyHC, as determined by electrophoretic and immunoblotting analysis, can increase up to 85% of the whole MyHC complement (Schiaffino et al., 1988; Ausoni et al., 1990). Accordingly, as shown in Fig. 10 *c*, high levels of 2X-MyHC transcripts are seen in most fibers of stimulated soleus muscles.

Discussion

This study shows that (*a*) 2X-MyHC is a distinct protein coded by a specific gene and not a posttranslational product of other MyHCs, thus giving definitive support to the notion of type 2X fibers as a major fiber type population in rat skeletal muscle; (*b*) the expression of 2X-MyHC gene is coordinately regulated with that of 2A- and 2B-MyHC genes so that only certain combinations of MyHC transcripts can be coexpressed within the same fiber; (*c*) the appearance around birth of 2X-, 2A-, and 2B-MyHC transcripts in different fiber populations defines a distinct neonatal phase of fiber type differentiation during muscle development.

Figure 6. Distribution of different MyHC mRNAs and proteins in type 2B (*B*), 2X (*X*), 2A (*A*), and type 1(*l*) fibers in rat EDL muscle. Serial cryosections were processed for in situ hybridization (*left column*) with 2B- (*a*), 2X- (*c*), 2A- (*e*), and β/slow-specific (*g*) cRNA probes, and for immunocytochemistry (*right column*) with anti-MyHC monoclonal antibodies reactive with 2B-MyHC (*b*), all MyHCs but 2X-MyHC (*d*), 2A-MyHC (*f*), and β/slow-MyHC (*h*). The circles in *a–d* mark a fiber coexpressing 2B- and 2X-MyHCs and the corresponding transcripts. The triangles in *c–f* mark a fiber coexpressing 2A- and 2X-MyHCs and the corresponding transcripts.
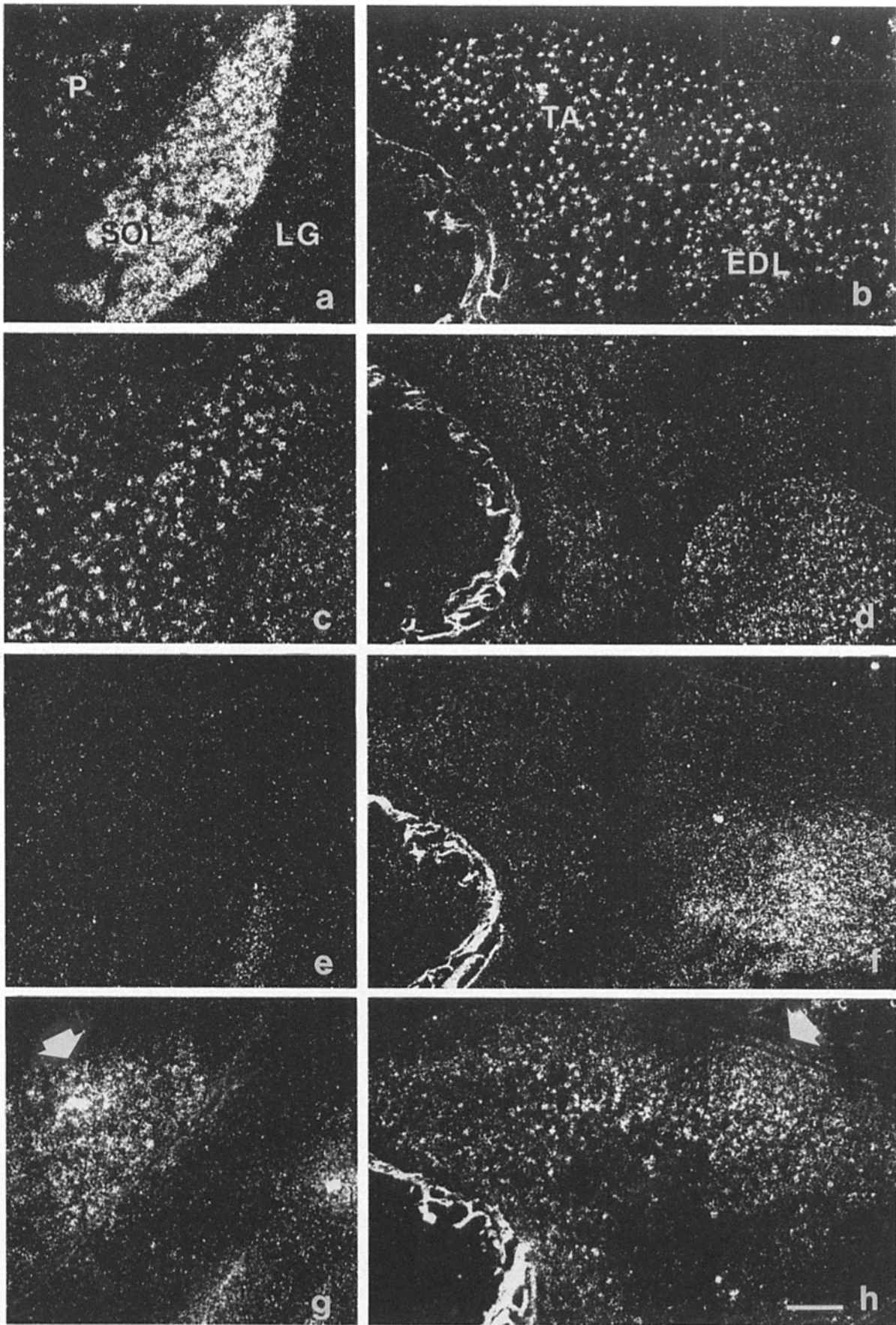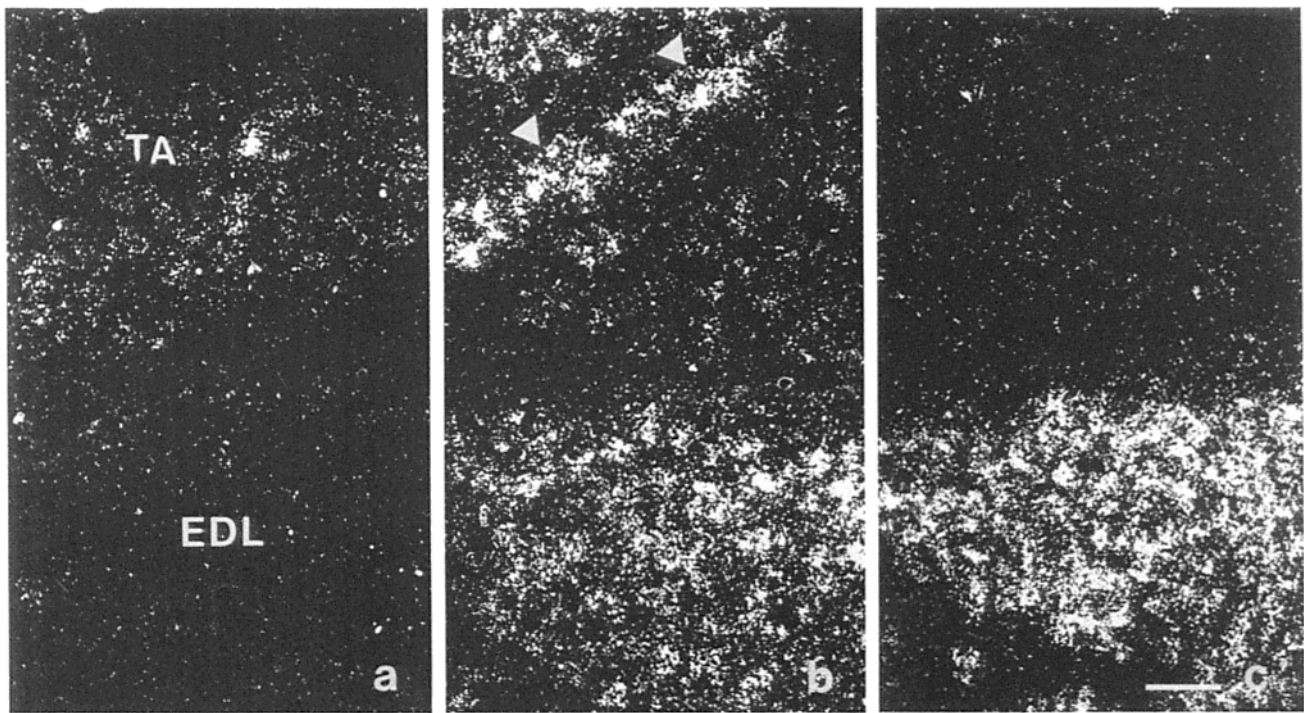
Figure 9. Expression of MyHC transcripts in hindlimb muscles from a 5-d-old rat. Serial sections of tibialis anterior (*TA*) and extensor digitorum longus (*EDL*) muscles hybridized with probes specific for 2B- (*a*), 2X- (*b*), and 2A- (*c*) MyHC mRNAs. Note that in this region 2B- and 2X- but not 2A-MyHC transcripts are expressed in the tibialis anterior and that 2A- and 2X- but not 2B-MyHC transcripts are expressed in the EDL. A bundle of tibialis anterior fibers reactive for 2X- and unreactive for 2B- and 2A-MyHC tmRNA is indicated by arrowheads.

2X-MyHC Is Encoded by a Distinct Gene Present in Different Mammalian Species

2X-MyHC was initially described in a specific subpopulation of type 2 muscle fibers showing a distinct pattern of reactivity with different anti-MyHC mAbs (Schiaffino et al., 1986, 1989*a*). Type 2X fibers were subsequently identified also in mouse and guinea pig skeletal muscles (Gorza, 1990; Schiaffino et al., 1990). In the rat, 2X fibers are especially numerous in the diaphragm muscle but are present as a major fiber population in fast-twitch leg muscles, whereas they are absent in the slow-twitch soleus muscle (Schiaffino et al., 1989*a*). By immunoblotting 2X-MyHC was found to migrate in the same electrophoretic band with 2A-MyHC (Schiaffino et al., 1989*a*), however using a modified electrophoretic procedure the 2X-MyHC isoform could be separated as a band of slightly higher mobility than 2A-MyHC but lower mobility than 2B-MyHC (LaFramboise et al., 1990). In this respect 2X-MyHC appears to correspond to the IId-MyHC isoform described by Pette and coworkers in rat and rabbit muscle (Termin et al., 1989*a*; Aigner et al., 1993). Until now it was not known whether 2X-MyHC is the

product of a distinct gene or results from differential RNA splicing or from posttranslational modification of 2A- or 2B-MyHC. The results reported here demonstrate that a distinct MyHC mRNA is expressed in type 2X fibers and that the level of this mRNA in different rat skeletal muscles closely matches that of 2X-MyHC. The finding that 2X-MyHC cDNA differs from 2A- and 2B-MyHC cDNA with respect to its restriction map and 3' coding and noncoding sequence indicates that 2X-MyHC transcripts are encoded by a distinct gene.

The end of the 3'-coding and the 3'UTR of the rat 2X-MyHC mRNA is very similar to that of MyHC genes expressed in human (Saez and Leinwand, 1986; Yoon et al., 1992) and in mouse skeletal muscle (Parker-Thornburg et al., 1992). Thus it appears that genes homologous to the rat 2X-MyHC gene are present in different mammalian species. In human skeletal muscle two type 2 fiber populations, referred to as type 2A and 2B fibers, can be distinguished by myosin ATPase histochemical staining (see Brooke and Kaiser, 1970) and two type 2 MyHC bands, referred to as 2A- and 2B-MyHC, have been identified by electrophoretic and immunoblotting analysis (Biral et al., 1988; Klitgaard et al.,

Figure 8. Expression of MyHC transcripts in hindlimb muscles from 5-d-old rat. (*Left column*) Serial sections of soleus (*SOL*), lateral gastrocnemius (*LG*) and peroneus digiti 4 (*P*) muscles. (*Right column*) Serial sections of tibialis anterior (*TA*) and extensor digitorum longus (*EDL*) muscles. In situ hybridization with probes specific for β/slow- (*a* and *b*), 2A- (*c* and *d*), 2X- (*e* and *f*), and 2B- (*g* and *h*) MyHC transcripts. Arrows in *g* and *h* point to areas in the peroneus and tibialis anterior where numerous fibers express 2B- but not 2A- or 2X-MyHC mRNAs. Note that a number of fibers in the soleus express 2A-MyHC transcripts, whereas 2X- and 2B-MyHC transcripts are not detectable in this muscle.
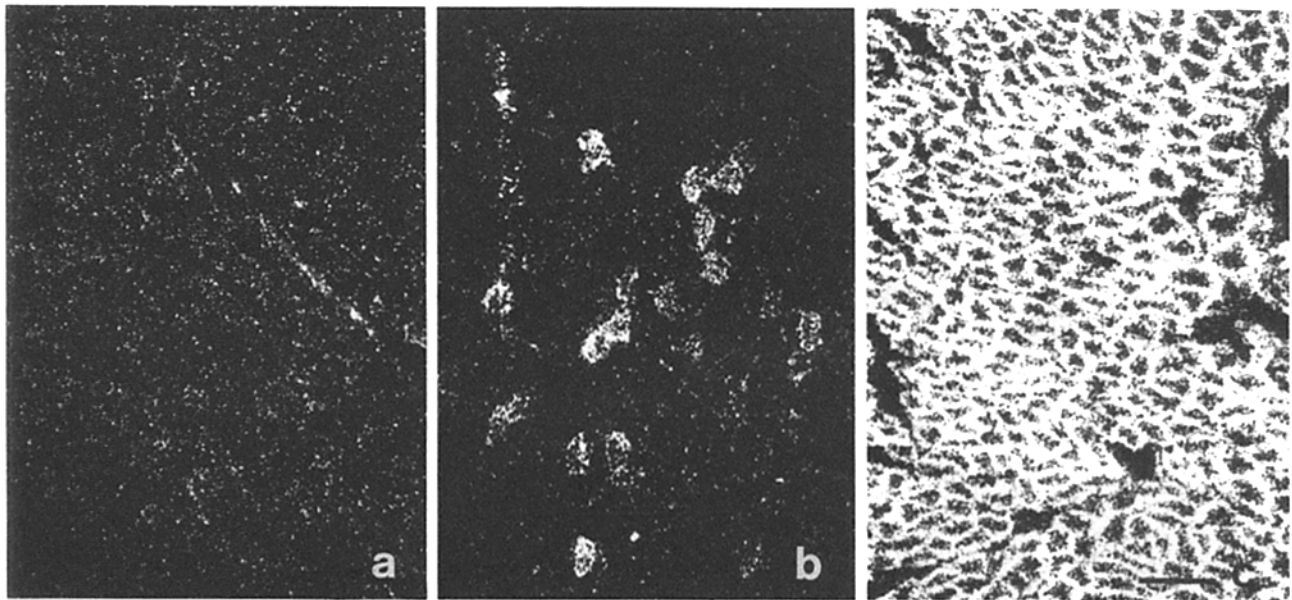
Figure 10. Distribution of 2X-MyHC transcripts in soleus muscles from normal (*a*) and hyperthyroid rats (*b*), and from soleus muscles denervated and stimulated for two months with a high frequency pattern of impulses (*c*).

1990). Studies aimed at defining the fiber type distribution of 2X-MyHC transcripts in human skeletal muscle are in progress (Schiaffino, S., and L. Leinwand, manuscript in preparation).

2X-MyHC Transcripts Can Be Coexpressed Either with 2A-MyHC or with 2B-MyHC Transcripts
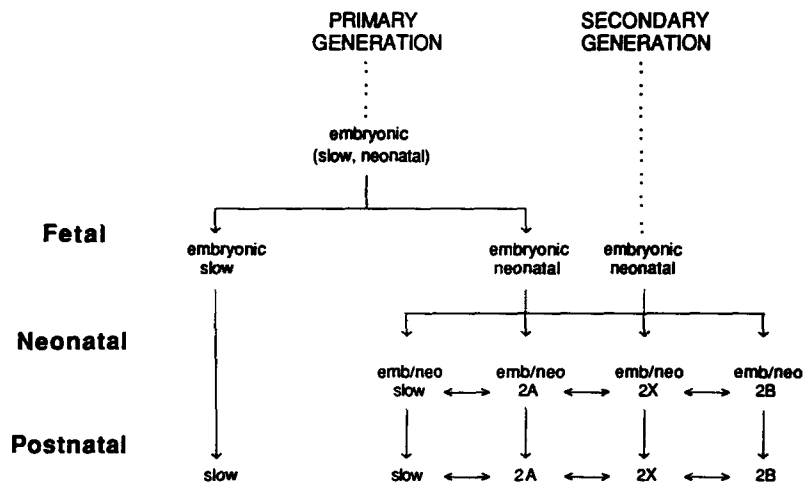
We have observed that 2X-MyHC transcripts represent the only MyHC transcript present in those fibers typed as 2X fibers based on anti-MyHC immunohistochemistry. In addition, they can be coexpressed in a number of fibers either with 2B- or with 2A-MyHC transcripts, whereas 2A- and 2B-MyHC transcripts are never expressed together in the same fiber. These observations are in agreement with our previous immunohistochemical studies on fibers with mixed MyHC composition: only certain combinations of MyHCs, i.e., β/2A, 2A/2X or 2X/2B, were detected within any single fiber (Schiaffino et al., 1990; Gorza, 1990). The MyHC transitions observed in electrically stimulated muscles also appear to reflect an obligatory pathway of MyHC gene expression, in the order 1 $\longleftrightarrow$ 2A $\longleftrightarrow$ 2X $\longleftrightarrow$ 2B (Ausoni et al., 1990). Similar findings were obtained by electrophoretic analyses on single muscle fibers in the rat (Termin et al., 1989*a,b*) and the rabbit (Aigner et al., 1993).

The factors responsible for fiber type–specific regulation of MyHC genes remain to be discovered. Sarcomeric MyHC genes are clustered on two chromosomes in mice and humans. The α and β/slow MyHC genes are closely associated on chromosome 14, whereas other skeletal MyHC genes are clustered on human chomosome 17 and mouse chomosome 11 (Leinwand et al., 1983; Weydert et al., 1985). The human skeletal MyHC cluster contains six MyHC genes, including one corresponding to the rat 2X-MyHC gene, located within a 500-kb segment of DNA (Yoon et al., 1992). The mouse skeletal MyHC cluster contains at least three MyHC genes, corresponding to the embryonic, perinatal and 2B-MyHC

genes, within a 370-kb segment (Cox et al., 1991). The localization of mouse 2A and 2X-MyHC genes remains to be determined, however it has recently been shown that the mouse MyHC gene homologous to the rat 2X-MyHC gene is located immediately 3' to the 2A-MyHC gene (Parker-Thornburg et al., 1992). In other gene clusters, such as the β globin and the *Hox* gene clusters, the linear order of the genes along the chromosome correlates with their temporal and spatial pattern of expression and it is therefore of importance to determine whether physical linkage and chromosomal order are also critical to the transcriptional regulation of MyHC genes influencing their pattern of expression. Recent results on the organization of the human skeletal MyHC genes show that their order is apparently not related to their developmental expression, since embryonic and perinatal MyHC genes are expressed sequentially during development but are not located adjacent to each other (Yoon et al., 1992).

Developmental Expression of 2X-, 2A-, and 2B-MyHC Transcripts and Differentiation of Type 2 Fiber Subpopulations

Changes in MyHC isoform expression define three major phases of fiber type differentiation in rat hindlimb muscles (Fig. 11). The first phase is characterized by the diversification of primary generation fibers and takes place during fetal development in the period between days 16 and 18 of gestation (Lyons et al., 1983; Dhoot, 1986; Narusawa et al., 1987; Harris et al., 1989; Condon et al., 1990; our unpublished observations). In the tibialis anterior muscle there is an early differentiation process within an apparently homogeneous population of primary generation fibers that express embryonic MyHC and low levels of β/slow- and neonatal-MyHCs: fibers in the outer region express embryonic and neonatal MyHC while fibers in deeper areas contain embryonic and β/slow-MyHCs (Condon et al., 1990). Secondary fibers that are formed in these muscles between days 18

PRIMARY
GENERATION

SECONDARY
GENERATION

embryonic
(slow, neonatal)

Fetal

embryonic
slow

embryonic
neonatal

embryonic
neonatal

Neonatal

emb/neo
slow ⟷ 2A ⟷ 2X ⟷ 2B

Postnatal

slow

slow ⟷ 2A ⟷ 2X ⟷ 2B

Figure 11. Major steps in muscle fiber diversification during rat hindlimb muscle development, based on MyHC isoform transitions (modified from Gunning and Hardeman, 1991). The scheme highlights three phases of fiber type differentiation. The fetal phase is characterized by the diversification of primary generation fibers into one population of fibers expressing embryonic and β/slow MyHCs and another population of fibers expressing embryonic and neonatal MyHCs. The emergence of secondary generation fibers expressing embryonic and neonatal MyHCs also takes place during fetal development. The neonatal phase is characterized by the diversification of primary and secondary generation fibers expressing embryonic and neonatal MyHCs with the emergence of different type 2 fiber subpopulations. The postnatal phase of muscle fiber differentiation is characterized by the disappearance of embryonic (*emb*) and neonatal (*neo*) MyHC and by further changes in MyHC expression. ⟷ indicates the existence of transitional fibers with mixed MyHC composition, i.e., coexpressing slow/2A or 2A/2X or 2X/2B MyHCs. The diagram is intended to depict phenotypic changes, not to define cell lineages: thus each ramification indicates the emergence of different fiber types from a precursor pool that appears homogeneous in terms of MyHC isoform expression but could already contain distinct cell lineages. Furthermore, the range of available options for each precursor pool may be restricted in certain muscles: for example the secondary generation fibers of soleus differentiate only into fibers expressing slow, slow/2A or 2A MyHCs.

and 21 of gestation express embryonic and neonatal but not β/slow-MyHC, independent of their regional distribution within the muscle. In the soleus all primary generation fibers express embryonic and β/slow-MyHC, whereas newly formed secondary fibers express embryonic and neonatal-MyHC (Condon et al., 1990).

The second developmental phase takes place during the neonatal period and is characterized by the diversification of both primary and secondary generation fibers expressing embryonic and neonatal-MyHC with the emergence of the various type 2 fiber subsets. Previous studies showed that in rat hind limb muscles 2A-MyHC transcripts can be detected by S1 nuclease at postnatal day 5 (Wieczorek et al., 1985; Mahdavi et al., 1991) and 2B-MyHC transcripts at 6–7 days (Russell et al., 1988). The timing of type 2 MyHC protein and mRNA accumulation differs between species and muscle types. In mouse limb muscles 2B-MyHC transcripts are already detectable in fetal stages and accumulate rapidly during the first 5 days postnatally (Weydert et al., 1987). In the rat diaphragm 2A-MyHC transcripts were detected at 21-d gestation (Kelly et al., 1991) as was the 2X-MyHC protein (LaFramboise et al., 1991) and transcripts (our unpublished observations). The results of this study show that 2A-, 2X-, and 2B-MyHC transcripts appear shortly after birth in hindlimb skeletal muscles and display from the outset a specific regional distribution. Each transcript accumulates in a specific subpopulation of developing muscle fibers with a differential pattern of distribution in different muscles. The factors responsible for the positional specification of type 2 fiber precursors and for the differential expression of different MyHC genes in limb muscles remain to be identified. The findings reported here point to an independent origin of type 2 fiber subsets and rule out schemes involving a common initial stage with coexpression of all three type 2 MyHC transcripts, or sequential activation of type 2 MyHC genes (e.g., 2X-MyHC preceding obligatorily 2A- and 2B-MyHC gene expression). Combinations of transcripts similar to

those seen in adult muscles, i.e., slow/2A-, or 2A/2X-, or 2X/2B-MyHCs, are also present from the outset in single fibers of neonatal muscles.

The third postnatal phase of muscle fiber differentiation is characterized by the downregulation of embryonic and neonatal MyHCs, which varies according to fiber type and muscle type (Schiaffino et al., 1988). In addition, further shifts in MyHC isoform expression take place at various postnatal stages. A number of type 2A soleus fibers progressively shift to β/slow-MyHC expression (see Butler-Browne et al., 1984). The expression of the 2X- and 2B-MyHC genes also changes with age. In old rats type 2X motor units become the predominant motor unit type in the rat tibialis anterior muscle, apparently as a result of an age related transition from type 2B to type 2X units (Larsson et al., 1991a). Electrophoretic and immunoblotting analyses show that the relative proportion of 2X-MyHC increases and that of 2B-MyHC correspondingly decreases with age (Larsson et al., 1993). In addition, MyHC changes can be induced by hormonal and neural influences: in particular, as shown here, the expression of 2X-MyHC transcripts is modulated by thyroid hormone and by electrical stimulation, in agreement with previous studies at the protein level (Ausoni et al., 1990).

Functional Significance of 2X-, 2A-, and 2B-MyHC Isoforms

The maximum velocity of muscle shortening reflects the turnover rate of cross-bridges and turnover rate is a property of the myosin isoforms present in the muscle fibers. Experiments with single fibers from rat and rabbit skeletal muscle indicate that maximum shortening velocity is correlated with MyHC composition. Slow fibers, containing β/slow-MyHC, have lower shortening velocity than fibers containing 2A-MyHC and these in turn have lower shortening velocity than fibers containing 2B-MyHC (Reiser et al., 1985; Eddinger

and Moss, 1987; Sweeney et al., 1988). The contractile properties of four different fiber types, including 2X fibers, were recently compared using rat single fibers in which the MyHC composition was determined by immunostaining with monoclonal antibodies (Bottinelli et al., 1991). The maximum shortening velocity of type 2X fibers was intermediate between that of 2A and 2B fibers, however large and overlapping ranges of variability were observed. It has been suggested that the velocity of muscle shortening is influenced by the alkali MyLC composition and varies as a function of the MyLC3f isoform content in type 2 fibers (Eddinger and Moss, 1987; Sweeney et al., 1988; Greaser et al., 1988). However, the relative contribution of MyHCs and MyLCs in determining the velocity of muscle shortening was not established in previous studies, since the fibers analyzed physiologically were not characterized as regards both MyHC and MyLC composition. Such a combined analysis has now been performed (Bottinelli et al., 1994; Bottinelli, R., R. Betto, S. Schiaffino, and C. Reggiani, manuscript submitted for publication): the results show that (a) both MyHCs and alkali MyLCs are important determinants of maximum shortening velocity and (b) the sensitivity of shortening velocity to changes in alkali MyLC composition depends on the MyHC isoform present, fibers containing 2X- and 2A-MyHC showing a lower sensitivity to variations in MyLC3f content than fibers containing 2B-MyHC.

References

Aigner, S., B. Gohlsch, N. Hamalainen, R. S. Staron, A. Uber, U. Wehrle, and D. Pette. 1993. Fast myosin heavy chain diversity in skeletal muscles of the rabbit: heavy chain IId, not IIb predominates. Eur. J. Biochem. 211:367–372.

Ausoni, S., L. Gorza, S. Schiaffino, K. Gundersen, and T. Lømo. 1990. Expression of myosin heavy chain isoforms in stimulated fast and slow rat muscles. J. Neurosci. 10:153–160.

Bär, A., and D. Pette. 1988. Three fast myosin heavy chain in adult rat skeletal muscle. FEBS (Fed. Eur. Biochem. Soc.) Lett. 235:153–155.

Biral, D., R. Betto, D. Danieli-Betto, and G. Salviati. 1988. Myosin heavy chain composition of single fibers from normal human muscle. Biochem. J. 250:307–308.

Bottinelli, R., S. Schiaffino, and C. Reggiani. 1991. Force-velocity relation and myosin heavy chain isoform composition in skinned fibres of rat skeletal muscle. J. Physiol. 437:655–672.

Bottinelli, R., R. Betto, and C. Reggiani. 1994. Maximum shortening velocity and myosin heavy chain and alkali light chain isoform composition of skinned fast fibres from rat skeletal muscle. J. Physiol. 473:90P.

Brooke, M. H., and K. K. Kaiser. 1970. Muscle fiber types: how many and what kind? Arch. Neurol. 23:369–379.

Butler-Browne, G. S., and R. G. Whalen. 1984. Myosin isozyme transitions occurring during the postnatal development of the rat soleus muscle. Dev. Biol. 102:324–334.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Anal. Biochem. 162:156–159.

Condon, K., L. Silberstein, H. M. Blau, and W. J. Thompson. 1990. Development of fiber types in the prenatal rat hindlimb. Dev. Biol. 138:256–274.

Cox, R. D., A. Weydert, D. Barlow, and M. Buckingham. 1991. Three linked myosin heavy chain genes clustered within 370 kb of each other show independent transcriptional and post-transcriptional regulation during differentiation of a mouse muscle cell line. Dev. Biol. 143:36–43.

Dhoot, G. K. 1986. Selective synthesis and degradation of slow skeletal myosin heavy chains in developing muscle fibers. Muscle & Nerve. 9:155–164.

Eddinger, T. J., and R. L. Moss. 1987. Mechanical properties of skinned single fibres of identified types from rat diaphragm. Am. J. Physiol. 253:C210–C218.

Feinberg, A., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6–13.

Gorza, L. 1990. Identification of a novel type 2 fiber population in mammalian skeletal muscle by combined use of histochemical myosin ATPase and anti-myosin monoclonal antibodies. J. Histochem. Cytochem. 38:257–265.

Gorza, L., K. Gundersen, T. Lømo, S. Schiaffino, and R. H. Westgaard. 1988. Slow-to-fast transformation of denervated soleus muscles by chronic high-frequency stimulation in the rat. J. Physiol. 402:627–649.

Greaser, M. L., R. L. Moss, and P. J. Reiser. 1988. Variations in contractile properties of rabbit single muscle fibres in relation to troponin T isoforms and myosin light chains. J. Physiol. 406:85–98.

Gubler, U. 1988. A one tube reaction for the synthesis of blunt-ended double-stranded cDNA. Nucleic Acids Res. 16:2726.

Gubler, U., and J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. Gene (Aust.). 25:263–269.

Gunning, P., and E. Hardeman. 1991. Multiple mechanisms regulate muscle fiber diversity. FASEB (Fed. Am. Soc. Exp. Biol.) J. 5:3064–3070.

Harris, A. J., R. B. Fitzsimons, and J. C. McEwan. 1989. Neural control of the sequence of expression of myosin heavy chain isoforms in foetal mammalian muscle. Development (Camb.). 107:751–769.

Izumo S., B. Nadal-Ginard, and V. Mahdavi. 1986. All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. Science (Wash. DC). 231:597–600.

Kavinsky, C., P. K. Umeda, A. M. Sinha, M. Elzinga, S. W. Tong, R. Zak, S. Jakovcic, and M. Rabinowitz. 1983. Cloned mRNA sequences for two types of embryonic myosin heavy chains from chick skeletal muscle. I. DNA and derived amino acid sequence of light meromyosin. J. Biol. Chem. 258:5196–5205.

Kelly, A. M., B. W. C. Rosser, R. Hoffman, R. A. Panettieri, S. Schiaffino, N. A. Rubinstein, and P. M. Nemeth. 1991. Metabolic and contractile protein expression in developing rat diaphragm muscle. J. Neurosci. 11:1231–1242.

Klitgaard, H., M. Zhou, S. Schiaffino, R. Betto, G. Salviati, and B. Saltin. 1990. Ageing alters the myosin heavy chain composition of single fibres from human skeletal muscle. Acta Physiol. Scand. 140:55–62.

LaFramboise, W. A., M. J. Daood, R. D. Guthrie, P. Moretti, S. Schiaffino, and M. Ontell. 1990. Electrophoretic separation and immunological identification of type 2X myosin heavy chain in rat skeletal muscle. Biochem. Biophys. Acta. 1035:109–112.

LaFramboise, W. A., M. J. Daood, R. D. Guthrie, S. Schiaffino, P. Moretti, B. Brozanski, M. P. Ontell, G. S. Butler-Browne, R. G. Whalen, and M. Ontell. 1991. Emergence of the mature myosin phenotype in the rat diaphragm muscle. Dev. Biol. 144:1–15.

Larsson, L., T. Ansved, L. Edstrom, L. Gorza, and S. Schiaffino. 1991a. Effects of age on physiological, immunohistochemical and biochemical properties of fast-twitch single motor units in the rat. J. Physiol. 443:257–275.

Larsson, L., L. Edstrom, B. Lindergren, L. Gorza, and S. Schiaffino. 1991b. Myosin heavy chain composition and enzyme-histochemical and physiological properties of a novel fast-twitch motor unit type. Am. J. Physiol. 261:C93–C101.

Larsson, L., D. Biral, M. Campione, and S. Schiaffino. 1993. An age related type IIB to IIX myosin heavy chain switching in rat skeletal muscle. Acta Physiol. Scand. 147:227–234.

Leinwand, L. A., R. E. K. Fournier, B. Nadal-Ginard, and T. B. Shows. 1983. Multigene family for sarcomeric myosin heavy chain in mouse and human DNA/localization on a single chromosome. Science (Wash. DC). 221:766–769.

Lyons, G. E., J. Haselgrove, A. M. Kelly, and N. A. Rubinstein. 1983. Myosin transitions in developing fast and slow muscles of the rat hindlimb. Differentiation. 25:168–175.

Maeda, K., G. Sczakiel, and A. Wittinghofer. 1987. Characterization of cDNA coding for the complete light meromyosin portion of a rabbit fast skeletal muscle myosin heavy chain. Eur. J. Biochem. 167:97–102.

Mahdavi, V., S. Izumo, and B. Nadal-Ginard. 1987. Developmental and hormonal regulation of sarcomeric myosin heavy chain gene family. Circ. Res. 60:804–814.

Nadal-Ginard, B., R. M. Medford, H. T. Nguyen, M. Periasamy, R. M. Wydro, D. Hornig, R. Gubits, L. I. Garfinkel, D. Wieczorek, E. Bekesi, and V. Mahdavi. 1982. Structure and regulation of a mammalian sarcomeric myosin heavy-chain gene. In Muscle Development: Molecular and Cellular Control of Muscle Development. M. Pearson and H. Epstein, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 143–168.

Narusawa, M., R. B. Fitzsimons, S. Izumo, B. Nadal-Ginard, N. A. Rubinstein, and A. M. Kelly. 1987. Slow myosin in developing skeletal muscle. J. Cell Biol. 104:447–459.

Parker-Thornburg J., B. Bauer, J. Palermo, and J. Robbins. 1992. Structural and developmental analysis of two linked myosin heavy chain genes. *Dev. Biol.* 150:99–107.

Reiser, P. J., R. L. Moss, G. G. Giulian, and M. L. Greaser. 1985. Shortening velocity in single fibres from adult rabbit soleus muscles is correlated with myosin heavy chain composition. *J. Biol. Chem.* 260:9077–9080.

Russell, S. D., N. Cambon, B. Nadal-Ginard, and R. G. Whalen. 1988. Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb skeletal muscle. *J. Biol. Chem.* 263:6370–6374.

Saez, L., and L. A. Leinwand. 1986. Characterization of diverse forms of myosin heavy chain expressed in adult human skeletal muscle. *Nucleic Acids Res.* 14:2951–2969.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Sassoon, D., I. Garner, and M. Buckingham. 1988. Transcripts of α-cardiac and α-skeletal actins are early markers for myogenesis in the mouse embryo. *Development (Camb.).* 104:155–164.

Schiaffino, S., L. Saggin, A. Viel, S. Ausoni, S. Sartore, and L. Gorza. 1986. Muscle fiber types identified by monoclonal antibodies to myosin heavy chains. *In* Biochemical Aspects of Physical Exercise. G. Benzi, L. Packer, and N. Siliprandi, editors. Elsevier, Amsterdam. 27–34.

Schiaffino, S., S. Ausoni, L. Gorza, L. Saggin, K. Gundersen, and T. Lømo. 1988. Myosin heavy chain isoforms and velocity of shortening of type 2 skeletal muscle fibres. *Acta Physiol. Scand.* 134:565–566.

Schiaffino, S., L. Gorza, S. Sartore, L. Saggin, M. Vianello, K. Gundersen, and T. Lømo. 1989a. Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J. Muscle Res. Cell Motil.* 10:197–205.

Schiaffino, S., J. L. Samuel, D. Sassoon, A. M. Lompré, I. Garner, F. Marotte, M. Buckingham, L. Rappaport, and K. Schwartz. 1989b. Non-synchronous accumulation of α-skeletal actin and β-myosin heavy chain mRNAs during early stages of pressure overload-induced cardiac hypertrophy demonstrated by in situ hybridization. *Circ. Res.* 64:937–948.

Schiaffino, S., L. Gorza, S. Ausoni, R. Bottinelli, C. Reggiani, L. Larsson, L. Edstrom, K. Gundersen, and T. Lømo. 1990. Muscle fiber types expressing different myosin heavy chain isoforms. Their functional properties and adaptive capacity. *In* The Dynamic State of Muscle Fibers. D. Pette, editor. Walter de Gruyter, Berlin. 329–341.

Strehler, E. E., M. A. Strehler-Page, J. C. Perriard, M. Periasamy, and B. Nadal-Ginard. 1986. Complete nucleotide and amino acid sequence of a mammalian myosin heavy chain gene. *J. Mol. Biol.* 190:291–317.

Sweeney, H. L., M. J. Kushmerick, K. Mabuchi, F. A. Sreter, and J. Gergely. 1988. Myosin alkali light chain and heavy chain variations correlate with altered shortening velocity of isolated skeletal muscle fibres. *J. Biol. Chem.* 263:9034–9039.

Termin, A., R. S. Staron, and D. Pette. 1989a. Myosin heavy chain isoforms in histochemically defined fiber types of rat muscle. *Histochemistry.* 92:453–457.

Termin, A., R. S. Staron, and D. Pette. 1989b. Changes in myosin heavy chain isoforms during chronic low-frequency stimulation of rat fast hindlimb muscles. A single fiber study. *Eur. J. Biochem.* 186:749–754.

Weydert, A., P. Daubas, M. Caravatti, A. Minty, G. Bugaisky, A. Cohen, B. Robert, and M. Buckingham. 1983. 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.* 258:13867–13874.

Weydert, A., P. Barton, J. Harris, C. Pinset, and M. Buckingham. 1987. Developmental pattern of mouse skeletal myosin heavy chain gene transcripts in vivo and in vitro. *Cell.* 49:121–129.

Wieczorek, D. F., M. Periasamy, G. S. Butler-Browne, R. G. Whalen, and B. Nadal-Ginard. 1985. Co-expression of multiple myosin heavy chain genes, in addition to a tissue-specific one, in extraocular musculature. *J. Cell Biol.* 101:618–629.

Yoon, S-J., S. H. Seiler, R. Kucherlapati, and L. Leinwand. 1992. Organization of the human skeletal myosin heavy chain gene cluster. *Proc. Natl. Acad. Sci. USA.* 89:12078–12082.