

Co-expression of Multiple Myosin Heavy Chain Genes, In Addition to a Tissue-Specific One, in Extraocular Musculature

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ABSTRACT We have investigated the developmental transitions of myosin heavy chain (MHC) gene expression in the rat extraocular musculature (EOM) at the mRNA level using S1-nuclease mapping techniques and at the protein level by polypeptide mapping and immunochemistry. We have isolated a genomic clone, designated λ 10B3, corresponding to an MHC gene which is expressed in the EOM fibers (recti and oblique muscles) of the adult rat but not in hind limb muscles. Using cDNA and genomic probes for MHC genes expressed in skeletal (embryonic, neonatal, fast oxidative, fast glycolytic, and slow/cardiac β -MHC), cardiac (α -MHC), and EOM (λ 10B3) muscles, we demonstrate the concomitant expression at the mRNA level of at least six different MHC genes in adult EOM. Protein and immunochemical analyses confirm the presence of at least four different MHC types in EOM. Immunocytochemistry demonstrates that different myosin isozymes tend to segregate into individual myofibers, although some fibers seem to contain more than one MHC type.

The results also show that the EOM fibers exhibit multiple patterns of MHC gene regulation. One set of fibers undergoes a sequence of isoform transitions similar to the one described for limb skeletal muscles, whereas other EOM myofiber populations arrest the MHC transition at the embryonic, neonatal/adult, or adult EOM-specific stage. Thus, the MHC gene family is not under the control of a strict developmental clock, but the individual genes can modify their expression by tissue-specific and/or environmental factors.

Myosin heavy chain (MHC),¹ the main component of the thick filament of the sarcomere, is encoded by a highly conserved multigene family of 7–10 members (47) of which several appear to be clustered onto a single chromosome in mouse (16, 35) and human (35). Each different sarcomeric MHC gene displays a pattern of expression that is both tissue specific and developmentally regulated (39, 43, 46, 50). Protein and mRNA studies have demonstrated that myosin isozymes follow an embryonic \rightarrow neonatal \rightarrow adult transition pattern during mammalian (50, 73) and avian (2, 23, 37, 66) skeletal muscle development. In the cardiac muscle of small mammals, myosin isozyme switches occur in an embryonic

\rightarrow adult pattern (24, 36, 59). The mechanisms regulating these myosin isozyme transitions and their relevance to muscle physiology are not fully understood (see reference 69 for discussion).

Cellular and hormonal environments of muscle are known to significantly affect the phenotypic expression and transition of MHC isoforms. Both the cardiac and skeletal muscle isomyosin switches are subject to control by thyroid hormone. Hypothyroidism of adult mammals leads to an increased transcription of the cardiac β -MHC isoform in ventricular cardiac tissue (14, 19, 20, 36, 60); slow muscle-type MHC isoforms are quantitatively increased in adult rat skeletal muscle after thyroidectomy (12, 22, 31, 67). Developmental isoform transitions are also inhibited by hypothyroidism and accelerated by hyperthyroidism (12, 14, 22, 74).

¹ *Abbreviations used in this paper:* EOM, extraocular musculature; MHC, myosin heavy chain; nt, nucleotide(s).

The type of muscle innervation, as well as its associated nerve activity, are thought to be specifically involved in controlling contractile protein isozyme transitions (8, 22, 26, 56). Available data suggest that multiple innervation and chronic stimulation induce and maintain slow myosin synthesis in adult animals (5, 33, 34, 52, 57). Cross-innervation studies on fast- and slow-twitch muscles have demonstrated the importance of neural factors in determining the speed of muscle contractions (18). However, not all myosin isozyme changes are caused by neural stimuli since some changes are independent of continued innervation (11, 22). Also, slow myosin can be found in fetal muscles and its presence may therefore be nerve independent (71).

To understand the molecular mechanisms responsible for the developmental transitions of MHC gene expression, we have previously characterized cDNA and genomic sequences corresponding to several MHC isozymes (39, 43, 46, 50, 77). In this study we show that a previously unidentified MHC gene is expressed in rat extraocular muscle tissue (recti and oblique muscles) but not in hind limb or cardiac muscles. We have isolated a genomic clone that contains nucleotide (nt) sequences coding for amino acid 1697 to the carboxy terminus of the protein, a region corresponding to the α helical light meromyosin portion of this myosin molecule. This MHC gene is induced in the first month of postnatal life and remains expressed through adult life.

The multiple fiber types in the extraocular musculature (EOM) and the complex physiological responses found in these muscles make this an excellent system in which to investigate the presence and developmental transitions of EOM isozymes. The phenotypic expression of different MHC mRNA transcripts was investigated in the extrinsic eye muscles by S1-nuclease analysis (3) whereas the heavy chain proteins present were studied by polypeptide mapping and immunochemistry. The results show that this musculature not only contains a unique MHC gene product, but also undergoes a very complex MHC transition scheme. Unlike most sarcomeric muscles which demonstrate a sequential activation and repression of MHC genes during development, the EOM exhibits a sequential activation of MHC genes without complete repression of any of them. This pattern of regulation results in the simultaneous expression of at least six different MHC genes at the mRNA level and the synthesis of at least four different MHC proteins. Localization through immunocytochemistry demonstrates that, in general, these different myosin heavy chain isozymes are segregated in different fibers.

MATERIALS AND METHODS

Screening of Phage Genomic Libraries: Rat genomic libraries constructed by partial Eco RI-digested spleen DNA cloned into Charon 4A phage λ vectors were screened as described (4, 77). Approximately 1.5×10^6 phage plaques were screened using radioactively labeled (53) cDNA clones (36, 43, 50) containing an amino acid coding sequence known to hybridize to most, if not all, of the sarcomeric MHC genes (77).

Restriction Endonuclease Mapping and DNA Sequence Analysis: Genomic clone λ 10B3 DNA was isolated and digested with the restriction endonucleases Eco RI, Hind III, Bam HI, Xba I, and Sac I. The relative position of each restriction site was determined by partial enzyme digests separated on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to 32 P-labeled fragments obtained from complete digests of the clone (62). After hybridization, the filters were washed in 0.015 M NaCl, 0.015 M sodium citrate, 0.1% SDS at 65°C. The DNA sequence analysis was performed as described by Maxam and Gilbert (40) and by the dideoxy chain

termination method of Sanger et al. (58).

RNA Isolation and S1-Nuclease Mapping Analysis: Total cellular RNA was isolated from the muscles of Wistar rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) using modifications of the hot phenol procedure (61) and from L₆E₉ cells (45) by the method of Favalaro et al. (21). In preparation of RNA from EOM, the recti muscles (superior, inferior, exterior, and interior) and oblique muscles (superior and inferior) from 1-month or 1-yr-old (adult) rats were pooled and classified as "rectus" and "oblique," respectively. EOM tissue from newborn pups was not separated into recti or oblique tissue, but, rather, was collectively gathered.

RNA-DNA hybridization was performed as described (3, 36, 50). The restriction fragments were 3' end-labeled with (α - 32 P)cordycepin (New England Nuclear, Boston, MA) using terminal transferase. After size separation by electrophoresis, the desired fragments were strand separated, and the strand complementary to the mRNA was purified (40). The probes were hybridized in DNA excess to 20 μ g of total RNA extracted from different muscle tissues. Hybridization was in 25 μ l of 80% deionized formamide, 0.4 M NaCl, 10 mM PIPES (pH 6.1), 1 mM EDTA, and 0.05% SDS. The hybridization mixture was incubated at 65°C for 1 h, the temperature adjusted to 42°C, and incubation continued for 20 h. S1-nuclease digestion was in 300 μ l for 1 h at 25°C with 150 U of enzyme (New England Nuclear) in 300 mM NaCl, 30 mM Na acetate (pH 4.5), 3 mM ZnSO₄. The reaction was terminated with 10 mM EDTA and precipitated with ethanol. Dried pellets were resuspended in 85% formamide and electrophoresed on 6% polyacrylamide, 8.3-M urea sequencing gels (40).

Electrophoretic and Immunological Analysis of Myosin Isozymes: The sources of the different hind limb myosin isozymes were described previously (13). Briefly, the gastrocnemius and soleus muscles of 1-month-old rats were the source of adult fast and slow myosin isozymes, respectively. Neonatal myosin was prepared from 7-8-d-old bulk hind leg muscle, and embryonic myosin was obtained from cultures of L6 myotubes (72); for the EOM biochemical analyses, the four recti and two oblique extraocular muscles were pooled. Extracts were prepared and the native myosin isozymes analyzed by electrophoresis in nondenaturing conditions as described (13). Myosins were purified (9) and subjected to partial proteolysis with chymotrypsin after denaturation by SDS (72). After separation of the polypeptides on SDS polyacrylamide gels, the gels were either stained with Coomassie Blue or the proteins were electrophoretically transferred to nitrocellulose filters as described by Burnette (10), except that the transfer buffer contained 0.1% SDS. Immunoblotting and indirect immunofluorescence were performed using rabbit antibodies to myosin prepared and characterized as described previously (13).

RESULTS

Isolation of MHC Genomic Clone λ 10B3

MHC genomic clones were isolated by screening a partial Eco RI genomic library of rat genomic spleen DNA (77) with previously characterized cDNA clones (39, 43, 50). One of the isolated clones, λ 10B3, was selected for further analysis because its restriction pattern differed from previously identified MHC genomic clones (77) (Fig. 1A). Genomic clone λ 10B3 was Eco RI restricted and blotted onto nitrocellulose after agarose gel electrophoresis and hybridized to the following radioactively labeled cDNAs: pMHC 25 (embryonic MHC [43]), pFOD 5 (neonatal MHC [50]), pMHC 62 (adult MHC [46]²), and pCMHC 21 (adult cardiac α -MHC [36, 39]). Each of these cDNA clones hybridized to two Eco RI fragments 2.3 kb and 8.9 kb from λ 10B3 (not shown).

The individual Eco RI fragments of λ 10B3 were hybridized to Northern blots (65) containing total RNAs from atrial, ventricular, and several different skeletal muscle tissues. A single hybridizing band (with varying intensities) corresponding in size to MHC mRNA (~32S) was observed with most muscle tissues (data not shown). The extensive cross-hybridization observed between λ 10B3 and the sarcomeric MHCs rendered Northern blot analysis unsuitable for defining tissue- and developmental stage-specificity of this MHC gene.

Sequence Analysis of MHC Clone λ 10B3

To determine the precise MHC region encoded in λ 10B3,

λ10B3

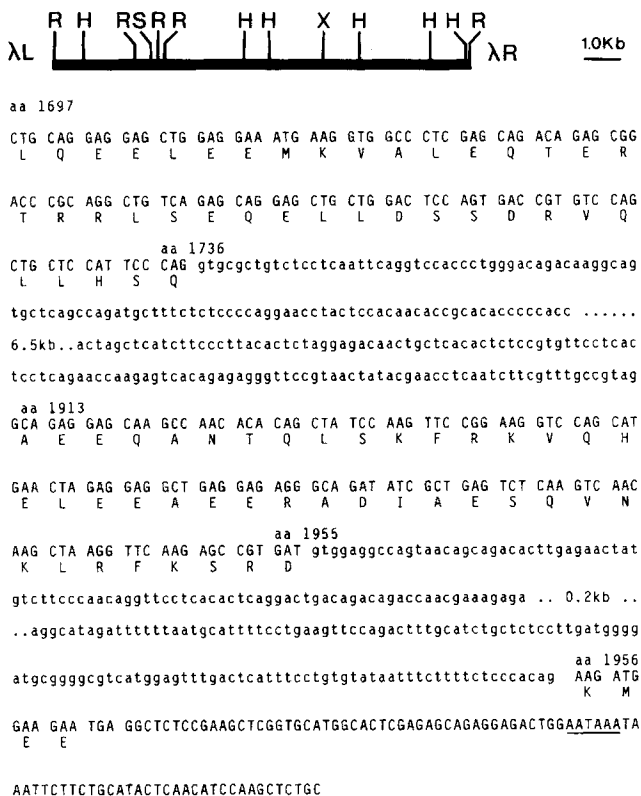


FIGURE 1 Restriction-endonuclease map (A) and partial nucleotide and amino acid sequences (B) of the λ10B3 MHC genomic clone. The restriction sites were mapped by analyzing the digests in ethidium-bromide-stained agarose gels and by transferring the digests to nitrocellulose, followed by hybridization to fully digested ³²P-labeled probes. The restriction sites are R (Eco RI), H (Hind III), S (Sac I), and X (Xba I). No Bam HI sites were detected. The nucleotide and derived amino acid sequences are written in the 5' to 3' (NH₂ to COOH) direction. The deduced amino acid sequence within exons is given in the one-letter code below the second nucleotide of the corresponding codon. The polyadenylation signal sequence is underlined. Dots indicate the undetermined sequences of this MHC.

DNA sequencing analysis was conducted on this genomic clone. Selected fragments which hybridized to MHC cDNA clones were sequenced by the Maxam-Gilbert method (40) and by the dideoxy chain termination method after subcloning into M13 phage (58). A comparison of the amino acid sequences derived from λ10B3 nucleotide sequences with other rat MHC amino acid sequences (39, 50) revealed that this genomic clone contains a sequence which codes for amino acid 1697 to the carboxy terminal amino acids (Fig. 1B). Further comparison of the nucleotide and amino acid sequences between λ10B3 and other skeletal and cardiac MHC cDNAs (39, 50)² demonstrate substantial regions of sequence conservation interspersed with divergent areas, sharing greatest amino acid homology with the gene encoding the adult slow skeletal/embryonic cardiac MHC (Table I). This homology clearly demonstrates that λ10B3 belongs to the sarcomeric MHC gene family yet is different from any of the previously characterized MHC sequences.

²Nadal-Ginard, B., M. Periasamy, D. Wiczorek, E. Strehler, S. Grund, S. Izumo, D. Bois, and V. Mahdavi, manuscript in preparation.

A comparison between the λ10B3 and the embryonic MHC gene sequence³ revealed exon-intron splice junctions at identical positions, following amino acid 1736 and amino acid 1955. Furthermore, the size of the exon encoding aa 1913-1955 (42 aa) is identical in both genes. Thus, there appears to be a general conservation in the exon-intron organization at the 3' end of MHC genes as has been observed for the 5' end (63).

A characteristic feature of the rat MHC multigene family is that the last few amino acids, together with the 3' untranslated region, reside in a separate exon (46). Similarly in the MHC gene, represented by the λ10B3 clone, the carboxy-terminal exon as identified by an acceptor splice consensus sequence (TTCTCCCACAG) proposed by Breathnach and Chambon (7) lies 438 bp downstream of the codon specifying aa 1955. It carries the characteristic MHC carboxy-terminal Glu Glu Stop codon sequence (46) followed by the 3' untranslated region and the poly (A) addition signal specified by AATAAA nucleotide sequence (Fig. 1B).

The MHC Gene Represented by Clone λ10B3 Is Expressed in the EOM

The nucleotide sequence divergence between the coding exons in the clone λ10B3 and other sarcomeric MHC cDNAs provides the gene-specific sequences with which to study the expression of this gene using the S1-nuclease mapping technique (3). In this technique, when the single, end-labeled DNA probe used hybridizes to its homologous mRNA, complete protection of the probe from S1-nuclease single strand-specific digestion is obtained, whereas hybridization to mRNAs partially homologous to the probe at the labeled end generates shorter labeled fragments. The λ10B3 probe used is a double-stranded, 296-bp long, Pst I-Eco RI fragment that maps at the 5' end of the genomic clone. It includes the codons for aa 1697-1736, and 179 bp of intervening sequence. Hybridization to the homologous mRNA should generate a protected fragment 117 nucleotides (nt) long, since at this point an exon-intron splice site occurs and the rest of the sequence in the probe corresponds to an intervening sequence (Fig. 1B). From the S1-nuclease protection pattern observed in Fig. 2A, it is apparent that the gene represented by λ10B3 is expressed in rat EOM (recti and oblique muscles) and not in cardiac or limb skeletal musculature, including the soleus muscle. Furthermore, this probe does not show any protection with RNA isolated from newborn cardiac or adult atrial tissues, 11-d or 13-d fetal skeletal musculature, adult diaphragm, or extensor digitorum longus muscles (data not shown). In addition, RNA isolated from the masseter muscle (a muscle which in carnivorous predators displays a hyperfast contractile myosin [55, 64]) does not show protection when hybridized with the above probe. The band at 296 bp seen in Fig. 2A is due to reannealing of the probe to itself; the faint band at ~155 bp is due to incomplete denaturation of the probe because of the high (60%) G-C content in the intervening sequence.

To ascertain the developmental stage specificity of λ10B3 MHC expression, total RNA was isolated from the extrinsic eye muscles at 3 d, 1 mo, and 1 y of postnatal life. As seen in Fig. 2B, the mRNA represented by the λ10B3 MHC gene is first detected between 3 d and 1 mo of postnatal life, and is

³Strehler, E. E., M.-A. Strehler, J.-C. Perriard, M. Periasamy, and B. Nadal-Ginard, manuscript in preparation.

TABLE I. Amino Acid Comparison of Rat Extraocular MHC λ 10B3 to Skeletal and Cardiac MHCs

MHC cDNA clone	Divergence in amino acid sequence aa 1697-1736	Nonconservative amino acid changes aa 1697-1736	Divergence in amino acid sequence aa 1913-1955	Nonconservative amino acid changes aa 1913-1955	Total divergence	Total nonconservative divergence
Embryonic skeletal (pMHC 25)	13/39 (33%)	3/13 (23%)	7/42 (17%)	1/7 (14%)	20/81 (25%)	4/20 (20%)
Neonatal skeletal (pFOD 5)	11/39 (28%)	3/11 (27%)	7/42 (17%)	3/7 (43%)	18/81 (22%)	6/18 (33%)
Adult skeletal fast oxidative (pMHC 40)	13/39 (33%)	3/13 (23%)	5/42 (12%)	1/5 (20%)	18/81 (22%)	4/18 (22%)
Adult skeletal fast glycolytic (pMHC 62)	11/39 (28%)	3/11 (27%)	7/42 (17%)	3/7 (43%)	18/81 (22%)	6/18 (33%)
Adult slow skeletal/cardiac β -MHC (pCMHC 5)	13/39 (33%)	2/13 (15%)	3/42 (7%)	0/3 (0%)	16/81 (20%)	2/16 (12%)

The derived amino acid sequences (aa 1697-1736; aa 1913-1955) of λ 10B3 were compared with the analogous coding regions from the following MHC cDNA clones: embryonic skeletal (pMHC 25)(43), neonatal skeletal (pFOD 5)(50), adult skeletal, fast oxidative (pMHC 40),² adult skeletal, fast glycolytic (pMHC 62),² and adult slow skeletal/cardiac β -MHC (pCMHC 5)(39).

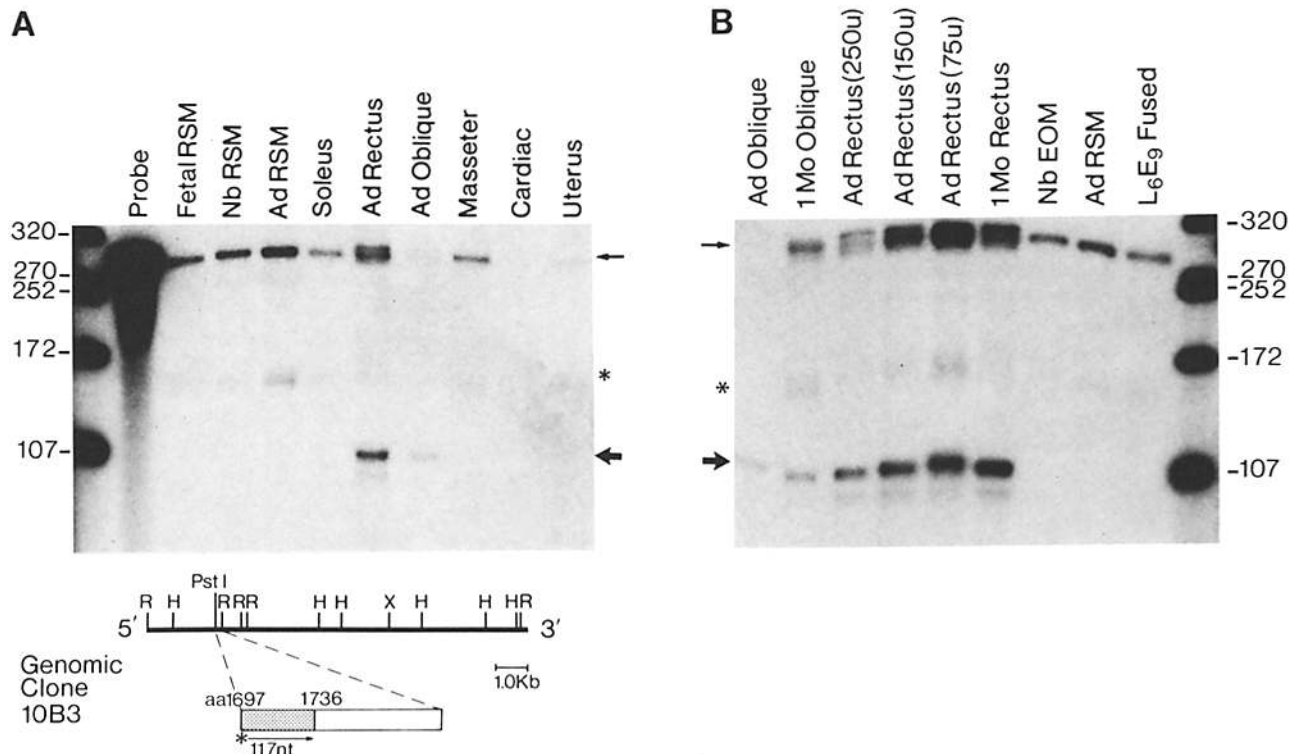


FIGURE 2 Detection of extraocular muscular MHC mRNA by S1-nuclease mapping. Total RNA was isolated from muscle tissues of various developmental stages. A and B show the pattern of S1 nuclease protection obtained using a double-stranded probe (296 bp) which is 3' end-labeled at the Pst I site corresponding to aa 1697. Full protection of the probe was 117 nt (aa 1679-1736), followed by 179 bp of intervening sequence. The artifactual band at 296 bp is due to self-reannealing of the probe; the faint band at 150 (*) is due to incomplete denaturation of the probe (the intron region has a 60% G-C content). Size markers are from selected digested fragments of the cDNA clones pCMHC5, -21, and -26 (36, 39). EOM, extraocular musculature; RSM, rat skeletal limb muscle; Nb, newborn; Ad, adult; Mo, month; u, units of S1.

present throughout adult life. The same results are obtained when higher concentrations of S1-nuclease (250 U) are used. It is unlikely that this gene is expressed in skeletal musculature since longer exposure of the gels shown in Fig. 2 did not reveal any full protection of the probe (data not shown). From these results it is reasonable to conclude that the MHC gene represented by λ 10B3 shows a narrow tissue-specific expression that is extraocular muscle-specific.

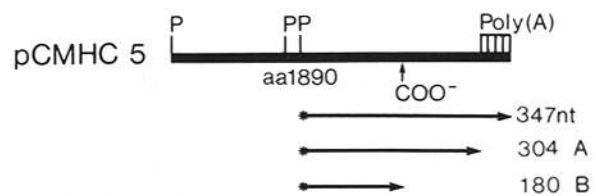
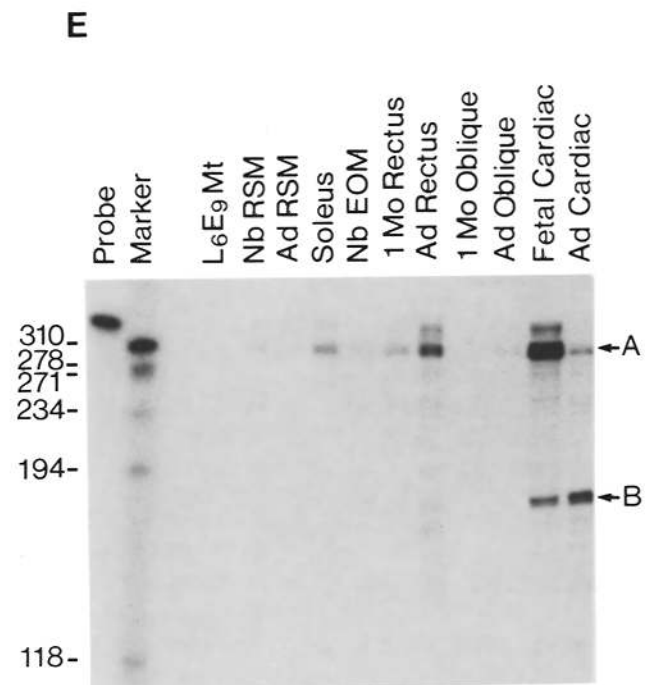
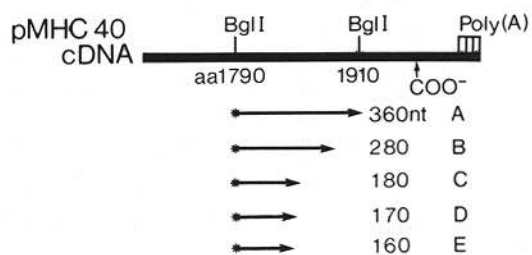
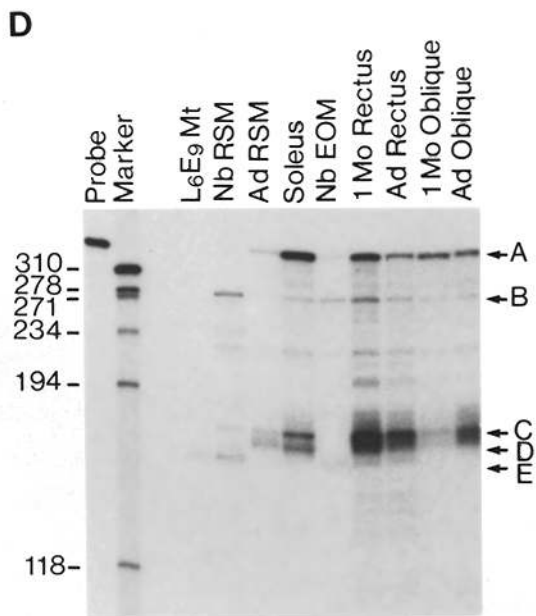
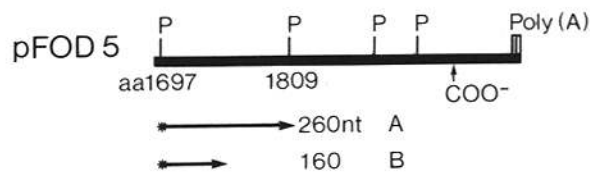
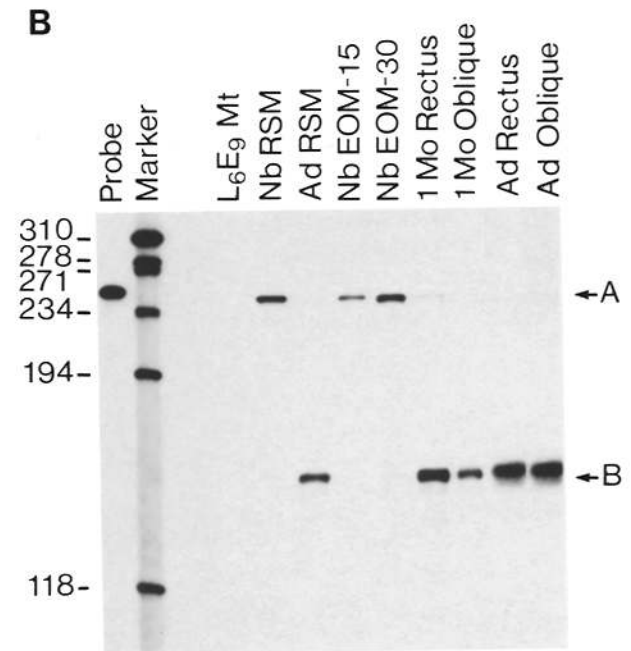
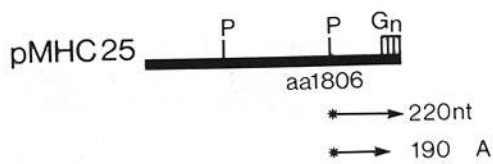
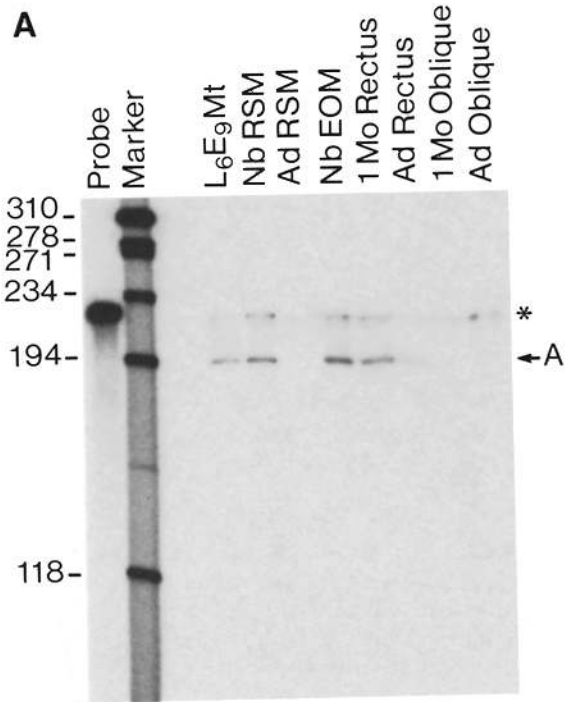
Multiple MHC Isozyme mRNA Sequences Are Expressed in the EOM

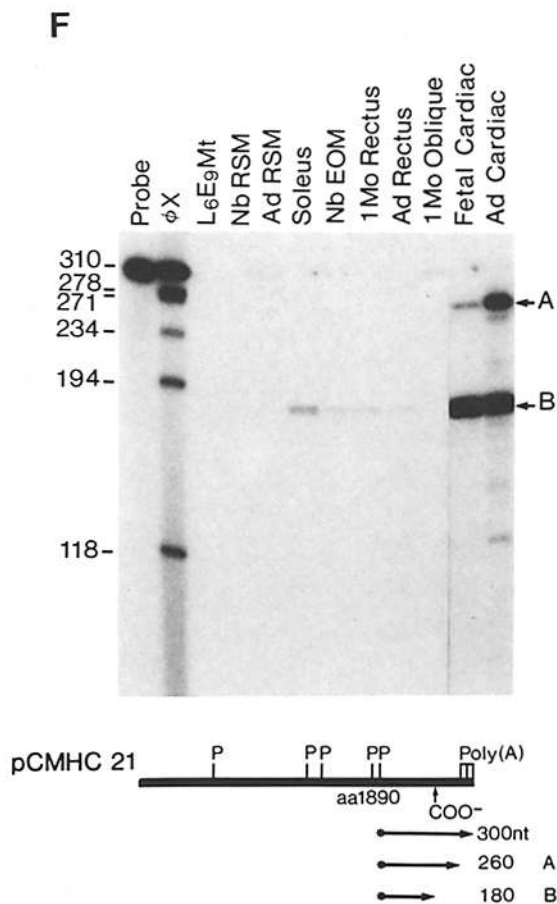
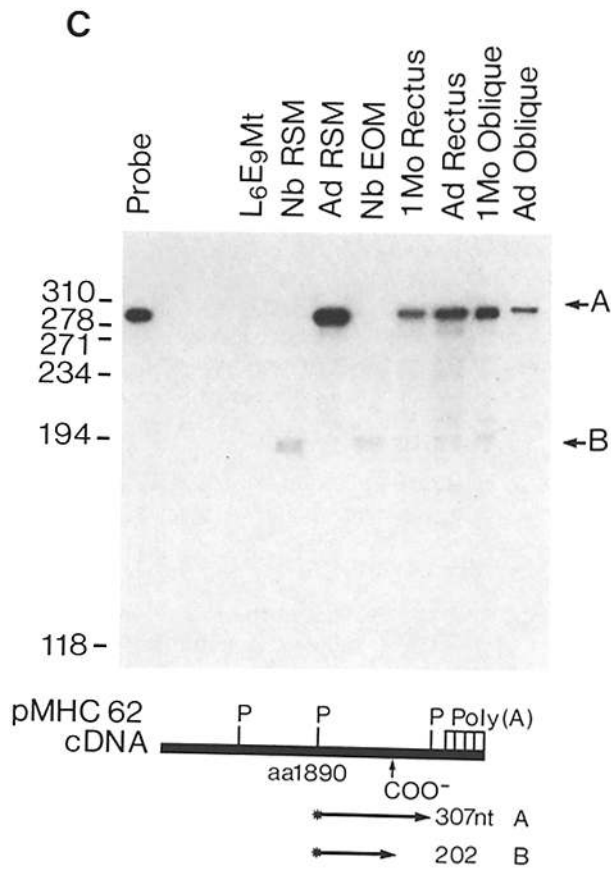
To determine the developmental expression of MHCs in EOM and whether there are multiple MHC isoforms in these muscles (in addition to the type represented by λ 10B3), we used probes from rat embryonic (39, 43), neonatal (50), and adult (36, 46)² specific sarcomeric MHC cDNA clones in S1 nuclease protection analyses. Single-stranded probes were pre-

pared from 3' end regions of cDNA clones and hybridized to extraocular, limb skeletal, and cardiac muscle RNAs.

An MHC gene represented by an embryonic skeletal muscle cDNA probe pMHC25 is expressed in embryonic life and continues to be present in neonatal rat skeletal muscle (43).⁴ However, by 2 postnatal wk, the gene has been de-induced and its transcripts are no longer detectable.⁴ The probe used in this analysis is 220 nt long (including the 16-bp poly G tail used in cDNA construction), maps at the 3' end of the cDNA pMHC25, and contains the codons for aa 1806-1869 of the MHC protein (Fig. 3A). As shown in Fig. 3A, the expected full length protection of the probe (190 nt) is obtained with RNA isolated from L₆E₉ myotubes (a rat muscle cell line [45]) where this gene is expressed (72), and in skeletal muscle and EOM isolated from animals in the pre- and perinatal period. Surprisingly, although full protection is not observed

⁴Periasamy, M., R. M. Wydro, and B. Nadal-Ginard, manuscript in preparation.





in adult limb skeletal muscle, it is detectable in 1 mo and adult (1 y) recti and oblique EOM RNAs; its amount, however, is diminished in the older muscle RNA samples. The presence of a weak artifactual band at 220 nt is due to incomplete S1 nuclease digestion of the poly G tail of the probe.

A cDNA clone, pFOD 5, has been characterized and found to contain sequences corresponding to a perinatal-specific skeletal MHC mRNA (50). Perinatal MHC mRNA is first detectable late in fetal life, reaches maximum levels of expression at the end of the first postnatal week, and is de-induced thereafter; its levels are almost undetectable at 28 d of postnatal life in hind limb muscles.

A single-stranded probe was prepared from pFOD5 and hybridized with total RNA from the various developmental stages of the extraocular muscle tissues. As shown in Fig. 3B, expected full-length protection (260 nt) of the probe (A) is obtained with skeletal muscle mRNAs isolated from animals in the perinatal period. L₆E₉ myotubes, which express an embryonic MHC mRNA, do not show any full-length protection of this probe. Full protection of the probe was not visible even with long exposure in adult rat skeletal limb muscle. In EOM tissue, a fully protected fragment was observed with RNA from all developmental stages, including adult. However, the intensity of the fully protected band greatly diminishes with the increasing age of the animal. A similar result has been obtained using as probe the 3' untranslated region of pFOD 5 (data not shown). Based on nucleotide sequence comparisons (39, 50)², the partially protected band appearing at 160 nt (B) represents the appearance of adult-specific MHCs.

Adult rat skeletal muscle MHC cDNAs were used in analyzing the MHC isoforms in extrinsic eye musculature. Based on tissue-specific expression, pMHC 62 has been found to be a cDNA representing an adult fast-glycolytic MHC gene (46)². Detection of mRNA transcripts from the gene represented by pMHC 62 are first detectable in 7-d postnatal skeletal muscle tissue, with maximum levels attained after 3 mo². A single-stranded probe was prepared from pMHC 62 and hybridized to total RNA from extraocular tissues and control RNAs. As seen in Fig. 3C, full protection of this probe (A) is seen only with RNAs from adult stages, including the EOM RNA. No protection was seen in L₆E₉ myotube RNA, and newborn tissues demonstrated only partial protection (B). The partially protected band may represent neonatal and/or other MHC mRNAs. This result demonstrates that the gene represented by pMHC 62 is actively expressed only in the adult EOM but

FIGURE 3 Detection of MHC mRNAs in extraocular mRNA by S1 nuclease analysis. Single-stranded MHC probes were prepared from various stage and tissue-specific cDNAs: (A) pMHC25, embryonic skeletal MHC (43); (B) pFOD5, neonatal skeletal MHC (50); (C) pMHC62, adult fast-glycolytic MHC²; (D) pMHC40, adult fast-oxidative MHC²; (E) pCMHC5, slow skeletal/cardiac β -MHC (36); (F) pCMHC21, cardiac α -MHC (36). Probes were labeled at the restriction site (*) which corresponds to the designated amino acid of the MHC protein. The bands labeled A demonstrate fully protected probes, whereas the bands labeled B-E correspond to partially protected fragments. The artifactual band in Fig. 3A at 200 nt is due to the poly G tail used in construction of the cDNA. In Fig. 3B, 15 μ g and 30 μ g of Nb EOM RNA was analyzed. The size markers were generated from ³²P-labeled ϕ X Hae III digests. Abbreviations are as designated in Fig. 2, with the following addition: Mt, myotubes.

not during the early perinatal stages of development.

The expression of another adult skeletal muscle MHC gene, represented by the cDNA pMHC 40, was investigated in EOM tissue. This cDNA contains sequences corresponding to fast-oxidative MHC mRNA (46).² These transcripts are first detectable in limb skeletal muscle during early postnatal development, reaching maximum levels by 3 mo. Muscles, such as the soleus, which have an increased amount of fast-oxidative myofibers, show a significant amount of fully protected transcripts when probed with this MHC cDNA.²

The pMHC 40 probe used is single stranded, 360 nt long, and contains codons for aa 1790–1910 of MHC. As shown in Fig. 3D, when pMHC 40 is used as a probe and hybridized to total RNA from various skeletal muscle tissues, full protection (360 nt) (*A*) was visible in all samples including rat skeletal muscle and EOM from all developmental stages except in L₆E₉ myotubes.

Based on nucleotide sequence comparison of pMHC 40 with the other MHC cDNAs characterized in our laboratory (39, 50)², the presence of some partially protected bands (*B–E*) can be assigned to specific MHC transcripts. *B* represents the neonatal MHC mRNA; *C* will be discussed later; *D* represents the fast-glycolytic MHC mRNA; and *E* corresponds to the embryonic MHC mRNA. These results are in agreement with those observed in Fig. 3, *A–C*. The presence of unidentified partially protected fragments in Fig. 3D may represent other MHC transcripts not yet identified or partially digested transcripts due to “nibbling” of the S1 nuclease enzyme.

To examine whether a slow twitch myosin might also be expressed in EOM muscles, we used a cDNA clone, pCMHC 5, specific for cardiac β -MHC/adult slow-twitch MHC mRNA predominantly expressed in fetal cardiac and adult skeletal muscle (36). The probe used is 347 nt long and maps at the 3' end of the cDNA containing the codons for the amino acids from 1890 to the carboxy-terminal end and the 3' untranslated region, including the poly A tail and the poly dG cloning linker at the 3' end.

Using the pCMHC 5 probe, a fully protected fragment 304 nt long of varying intensities is visible in all muscle RNA samples used with the exception of L₆E₉ myotubes (Fig. 3E, band *A*). The rat EOM also demonstrates fully protected transcripts in all developmental stages. The varying intensities of the fully protected band in the RNA samples reflect the myofiber composition of the particular muscle under analysis. From the nucleotide sequence comparison it was determined that band *C* of Fig. 3D represents the slow-twitch MHC mRNA transcript.

A previous study (36) has demonstrated that when pCMHC 5 probe is used in S1 hybridization studies, a partially protected band of 180 nt corresponds to the adult cardiac α -MHC transcript. The correct inverse relationship with respect to intensities of the fully and partially protected bands (*A* and *B*, Fig. 3E) is reflected in the fetal and adult cardiac samples (36). An interesting finding was that the extraocular tissues also showed a faint, partially protected fragment of the appropriate size as the adult cardiac α -MHC. This protected band might represent the presence of the cardiac α -MHC, or alternatively, a different MHC isoform with a similar molecular structure. Evidence for additional MHC isoforms with this structure has been suggested in a previous study (36).

To test whether the EOM synthesizes the adult cardiac α -MHC transcript, the 3' end of pCMHC 21 was used to probe

its expression in varying skeletal and cardiac tissues (36). The probe used is 300 nt long, maps at the 3' end of the cDNA, and contains the codons for the amino acids 1890 to the carboxy-terminal end, including the poly A tail (Fig. 3F). The results show that a fully protected transcript was detected in the cardiac muscle RNAs and not in the EOM RNA samples. However, a partially protected band (possibly representing the slow-twitch MHC gene transcript detectable by pCMHC 5 in Fig. 3E) is clearly visible in the EOM samples. The absence of the fully protected band with the pCMHC 21 probe might be due to the low level of this RNA transcript in EOM tissue. Alternatively, it is possible that the partially protected band (*B*) in Fig. 3E represents an mRNA with a coding region similar to the α - and β -MHC cardiac genes but a diverse 3' untranslated region. This undefined MHC transcript may represent another slow-twitch MHC different from that coded for by the gene represented by pCMHC 5.

Multiple MHC Proteins Are Expressed in EOM

We carried out an analysis of the myosin protein species found in the EOM in order to determine if the multiple mRNA isoforms detected were also expressed at the protein level and to evaluate the relative amounts of each isozyme.

Native myosin isozymes were examined using electrophoresis in pyrophosphate-containing buffer; this method separates isozymes composed of different heavy chains as well as those formed by the possible combinations of light chains with individual heavy chains (17, 29). Adult EOM (Fig. 4c) shows a pattern of native myosin isozymes which is more complex than those exhibited by hind limb muscles, either predominantly fast-type (Fig. 4d) or predominantly slow-type (Fig. 4e). Part of the complexity can be explained by the light chain content of EOM which was determined by two-dimensional gel electrophoresis (results not shown); the EOM contains predominantly the three fast-type light chains and only traces of the slow forms, with no evidence of the embryonic light chain (70). This light chain content therefore should result in the formation of two homodimer and one heterodi-

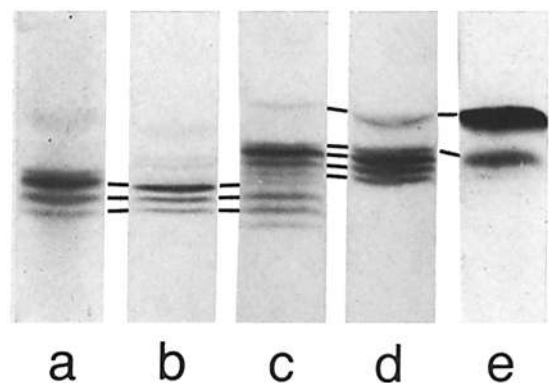


FIGURE 4 Electrophoresis of native myosin isozymes from EOM and hind limb muscles. Results are shown for the analysis of myosin-containing extracts prepared from EOM of 7-d-old rats (*a*), hind limb muscle of 8-d-old rats (*b*), adult EOM (*c*), gastrocnemius muscle of 1-mo-old rats (*d*), and soleus muscle of 1-mo-old rats. Analysis of mixtures of these extracts (not shown) established the correspondence of the bands among different samples; this is indicated by the horizontal bars between bands. The three neonatal myosin bands are indicated in *a–c*, the four fast myosin bands are indicated in *d*, and the major slow myosin band and minor fast myosin band in soleus muscle are indicated in *c–e*.

mer species for some heavy chain type(s), as shown previously for fast myosin (38).

Since the migration positions and relative separation of native myosin bands are sensitive to protein load and time of electrophoresis, we analyzed mixtures of EOM extracts and samples containing adult fast (Fig. 4*d*), slow (Fig. 4*e*), and neonatal isoforms (Fig. 4*b*). In this way, we established the correspondence between the different bands, and this is indicated by the horizontal bars in Fig. 4. Adult EOM contains bands which co-migrate with four adult fast-type bands, probably representing a mixture of type IIA and IIB myosins (13). Three other bands are also seen which co-migrate with the neonatal skeletal myosin isoforms. The fastest migrating band in the adult EOM sample does not correspond to any of the known adult fast and slow, neonatal, or embryonic bands found in rat hind limb muscles (13, 73). Finally, one minor band in the EOM sample (the slowest migrating band) co-migrates with hind limb slow myosin.

Analysis of EOM from 7-d-old rats (Fig. 4*a*) shows a simpler pattern than that of the adult EOM sample, and one which resembles the pattern given by neonatal hind limb muscle (Fig. 4*b*). In the 7-d EOM muscle, three major bands are seen which were found to co-migrate with the three hind limb neonatal bands (horizontal bars in Fig. 4, *a* and *b*). A fourth band, immediately above the slowest-migrating neonatal band, corresponds to the position of embryonic myosin. Thus, the EOM of 7-d-old rats has a myosin content equivalent to that of neonatal hind limb muscle (73). In contrast, the adult EOM contains more native myosin species than are found in adult hind limb muscles. The analysis of EOM of 1-mo-old rats demonstrated that the adult EOM pattern was already established by that time (results not shown). This is in full agreement with the results from the S1 nuclease analysis where no qualitative differences have been detected between 1-mo and adult EOM MHC mRNA transcripts.

We used polypeptide mapping (72) to examine directly the MHC types present in adult EOM. Myosins were purified from adult EOM, adult fast- and slow-type hind limb muscles, neonatal hind limb muscles, and from L6 myotubes in culture as a source of embryonic myosin (13, 72). After denaturation with SDS and treatment with chymotrypsin, the heavy chain cleavage products were analyzed on SDS polyacrylamide gels. Comparison of the cleavage patterns showed that the adult EOM MHC (Fig. 5*c*) is most similar to adult fast-type MHC (Fig. 5*b*) as judged by the presence of individual polypeptides as well as their relative intensities. The small differences between the EOM and fast MHC patterns do not seem to be due simply to the presence of embryonic, neonatal, or slow heavy chain polypeptides (Fig. 5*a*, *d*, and *e*, respectively) in the EOM pattern but, rather, to the presence of other uncharacterized heavy chains. The close correspondence between the EOM and fast heavy chain cleavage patterns means either that skeletal fast-type isoforms are the predominant heavy chain species or that the specific EOM heavy chain, represented by the clone λ 10B3, is also a major form which may be very homologous to the fast myosin types.

We combined the polypeptide mapping technique with immunoblotting using myosin antibodies (13) specific for embryonic, neonatal, and adult fast and slow MHC in an attempt to confirm the results of Fig. 5 and also to identify minor heavy chain isoforms in the adult EOM myosin. The particular advantage of this immunopolypeptide mapping approach is that the specificity of a positive antibody reaction

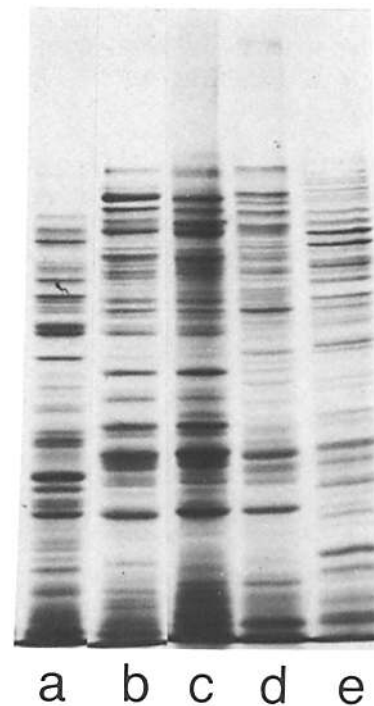


FIGURE 5 Polypeptide mapping of adult EOM myosin and the four major hind limb muscle isoforms. Approximately 15 μ g of each SDS-denatured myosin was digested with chymotrypsin and separated on an SDS-containing polyacrylamide gel which was subsequently stained with Coomassie Blue. Results are shown for embryonic myosin (*a*), adult fast myosin (*b*), adult EOM myosin (*c*), neonatal myosin (*d*), and adult slow myosin (*e*).

is confirmed by the pattern of polypeptides which react with the antibody. The results of this approach using an antibody to adult fast myosin are shown in Fig. 6*A*. The antibody reacts with polypeptides derived from the homologous fast MHC (lanes *d* and *e*) and with the adult EOM MHC (lane *c*). Neither slow, neonatal, or 7-d-old EOM myosins (lanes *f*, *a*, and *b*, respectively) show substantial reactivity with this antibody. Thus, the results of Fig. 6*A* demonstrate that the adult EOM heavy chains are identical or highly homologous to hind limb fast myosins, confirming the polypeptide and S1 nuclease mapping results of Figs. 3 and 5.

It can be demonstrated that both the 7-d-old and adult EOM myosins contain an MHC isoform which reacts with an antibody to neonatal heavy chain. The antibody-stained polypeptides of the 7-d EOM sample (Fig. 6*B*, lane *d*) are nearly identical to those of the neonatal myosin preparation from hind limb muscle (lanes *e* and *f*). This antibody reacts with the adult EOM sample (lane *c*) more strongly than with the slow (lane *a*) or fast (lane *b*) myosin cleavage products. The pattern of adult EOM polypeptides detected by the antibody shows several bands in common with the neonatal myosin, although some differences are also apparent. These differences concern mostly the relative intensities of the individual bands, and this may result from a slight difference in the extent of chymotryptic degradation between samples where the reacting species is the major form (i.e., neonatal myosin and 7-d EOM myosin) or a minor form as in the adult EOM sample (unpublished observations). Therefore, these results probably reflect the presence of neonatal MHC as a minor component of the adult EOM myosin.

Fig. 6*C* shows that slow MHC can be detected in adult

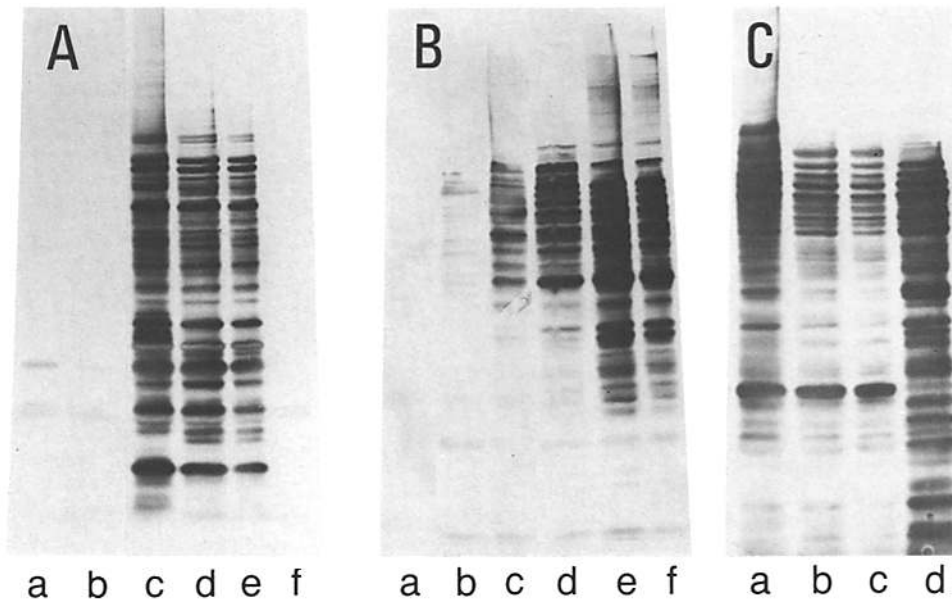


FIGURE 6 Immunopolypeptide mapping of myosin isoforms from EOM and hind limb muscles. After electrophoresis, the polypeptide maps were transferred to nitrocellulose paper and reacted with specific myosin antibodies. Panel A shows the reaction of fast myosin antibody with polypeptides derived from neonatal myosin (a), EOM myosin from 7-d-old rats (b), adult EOM myosin (c), adult fast myosin (d), adult fast myosin (half the amount loaded in lane d) (e), and adult slow myosin (f). Panel B shows the reaction of neonatal antibody with polypeptides from adult slow myosin (a), adult fast myosin (b), adult EOM myosin (c), 7-d EOM myosin (d), neonatal myosin (e), and neonatal myosin (half the amount loaded in lane e) (f). Panel C shows the reaction of slow antibody with polypeptides from adult EOM myosin (a), adult fast myosin (b), adult fast myosin (half the amount loaded in lane b) (c), and adult slow myosin (d).

EOM (lane a) and that the polypeptides reacting with the antibody are nearly identical to those in slow myosin (lane d) and those of the minor slow component found in the fast myosin preparation (lanes b and c). Finally, immunoblotting of cleavage products using an embryonic myosin antibody show a barely detectable reaction with adult EOM myosin; however, the bands that were detected did correspond to the embryonic pattern (results not shown). This would indicate that embryonic MHC is present only as a very minor component.

Immunocytochemistry of Adult EOM

Indirect immunofluorescence using antibodies to different MHCs was carried out to determine to what extent the individual isoforms were segregated into different fiber populations in the adult EOM. Fig. 7 shows the results of reacting serial sections of the superior rectus extraocular muscle with each of the four antibodies. Clear differences in myosin content are apparent between fibers found in the central region of the muscle and those in the peripheral region. Reactivity with the fast antibody (Fig. 7A) is strong on central fibers, whereas on the periphery of the muscle the reactivity is weaker or, in some fibers, negative. Neonatal myosin is found predominantly in the peripheral region of the muscle (Fig. 7B) whereas antibody to embryonic myosin reacts only peripherally (Fig. 7D). Antibody to slow myosin reacts with a minority of the fibers but also reveals a difference between the central and peripheral regions: the slow myosin-containing fibers in the periphery are substantially smaller than the centrally located slow fibers.

From the comparison of the serial sections, it appears that slow myosin-containing fibers do not react with any other myosin antibody. This is also true for most of the fast myosin-

containing fibers in the central region of the muscle. However, in the peripheral region, fibers are seen which clearly react with both fast and neonatal antibodies (Fig. 7, A and B) or with neonatal and embryonic antibodies (Fig. 7, B and D). Although the immunopolypeptide mapping results of Fig. 6 suggest that each antibody is reacting with the hind limb myosin form to which it was prepared, we cannot determine whether the specific EOM heavy chain (represented by $\lambda 10B3$) is also detected by one or more of the antibodies. Nonetheless, the results of the immunocytochemical analysis seem to clearly indicate that there is a general tendency toward segregation of the different myosin types into different fiber populations.

DISCUSSION

The results presented here demonstrate that the adult rat extraocular muscles express all known sarcomeric MHC genes (with the possible exception of the α -cardiac gene), including an isoform which is specific for these muscles. These muscles have a more complex set of MHC mRNA transcripts and native myosin isoforms than either fast- or slow-type skeletal muscles in the hind limb. This complexity extends to the distribution of the four major hind limb isoforms; in extraocular muscles there is a tendency for the myosin isoforms to segregate into different fibers with specific myosin types localized either to the peripheral or central part of the muscle.

The fact that the extraocular muscles simultaneously express multiple sarcomeric MHC mRNAs and proteins illustrates new aspects concerning the regulation and expression of the MHC gene family. Adult extrinsic eye muscles transcribe a previously unidentified MHC gene ($\lambda 10B3$) concomitantly with the genes previously known to be expressed in adult skeletal musculature: slow, fast oxidative, and fast gly-

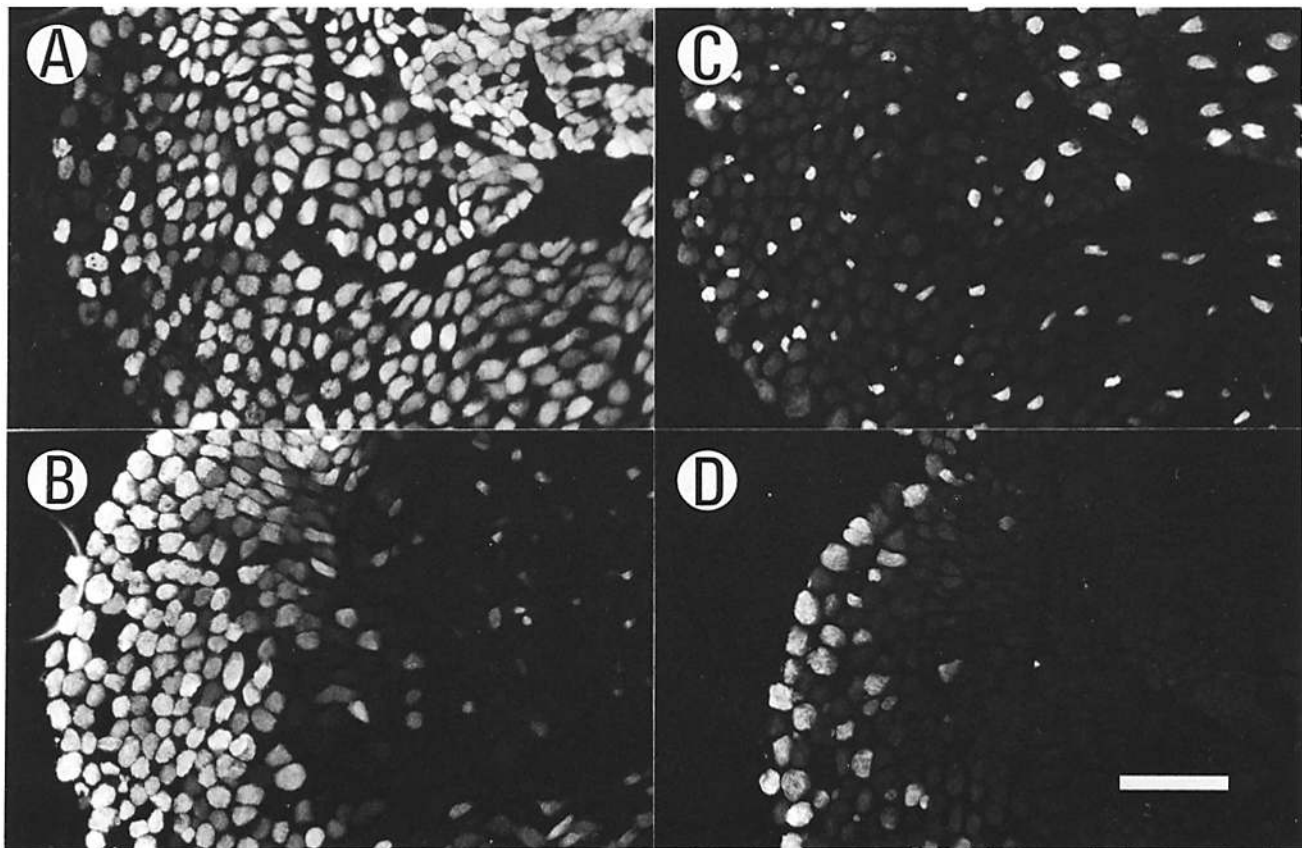


FIGURE 7 Indirect immunofluorescence on the adult superior rectus muscle using antibodies to hind limb myosin isozymes. Serial sections of adult EOM were reacted with antibody to adult fast (a), neonatal (b), adult slow (c), or embryonic (d) myosin. The antibody reaction was detected using a rhodamine-conjugated goat anti-rabbit immunoglobulin second antibody. Photos of the same region of each serial section are shown to allow comparison of the antibody reactivity of individual fibers. The peripheral region of the muscle is on the left of each photo, while the central region is on the right. Bar, 100 μ m.

colytic. In addition, transcripts of the embryonic and neonatal skeletal MHCs have also been detected in the adult recti and oblique muscles, whereas in hind limb muscles they are only transiently and sequentially expressed in fetal and neonatal life, but not in the adult. Furthermore, another MHC mRNA transcript similar in the carboxy terminus to the slow-twitch myosin clone pCMHC 5, but containing a different 3' untranslated region, has also been identified in these muscles.

During the neonatal stage, five different MHC mRNAs are expressed in EOM: embryonic, neonatal, fast oxidative, slow-twitch/cardiac β -MHC, and "slow twitch-like." However, electrophoresis of native myosin demonstrates that the predominant myosin protein accumulated at this time is the neonatal form. In the adult stages, the transcripts from these genes are still retained and supplemented with at least two additional ones: a fast-glycolytic and an EOM-specific MHC. Polypeptide and immunopolypeptide mapping results demonstrate that the predominant adult EOM forms are fast MHCs, or forms very homologous to these isozymes. It must be noted that, although mRNA transcripts and protein products of the embryonic and neonatal skeletal muscle MHC genes are still detectable in the adult extraocular muscle, they are present in decreased amounts compared with the perinatal stages. A quantitative estimate of these transcripts or proteins was not performed; however, these levels in the adult are greater than the amount which might be due a population of regenerating myotubes in untraumatized muscle, as deter-

mined by comparison to the adult hind limb muscles used in the various experiments. Furthermore, there is no evidence (i.e., central nuclei) for a regenerating muscle fiber population in the EOM (41, 68). The simultaneous expression of these multiple MHCs demonstrates that in the rat EOM there is a very high degree of plasticity at the level of MHC gene transcription. Expression of multiple MHC genes has been detected in other muscles; for example, cardiac muscle transcribes both α - and β -MHC, and soleus muscle produces slow-twitch, fast-oxidative, and fast-glycolytic myosins.² However, the high number of MHC transcripts present in the adult EOM is unique.

The expression of a full complement of sarcomeric MHC genes in adult EOM tissue, in addition to embryonic and neonatal isoforms, forces a re-evaluation in defining the developmental and tissue specificity of MHC isoforms. That there is protein synthesis and regional localization of the embryonic and neonatal isoforms strongly suggests that the production of these mRNA transcripts is not merely a product of gene transcription leakage, but is a controlled and regulated process. Furthermore, the constitutive expression of the embryonic and neonatal myosin isozymes during the adult developmental stage clearly demonstrates that their expression is not solely regulated by developmental stage-specific factors. Although there are predominant MHC isoforms in an isolated muscle at a specific stage of development, with the exception of the EOM-specific MHC, there are no sarcomeric MHC

isoforms which exhibit complete developmental- or tissue-specific expression.

The complex pattern of MHC gene expression in EOM is consistent with morphological, histochemical, and physiological demonstrations of multiple distinct fiber types in these muscles, ranging from fiber layers exhibiting slow- to fast-twitch contractions (1, 41, 44, 68). Slower fibers are located primarily on the periphery of the recti and oblique muscles with fast fibers being internal. One subpopulation of extrinsic eye fibers is multiply innervated, distributed throughout the EOM, and displays electrophysiological properties indicative of slow-tonic contractures (27, 28, 34), an electromyographic pattern generally associated with multiply innervated amphibian musculature (34). Furthermore, antibodies prepared against avian slow myosin exhibit differential staining between mammalian extraocular muscles and slow-twitch contracting body musculature (i.e., soleus muscle) (5, 6), thereby suggesting the existence of two distinct mammalian slow muscle fiber populations. Some EOM fibers also display a "hyper-fast" contractile response, known to be among the fastest contracting mammalian muscle fibers (15). The confined expression of λ 10B3 to the EOM and its absence in the hind limb and cardiac musculature suggests that the gene represented by clone λ 10B3 may be coding for the MHC component associated with either the slow-tonic contracture or the "hyper-fast" contraction of the EOM; current datum, however, is not sufficient to resolve this issue. Interestingly, the only mammalian muscles which exhibit slow-tonic contractures are those which have the "en grappe" neuromuscular junction indicative of multiple innervation (28). These muscles are the extraocular, esophageal, and tensor tympani muscle of the middle ear (49, 54). It would be interesting therefore to determine whether these muscles also express the λ 10B3 MHC gene.

Although earlier studies (30, 32) suggested that mesodermal mesenchyme alone gives rise to the extrinsic ocular muscles, recent investigations have indicated that numerous muscles of the face and neck actually originate from ectodermal primordial tissue (neural crest) (48). Therefore, it is unlikely that the unusual MHC phenotype in this muscle is a reflection of its embryonic origin, since other facial muscle (i.e., masseter) have an MHC pattern similar to limb musculature.² Regardless of the site of origin of the EOM, it is interesting that the developmental transition of MHC isoforms is similar to that of hind limb muscles until early postnatal life when neonatal myosin is the major form. Subsequently, a number of isozymes are accumulated which render the adult myosin component of the extraocular muscles more complex than that of the hind limb muscles.

From the point of view of gene regulation, it is interesting to determine whether a specific fiber synthesizes only one particular myosin isozyme, or alternatively, whether multiple myosin isozymes are expressed within the same muscle fiber. The immunocytochemical results presented here suggest that in EOM different myosin isozymes are segregated into different fiber populations although some fibers appear to contain both neonatal and fast myosin or embryonic and neonatal myosin in the peripheral region of the muscle. The distribution of the various myosin isozymes into such discrete locales suggests the presence of myofiber populations which arrest the MHC transition at various developmental stages. These terminal isoform stages appear to be embryonic, neonatal/adult, adult-similar to hind limb musculature, and adult

EOM-specific. Thus, these fiber types reflect different times of arresting the MHC developmental program which is presumably subject to and controlled by environmental influences. Although this MHC transition process could be regulated at a translational level, current evidence demonstrates that MHC synthesis is controlled mainly at the pre-translational level (42, 75).

The developmental and tissue-specific expression of the MHC gene family is reminiscent of the regulated expression of the β -globin (25, 76) and immunoglobulin (51) gene families. All these families exhibit sequential activation of genes clustered on a single chromosome, however the MHC gene family exhibits some unique features concerning the ability to reversibly modulate the expression of these genes by various influences. For example, during development, MHC transitions can be modified in response to hormonal influences such as thyroid hormone levels (12, 14, 22, 74). Furthermore, in adult muscle, transitions can also be induced by hormonal (12, 36)⁵ and neural (18, 33) influences. Another salient feature of the MHC gene family is the differential regulation and expression of specific MHC isoforms in different striated muscles. For example, the β -MHC gene is both the adult slow skeletal form and the principle form of the rat fetal myocardium; the skeletal embryonic and neonatal MHC are expressed only during fetal and early postnatal life in the hind limb muscles while, as shown here, they remain expressed through adult life in certain EOM fibers. These data strongly suggest that the development and tissue-specific expression of the MHC gene family is not under the control of a strict developmental clock, but is responsive to hormonal and/or neuronal factors that modulate its expression either directly or through the availability of the corresponding receptors on particular fibers.

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