Three Slow Myosin Heavy Chains Sequentially Expressed in Developing Mammalian Skeletal Muscle

SIMON M. HUGHES,1 MILDRED CHO, ILENE KARSH-MIZRACHI,2 MARILYN TRAVIS, LAURA SILBERSTEIN,3 LESLIE A. LEINWAND,4 AND HELEN M. BLAUT5

Department of Pharmacology, Stanford University Medical Center, Stanford, California 94305-5332

Accepted February 2, 1993

Myosin heavy chain (MyHC) isoforms show a striking diversity of expression patterns during mammalian development. Using a set of monoclonal antibodies that recognize different epitopes on myosin heavy chain isoforms we show that there exist in human and rat skeletal muscle at least three isoforms of slow twitch myosin heavy chain. To facilitate a comparison of our results to others obtained using different antibodies or species, we have identified cDNAs encoding the epitopes recognized by the three slow antibodies. Using these reagents, we show that the onset of expression of three slow MyHC isoforms is temporally distinct during early gestation. This result suggests that a sequence of MyHC transitions plays an important role in determining muscle fiber function at fetal, neonatal, and adult stages.

INTRODUCTION

Myosin heavy chain (MyHC) is the molecule primarily responsible, in collaboration with actin, for the contraction of muscle fibers. MyHC is composed of two major domains, a rod that forms an α-helical coil with the rod of another MyHC and anchors the MyHC dimers into the matrix of the thick filament and a head that binds to actin, has actin-catalyzed ATPase activity, and ratchets along the thin filament to effect movement. Each muscle fiber is specialized to be a particular fiber type that contracts at a characteristic rate. Different fiber types contain different isoforms of MyHC (Reiser et al., 1988; Klitgaard et al., 1990) and it has been hypothesized that the different ATPase rates of these MyHC isoforms are in large part responsible for the different intrinsic rates of shortening of these fibers. Accordingly, slow fibers characteristically have low ATPase activity and fast fibers have high MyHC ATPase activity (Barany, 1967; Reiser et al., 1988).

In vivo, muscle fibers of different types tend to be clustered in different proportions so that some muscles, generally those involved in maintaining posture, contain predominantly, but not exclusively, slow fibers. Conversely, in other muscles, fast fibers are in the majority (Schmalbruch, 1985). However, all muscles contain a mixture of fiber types which, in most regions, appear to be randomly interspersed.

Muscle fibers are formed and then grow by the fusion of myoblasts, mononucleate precursor cells (Mauro, 1961). Different MyHC isoforms are expressed over time as multinucleate muscle fibers are initiated, grow, and then attain their adult characteristics (Lyons et al., 1988; reviewed in Pette and Staron, 1990). Initially, an embryonic MyHC isoform is expressed. Later this isoform declines in most muscles and is replaced by neonatal isoforms that are eventually superseded by adult isoforms (Schiappino et al., 1989; LaFramboise et al., 1991). Superimposed upon the expression of stage-specific isoforms is the expression of fiber-type-specific isoforms. Fiber-type distinctions begin in the embryo and persist into adulthood. In normal adult muscle, specific MyHC isoforms are prevalent in either slow-twitch or fast-twitch fibers, but not usually found together in the same fiber (Klitgaard et al., 1990). The function of the complex changes in MyHC isoform expression is unclear but has been suggested to reflect either different physiological needs of fetal, neonatal, and adult muscles (Reiser et al., 1988) or a route to the accurate construction of sarcomeres, the regular arrays into which thick and thin filaments are organized (Taylor and Bandman, 1989; Epstein and Fischman, 1991; Lowey et al., 1991).

To date, most evidence suggests that MyHC diversity in mammalian development is due to separate genes...
that are expressed in temporally and spatially distinct patterns. In mammals, seven different fast MyHC isoforms have been identified (Pette and Staron, 1990) and the genes that encode them cloned (Leinwand et al., 1983; Strehler et al., 1986; Karsch-Mizrachi et al., 1989, 1990; Bober et al., 1990; Stedman et al., 1990). The skeletal fast MyHC genes are clustered at a single chromosomal locus (chr. 11 near the nude locus (nu) in the mouse and the short arm of chr. 17 in humans) (Leinwand et al., 1983; Weydert et al., 1985; Buckingham et al., 1986; Cox et al., 1991). The two mapped α and β cardiac MyHC genes are clustered in a separate locus (14q12 in the human (Matsuoka et al., 1989; Qin et al., 1990) and chr. 14 in mice (Weydert et al., 1985), near the nucleotide phosphorylase locus (Np-1)). The α-cardiac MyHC is expressed exclusively in atrial and masseter muscle (Bredman et al., 1991) and is a fast MyHC. The β-cardiac MyHC is expressed in ventricular, atrial, and skeletal muscle (Mahdavi et al., 1987). The β-cardiac MyHC is considered to be a slow isoform because it has a lower ATPase activity than the α-cardiac isoform and is found in slow-twitch fibers of skeletal muscle. There are two reports of a second β-cardiac MyHC in humans (Jandreski and Liew, 1987; Tsuchimochi et al., 1988), one of which appears to be expressed in skeletal muscle (Jandreski and Liew, 1987). In chicken skeletal muscle, two developmentally regulated isoforms of slow MyHC have also been found (Kilby and Dhoot, 1988). Although these studies suggest that there may be more than one slow MyHC, the relationships between mammalian and chicken slow MyHC isoforms have been unclear because the genes encoding them have not been identified. Moreover, although suggestive, compelling evidence for multiple slow MyHCs in mammalian skeletal muscle has been lacking.

Our results provide evidence that the generation of slow muscle fibers in human and rat skeletal muscle involves at least three separate slow MyHC isoforms which differ in their onset of expression during development. We used monoclonal antibodies to investigate the diversity of human and rat MyHC isoforms in an attempt to understand better both the diversity of muscle fiber types and the developmental MyHC isoform transitions of muscle. In order to compare our results readily to those of others which were obtained with other antibodies or species, we identified cDNA sequences encoding epitopes recognized by the three antibodies to slow MyHCs. We conclude that three different slow MyHCs are developmentally regulated during mammalian muscle development.

MATERIALS AND METHODS

Muscle Tissue

Human muscle tissue was obtained from autopsy material through the Stanford Department of Pathology. Surgical specimens were obtained at Stanford University, or the International Institute for the Advancement of Medicine (Essington, PA) in accordance with the guidelines of the Stanford University Human Subjects Committee. For analyses of adult muscle, frozen tissue sections and MyHC extracts were from vastus lateralis muscles in general. For analyses of early gestation and midgestation muscle, upper leg and lateral thigh muscles were obtained, respectively. Adult diaphragm muscles were also obtained for production of MyHC extracts and gave the same results as from vastus lateralis. Tissues collected for extraction of MyHCs were snap-frozen in liquid nitrogen as soon as possible after removal from the body. Tissues to be used for cryostat sections or primary culture were stored on ice until processed.

Rat muscle was obtained from Wistar rats (Simonsen Laboratories Inc., Gilroy, CA) and killed by CO₂ inhalation in accordance with the guidelines of the Stanford Administrative Panel on Laboratory Animal Care. Fetal ages were determined from the pluck date, designated as Embryonic Day 0 (E0). Pups were usually born on the night of E21/22.

Monoclonal Antibodies

Mice were immunized by subcutaneous injection of partially purified MyHC antigens from neonatal (Day 5 postnatal) or adult (21-year) human skeletal muscle myosin preparations (Silberstein and Blau, 1986; Webster et al., 1988). Hybridomas were produced 3 days after a final intravenous boost of antigen. Colonies were screened for production of antibodies that reacted with MyHC in ELISA and further tested for specific reaction with subpopulations of human skeletal muscle fibers in immunofluorescence assays on tissue sections (Webster et al., 1988) and reacted with a 220-kDa MyHC band on Western blots of the immunogen (data not shown) or of whole cell lysates of human muscle cells (Fig. 1). All hybridoma lines reported in these studies were subcloned by limiting dilution a minimum of three times. Antibody N2.261 (IgG1) resulted from immunization with neonatal myosins. Antibodies A4.840 (IgM), A4.951 (IgG1), and A4.74 (IgG1) resulted from immunization with adult human myosins and were previously named 4A.840, 4A.951, and 4A.74 (Webster et al., 1988). All of these monoclonal antibodies are available from the American Type Culture Collection.

SDS-PAGE of Bacterial or Mammalian Expression Products of Cloned MyHC Genes or of Extracts of Rat MyHCs

A cDNA encoding the N-terminal 100 kDa of the human β-cardiac/slow MyHC was cloned into a Dictyostelium expression vector, introduced into B2 Dictyoste-
Mammalian Slow Myosin Heavy Chains

Hughes et al.

lium, and generously provided by D. Manstein (National Institute for Medical Research, London, England). For preparation of whole-cell lysates, cells were washed in 10 mM Tris, pH 7.4/1 mM EDTA and lysed in a buffer containing 50 mM Tris, pH 7.5, 20 mM sodium pyrophosphate, 20 mM sodium sulphite, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 20 µg/ml trypsin inhibitor, 0.1 µM pepstatin A, 0.1 mM leupeptin, and 0.5% Triton X-100. Lysates were immediately diluted 1:1 in boiling Laemmli SDS sample buffer and loaded onto polyacrylamide gels as described below.

Fragments of all other human or rat MyHCs described here were isolated and cloned into bacterial or mammalian expression vectors and expressed in E. coli or cos cells (see Table 1). Purified MyHC protein fragments or crude extracts were solubilized in Laemmli sample buffer for electrophoresis. In addition, the endogenous MyHCs expressed in cardiac tissue from hypothroid rats and L6E9 rat myotube cultures were analyzed. All samples were electrophoresed through standard 0.1% SDS-12.5% polyacrylamide gels (made from a polyacrylamide stock of 29.2% acrylamide and 0.8% bis-acrylamide; Laemmli, 1970) on a Bio-Rad minigel apparatus for Western blotting (described below).

Extraction of MyHCs from Human and Rat Muscle Tissues

MyHCs were extracted from human and rat muscle tissues essentially as described in Butler-Browne and Whalen (1984). Briefly, 50-200 mg of frozen muscle tissue was weighed and minced with scissors for 4-5 min in four times the tissue weight of a high salt buffer with 0.1% β-mercaptoethanol (β-ME) and protease inhibitors (20 µg/ml trypsin inhibitor, 0.1 µM pepstatin A, and 0.1 mM leupeptin). After 20-40 min of extraction on ice, debris was removed by centrifugation at 13,000g for 30 min at 4°C. Supernatants were dialyzed 10-20 fold in a low salt buffer with 0.1% β-ME and protease inhibitors and incubated overnight on ice. Precipitated MyHCs were pelleted by centrifugation at 13,000g for 30 min and resuspended in 0.5 M NaCl, 10 mM NaH2PO4, pH 7.0, with protease inhibitors and allowed to dissolve overnight on ice. Samples were then boiled for 3 min in Laemmli SDS sample buffer and stored in 10-µl aliquots at -80°C for up to 3 months.

Whole-Cell Lysates

Whole-cell lysates were made from cultured human muscle cells. Primary cells composed of >90% myoblasts were cultured in Ham’s F10 medium with 20% fetal calf serum (Hyclone), 0.5% chick embryo extract (GIBCO), and penicillin/streptomycin (GIBCO) as previously described by Blau and Webster (1981). Differen-
tiated myotubes were obtained by changing the medium to a differentiation medium (DM; DMEM with 5% horse serum (Hyclone), 10 µM bovine insulin (Sigma), 10 µM dexamethasone (Sigma), and antibiotics). After 1 week in DM, cells were washed two times, scraped off culture dishes, and transferred to Eppendorf tubes in ice-cold phosphate-buffered saline (PBS). After centrifugation at 1000g for 5 min, pelleted cells were resuspended and boiled in Laemmli SDS sample buffer (Laemmli, 1970).

High-Resolution SDS-Glycerol PAGE and Western Blotting of MyHC Extracts

MyHCs were separated by SDS-glycerol PAGE essentially as described by LaFramboise et al. (1990) but with modifications for optimal resolution of human isoforms. Samples were electrophoresed through a 3% polyacrylamide stacking gel and 5.5% polyacrylamide, 35% glycerol separating gel for 22-24 hr at 15°C at constant voltage (80 V through stacking gel, 160 V through separating gel) in a Tris-glycine buffer system (pH 8.3). Stock solutions of acrylamide contained 28.5% acrylamide (Bio-Rad) and 1.5% bis-acrylamide (Bio-Rad). Portions of the gel were either stained (Bio-Rad Silver Stain Plus Kit) or transferred to PVDF membrane (Immobilon-P; Millipore).

For Western blot analysis, proteins were transferred in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma), 5% methanol for 16–18 hr at 4°C at constant current (50 mA). Membranes were blocked for 2–4 hr in blocking buffer (PBS, 5% HS, 5% nonfat dry milk, 0.02% sodium azide) and air dried at room temperature prior to antibody staining. Tissue culture supernatants from mouse hybridomas producing antibodies specific for MyHCs were diluted 1:3 or 1:4 in blocking buffer immediately before incubating with membranes for 1–2 hr at room temperature. Membranes were washed three times in PBS, 0.05% Tween 20, incubated with peroxidase-conjugated goat anti-mouse secondary antibodies (F(ab)2 anti-IgG or anti-IgM, Cappel; diluted 1:1000 in PBS, 5% HS, 0.02% sodium azide) for 1 hr at room temperature, and washed again, as described above. Proteins detected by the antibodies were visualized by incubation of the membranes with ECL luminescent peroxidase substrate (ECL, Amersham) for 1 min and exposure to X-ray film (XAR-5, Kodak) until bands were visible (1 sec–1 min). Subsequently, all MyHC isoforms bound to the membranes could be detected by staining with antibody A4.1025 and peroxidase-anti-IgG, followed by visualization with the ECL substrate.

Staining of Tensed Myofibrils

Myofibrils were isolated from glycerinated human muscle fibers according to Pepe (1967) and incubated
with monoclonal antibody for 1 hr at room temperature, washed in PBS, and incubated with fluorochrome-conjugated goat anti-mouse IgG for 1 more hr. Myofibrils were washed again, placed on microscope slides, and viewed and photographed immediately.

**Immunohistochemistry**

Sections of human muscle were cut in a Bright cryostat at 10-15 μm and mounted on gelatin-coated glass slides. All steps were performed in a humidified box at room temperature with 0.05 ml of antibody solution per section. Sections were rinsed with a PAP pen (RPI) and preincubated with PBS, 5% HS, 0.02% sodium azide for 30 min to prevent nonspecific binding. Slides were then incubated with a 1:1 mixture of IgG and IgM anti-MyHC monoclonal antibodies (tissue culture supernatants) for 1 hr, then washed three times for 10 min each in a large volume of PBS, 0.05% Tween 20. To detect IgM or IgG, slides were then incubated in either 1:400 rhodamine-conjugated goat anti-mouse IgM (μ-chain specific; Cappel) or 1:100 FITC-conjugated goat anti-mouse IgG (γ-chain specific; Cappel), diluted in PBS, 5% HS. Slides were washed three times in a large volume of PBS, 0.05% Tween 20 for a total of 30 min and then mounted under a coverslip in Gelvatol (Monsanto). Slides were viewed and photographed under epi-illumination using a Zeiss Axiopt microscope.

The procedures for the immunohistochemistry of rat muscle were essentially identical to those for human, with the following modifications which proved optimal for staining of rodent tissue: Sections were preincubated with 0.1 mg/ml goat anti-mouse IgG (heavy and light) Fab (Cappel), 5% HS in PBS for 30 min to prevent nonspecific binding. Sections were then incubated with undiluted tissue culture supernatant containing the anti-MyHC monoclonal antibody for 1 hr, and then washed as described above. Sections were incubated in PBS, 5% HS for 1 hr before replacement with either 1:100 biotin-conjugated goat anti-mouse IgM (μ-chain specific; Kirkegaard and Perry) or 1:400 biotin-conjugated horse anti-mouse IgG (heavy and light chain specific; Vector) to detect IgM and IgG, respectively, diluted in PBS, 5% HS. Slides were washed as described above and then endogenous peroxidase was blocked using 5% H2O2 in methanol for 20 min. After three 10-min washes in PBS alone, avidin-biotin complex (Vectastain ABC Elite kit; Vector) was applied to the sections for 1 hr. Slides were washed for 20 min in two changes of PBS, 0.2% Tween 20 and then in PBS alone for two 10-min periods. Horseradish peroxidase reactivity was developed for approximately 1 min with 0.6 mg/ml diamobenzidine, 50 mM Tris–HCl, pH 7.2, 0.03% CoCl2, and 0.05% H2O2 mixed immediately prior to staining.

**In Situ mRNA Hybridization**

Sections of human Embryonic Week 17 vastus lateralis muscle were cut at 10 μm onto TESPA-coated slides. After drying, sections were fixed by application of 4% paraformaldehyde in 100 mM NaH2PO4, pH 7.4, for 20 min at room temperature. Slides were washed, treated with 20 μg/ml proteinase K in 50 mM Tris–HCl, pH 8.0, 5 mM EDTA for 15 min at room temperature, washed again, and then postfixed in the same fixative. After washing with PBS, 5 mM glycine the samples were acetylated in 0.5% acetic anhydride in 0.1 M triethanolamine, with continuous stirring, and then dehydrated through an alcohol series. Samples were prehybridized, hybridized, and washed according to Tautz and Pfeifer (1989). Digoxigenin-labeled random primed probes were produced from the unique 3'-untranslated portions of the human slow β-cardiac (0.3 kb of pSMHCZ; Saez and Leinwand, 1986) and human fetal MyHCs (0.1 kb of pSMHCZ; Karchs-Mizirachi et al., 1989) or from the coding region of human cardiac actin (pHMαAPx) using the Boehringer Genius kit. After washing, sections were incubated for 1 hr with alkaline phosphatase-coupled rabbit anti-digoxigenin Fab fragments (Boehringer), washed, and developed overnight in NBT/X-phosphate.

**RESULTS**

The derivation and function of diverse muscle fiber types in mammalian muscle during development is not well understood. As an approach to this problem, we generated a series of monoclonal antibodies directed against MyHC, a key contractile protein. Critical to the use of these reagents is a detailed characterization of their specificity. Here we first characterize three of our antibodies: A4.840, A4.951, and N2.261. As shown below, this characterization reveals that there are three distinct developmentally regulated isoforms that had not previously been described.

**Antibodies React to Epitopes on MyHC**

To ensure that the monoclonal antibodies specifically labeled epitopes on MyHC and not on other proteins present in muscle tissue, several tests were performed. First, each antibody was shown to recognize the purified myosin immunogen in ELISA and in Western blots (data not shown). Second, Western blots of whole-cell lysates of human primary muscle cells differentiated in tissue culture showed that a protein of approximately 220 kDa was the only protein detected with the antibodies (Fig. 1). Third, Western blots (Fig. 2) of muscle myosins separated on high resolution SDS–PAGE gels that resolve specific MyHC isoforms (Klitgaard et al., 1990) demonstrated that each antibody recognizes a particu-
lar subset of the MyHC bands revealed by silver staining. The three bands in adult muscle resolved on these gels, in order of migration from most to least rapid, are type I (slow), type IIa (fast), and type IIb (fast). The following criteria were used by Klitgaard et al. (1990) to assign each band an identity: (1) presence of the band in extracts made from a single, histochemically typed fiber, and (2) reactivity with monoclonal antibodies for which fiber-specificity had been determined based on ATPase activity in a histochemical assay. As shown in Fig. 2, two of the monoclonal antibodies, A4.840 and A4.951, specifically recognize slow (type I) MyHC: one antibody (A4.74) specifically recognizes fast (type IIa) MyHC and one antibody (N2.261) recognizes both fast (type IIa) and slow (type I) MyHCs. None of these antibodies reacts with type IIb fast, embryonic (E), or neonatal (N) isoforms (Fig. 2). Taken together these results indicate that each of these antibodies detects a distinct subset of skeletal MyHC isoforms.

**Three Antibodies React with Different Epitopes on Slow MyHCs**

Three lines of evidence suggest that the three antibodies to slow MyHC (Fig. 2) react with different epitopes on slow MyHC isoforms. First, each antibody showed a distinct reactivity in Western blots of slow MyHCs expressed from cDNAs and/or MyHCs extracted from cells or tissues (Figs. 2 and 3). Furthermore, in situ hybridization localized the transcripts encoded by the unique 3'-untranslated region of a slow cDNA to slow fibers (Fig. 4). Second, the antibodies differed in their binding to regions of myofibrils isolated from muscle tissue (Fig. 5). Third, the pattern of expression of MyHCs recognized by each antibody differed in muscle tissue sections (Figs. 6, 7, 8, and 10).

1) **Distinct cDNA fragments encode epitopes recognized by the three antibodies.** Strong evidence that our MyHC monoclonal antibodies recognize distinct epitopes comes from Western blot analysis of the products of MyHC cDNA fragments expressed in bacteria, COS cells, or Dictyostelium, as compared with full-length MyHCs in extracts of tissues (see Table 1). All three antibodies react with MyHC purified from cardiac tissue of hypothyroid rat (uppermost band, Fig. 3, lane 2) in which β-cardiac/slow MyHC is the only detectable MyHC isoform (Mahdavi et al., 1987), confirming that these antibodies detect epitopes on the β-cardiac/slow MyHC. Because lane 2 contains purified MyHC, the lower molecular weight bands that react with the antibodies are likely to be degradation products of MyHC. Further evidence that the antibodies are specific to
### TABLE 1
CHARACTERIZATION OF FOUR MONOCLONAL ANTIBODIES TO ISOFORMS OF MYOSIN HEAVY CHAIN

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Source of MyHC</th>
<th>A4.840</th>
<th>A4.951</th>
<th>N2.261</th>
<th>A4.74</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>embryonic</td>
<td>slow (1)</td>
<td>slow (1)</td>
<td>slow (1)</td>
<td>fast (1a)</td>
</tr>
<tr>
<td>S2</td>
<td>neonatal head</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMM</td>
<td>neonatal rod</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-cardiac/slow</td>
<td>rat cDNA in bacteria</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-cardiac/slow</td>
<td>human cDNA in dictyostelium</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>fast</td>
<td>human cDNA in bacteria</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(1) Specificity for distinct MyHCs: Western analysis of MyHCs from extracts of human muscle tissue, separated by glycerol/SDS-PAGE.

(2) Specificity for cloned gene products:  
- **S**: S1 S2 LMM  
- **Isoform**: embryonic, neonatal head, neonatal rod, β-cardiac/slow, fast  
- **Source of MyHC**: human cDNA in bacteria, human cDNA in cos cells, human cDNA in bacteria, rat cDNA in bacteria, human cDNA in dictyostelium

(3) Specificity for MyHCs in cells expressing a single isoform:  
- **embryonic**: rat L6E9 muscle cultures  
- **β-cardiac/slow**: rat cardiac tissue, hypo-T3

(4) Specificity for human muscle fibers by immunohistochemistry: Time course of MyHC expression during early development, showing proportions of positive primary (1*) and secondary (2*) fibers. Assays were performed at all time points indicated on X-axis.

**Note:** (1) Isoform specificities of antibodies to MyHCs in extracts of adult human skeletal muscle. (2) Specificities of antibodies in Western blots to MyHC fragments expressed from cDNAs. A schematic diagram of the portions of the MyHC protein represented by each cDNA is shown at left. S1 and S2 denote subfragments 1 and 2, respectively, of the head region of the MyHC protein. S1 contains the ATPase activity. LMM, or light meromyosin, represents the α-helical rod region which contains the sequences necessary for dimerization. (3) Reactivities of antibodies in Western blots to endogenous MyHCs in lysates of muscle cells which are thought to contain only one species of MyHC. (4) Diagram of the developmental time course of reactivity of each antibody in vivo. For each antibody, a pair of bars indicates the stages of development in which reactivity is detected in primary and secondary fibers, separately, and the percentage of positive fibers in each group of fibers.

* See legend to Fig. 3 for detailed descriptions of cDNAs.

* Schematic diagram of portions of MyHC protein represented by each cDNA. S1 and S2 denote subfragments 1 and 2, respectively. S1 corresponds to the head region and contains ATPase activity. S2 and LMM compose the α-helical rod region, which contains the sequences necessary for dimerization.
**Fig. 3.** A4.840, A4.951, and N2.261 recognize MyHCs encoded by β-cardiac/slow cDNAs. Samples containing MyHC proteins were electrophoresed through 12.5% polyacrylamide gels and either stained with Coomassie blue (top panel) or transferred to PVDF transfer membrane and reacted with antibodies as described in the legend to Fig. 1. Lane 1, an approximately 130-kDa N-terminal fragment of human β-cardiac/slow MyHC expressed in Dictyostelium from a cDNA. A protein of the mobility of the uppermost band in the A4.951 panel shows reaction with the positive control antibody, 9E10, which was raised against a c-myc peptide which is contained in this approximately 130-kDa fusion protein; lane 2, MyHC purified from cardiac tissue of a hypothyroid rat; lane 3, a rat β-cardiac MyHC (approximately 190 kDa) expressed in bacteria from a cDNA encoding all but approximately 100 amino acids of the N-terminal end; lane 4, whole-cell lysates of rat L6/E9 cultured myotubes; lane 5, a 45-kDa purified fragment of fast MyHC expressed in bacteria from a cDNA (pSMHCA); lane 6, whole-cell lysates of bacteria expressing a 100-kDa C-terminal portion of the human perinatal rod (pSMHZP); lane 7, bacteria only control; lane 8, whole-cell lysates of cos cells expressing a 90-kDa N-terminal portion of the human perinatal rod; lane 9, cos cell only control. Panels A, B, and C represent three separate experiments, respectively. Positions of the molecular weight markers are shown in the lanes marked MW and relative molecular weights are indicated in kilodaltons.

**MyHC is shown in Fig. 1:** all three antibodies react exclusively with a single 200-kDa band in whole-cell lysates of muscle cells.

Antibody A4.840 also reacts with an epitope on the polypeptide encoded by a large C-terminal fragment of the rat β-cardiac/slow MyHC cDNA (McNally et al., 1989) whereas the other antibodies do not detect this polypeptide (uppermost band, Fig. 3, lane 3). Again, the lower molecular weight bands are likely to be breakdown products of MyHC. Although human and rat β-cardiac/slow MyHC proteins are 98.5% identical in their C-terminal regions, we could not be certain that A4.840 detects the human β-cardiac/slow MyHC. Confirmation that A4.840 reacts with human β-cardiac/slow MyHC gene was therefore obtained by in situ hybridization studies (Fig. 4A). mRNA containing the unique S-untranslated region of human β-cardiac/slow MyHC cDNA (Saez and Leinwand, 1986) is present in the large
primary fibers that also contain A4.840-reactive MyHC (Fig. 4C). By contrast, embryonic MyHC mRNA is mainly expressed in the small newly forming secondary fibers at this stage (Fig. 4B) and actin mRNA is present in all muscle fibers (Fig. 4D). Taken together, these results indicate that A4.840 detects an epitope on the protein encoded by the β-cardiac/slow MyHC in both rat and human.

By contrast with A4.840, the other two antibodies to slow MyHC, A4.951, and N2.261 reacted with the N-terminal part of human β-cardiac/slow MyHC (Diederich et al., 1989) expressed from a cDNA (Fig. 3, lane 1). Because we do not have a full-length β-cardiac/slow clone we cannot determine with certainty that the N-terminal epitopes A4.951 and N2.261 are ever on the same MyHC molecule as the C-terminal A4.840 epitope. However, as all three antibodies react with Week 17 human primary muscle fibers in tissue sections (data not shown) that contain β-cardiac/slow MyHC mRNA (Fig. 4) and with β-cardiac/slow MyHC from cardiac tissue of hypothyroid rat (Fig. 3, lane 2), there may exist a molecule that contains all three epitopes.

None of the three monoclonal antibodies to slow MyHC recognizes the expression products of human perinatal head (N-terminal) or rod (C-terminal) MyHC cDNAs (Fig. 3, lanes 6 and 8) or the light meromyosin portion of a human adult fast MyHC cDNA (Fig. 3, lanes 5 and 8; for reactivity to fast MyHC; Cho et al., 1993a). Moreover, none of these antibodies reacts with the embryonic MyHC(s), the only MyHC(s) detectable in differentiated rat L6E9 cells in tissue culture (Mahdavi et al., 1987; see also Fig. 3, lane 4). These results suggest that the three antibodies recognize at least two distinct slow MyHC epitopes; A4.951- and N2.261-reactive epitopes are located in the N-terminus, whereas the A4.840-reactive epitope is in the C-terminus. Taken together with our finding that the N2.261 epitope is distinct from the others in that it is present on both type I and type IIa MyHCs (Fig. 2), we can further conclude that the three antibodies recognize three distinct slow MyHC epitopes.

(2) Staining of myofibrils suggests that three antibodies recognize distinct epitopes. Staining of teased myofibrils after the method of Pepe (1967) showed that each antibody bound preferentially to different regions of the sarcomere: Antibody A4.840 detects two stripes per sarcomere which correspond to the region of overlap between thick and thin filaments (Fig. 5A). Such a pattern of staining is suggestive of an epitope on a part of the MyHC molecule, possibly the rod, that is only revealed upon interaction of the head with actin, consistent with the binding of A4.840 to the expression product of the rat β-cardiac/slow MyHC cDNA that contains the entire 3'-rod region (Fig. 3, lane 2) but not the peptide encoded by the 5'-head region cDNA (Fig. 3, lane 1). In contrast, A4.951 detects the central H zone most intensely with weaker staining in the region of the A band where the thick and thin filaments overlap (Fig. 5). This pattern suggests that A4.951 may bind to an epitope in the head region that is at least partially hidden when MyHC binds to actin. This interpretation is in accordance with the finding that this antibody recognizes an epitope encoded by the 5'-head region of β-cardiac/slow MyHC (Fig. 3, lane 1). Finally, N2.261 labels a single H zone stripe but does not show the same weak reaction as A4.951 in the overlap zone (Fig. 5). The binding of the antibodies to particular parts of the A band is further confirmation that the epitopes within the muscle fiber recognized by these antibodies are on MyHC molecules. Differences in the staining patterns of these antibodies on myofibrils suggests that they recognize distinct epitopes (Pepe, 1967).

(3) Epitopes recognized by three antibodies to slow MyHC appear at different times in development. A4.840*, A4.951*, and N2.261* MyHCs appear sequentially at distinct times in development (Figs. 6, 7, 8, and 10). The reactivity of these antibodies at different stages in developing human and rat muscle, which is described in detail below, is strong evidence that the antibodies recognize distinct epitopes.

Three Antibodies Detect Distinct Isoforms of Slow MyHC in Developing Human Muscle

To examine the distribution of slow MyHC isoforms during human muscle development we investigated the location and timing of expression of the MyHCs recognized by the antibodies. First, the antibodies were
shown to be specific for type I slow fibers in vivo. Fibers were identified in adult muscle as type I slow fibers by histochemical analysis of ATPase activity (Webster et al., 1988), by their lack of labeling with several antibodies to fast MyHC isoforms (Cho et al., 1993a) and by their characteristic frequency and size in adult human vastus lateralis muscle (Wohlfart, 1997). By these criteria, all three antibodies recognize slow type I fibers in adult muscle (Figs. 6t and 6u; 7q and 7r).

The three epitopes recognized by antibodies A4.840, A4.951, and N2.261 appear at different times in early human embryonic development. During Embryonic Weeks 6 to 8, primary fibers are forming and these fibers express readily detectable levels of A4.840-reactive slow MyHC but no detectable A4.951 immunoreactivity (Fig. 6). By Embryonic Week 10, when primary muscle fiber formation is nearly complete, A4.951-reactivity is detected in all fibers that label with A4.840. The third antibody to slow MyHC, N2.261, first detects MyHC at yet a later stage of embryogenesis. Fibers recognized by both A4.840 and A4.951 at Week 11 are not recognized by N2.261 (Fig. 7). N2.261+ MyHC is not detected until Fetal Week 14. Beyond this early stage, until 22 weeks of gestation, the fibers recognized by the three antibodies are identical (Figs. 6 and 7). First, the three antibodies label the majority of large diameter, primary fibers and later in gestation they also label a proportion of smaller, secondary fibers. However, late in gestation, another difference is evident: N2.261 not only labels slow type I fibers, but also fast type IIa fibers (see below), presumably because the epitope is common to both MyHC molecules. Thus, N2.261 recognizes a slow MyHC isoform (Figs. 2, 3, and 7), the expression of which is temporally distinct from the other two slow isoforms.

Taken together, these results indicate that the three antibodies to slow MyHC recognize different epitopes that are differentially expressed both spatially and temporally. Furthermore, these results suggest that the antibodies recognize three different slow MyHC isoforms in developing human muscle.

Three Isoforms of Slow MyHC in Developing Rat Muscle

Studies in the rat confirmed our finding in human muscle that suggested the existence of three slow MyHC isoforms, although the time course of their expression differed. The smaller size of rat leg muscles allowed us to examine several different muscles of the lower hindlimb at many stages of development. In agreement with the findings of others (Harris et al., 1989; Condon et al., 1990), from the time that muscles first form until Embryonic Day 17 (E17), all primary fibers contain slow MyHC detected by reactivity with A4.840 (Fig. 8A). As in human development, as secondary fibers form they do not express slow MyHC in most muscles (Fig. 8E). However, virtually all secondary fibers in addition to the primaries in the soleus and red strip of the lateral gastrocnemius eventually express A4.840+ MyHC (Fig. 8E). After birth, few changes occur in this pattern. The expression of A4.840+ MyHC in a small percentage of fibers predominantly in the deep regions of muscles is maintained (Fig. 8I). The soleus and red strip of the lateral gastrocnemius continue to express slow MyHC in most fibers (Fig. 8M), whereas about 10% of fibers in the soleus lose A4.840-reactivity.

As in the human, the A4.951-reactive slow MyHC epitope is expressed after the A4.840 epitope early in development. A4.951+ MyHC was first detected in slow fibers 4 weeks after birth (P27), after all fibers had been formed. Its expression continues into the adult (Figs. 8B, 8F, 8J, 8N, and 8R). In the rat, A4.951+ fibers are a subset of A4.840+ fibers, providing evidence that the antibodies distinguish two kinds of slow fibers in many adult muscles (Figs. 8Q, 8R, and 9). A second molecular difference distinguishes these fibers in the adult. The antibody N3.36, which detects epitopes on many adult fast MyHC isoforms, is expressed in A4.840+ A4.951+ fibers but not in A4.840+ A4.951+ fibers. This is true of all muscles of the adult (Figs. 8Q and 8R; Cho et al., 1993a). Thus, in the adult, two populations of slow fibers can be distinguished by their expression of the A4.951 and A4.840 epitopes.

As in the human, N2.261 also distinguishes a third developmentally regulated slow MyHC isoform in the rat, where this isoform is first expressed slightly after the start of secondary fiber formation. N2.261+ MyHC is first detected weakly at E21 in A4.840+ slow fibers of all muscles (Figs. 8C and 8D). Expression increases in slow fibers in the adult where all A4.840+ fibers also express N2.261+ MyHC (Figs. 8K, 8O, 8S, and 9).

Thus, our antibodies appear to distinguish three slow MyHC isoforms during development. One isoform, expressed by embryonic rat muscles, contains the A4.840 epitope but not the epitopes recognized by A4.951 and N2.261 (Fig. 9). Another is a distinct slow MyHC isoform that is first expressed around birth and which contains the N2.261 epitope (with or without the A4.840 epitope). The third is expressed in adult rat muscles and carries the A4.951 epitope (with or without the N2.261 and A4.840 epitopes).

The transitions in slow MyHC expression detected by these three antibodies during development could either be due to differences in the primary sequence of MyHC, to changes in the expression of associated proteins, or to post-translational modifications. Myosin light chain isoforms that associate tightly with MyHC could mask the N2.261 and A4.951 epitopes at early stages. To test
**Fig. 6.** A4.840 and A4.951 recognize two distinct slow MyHCs in early development. Transverse sections of human limb muscle at weeks 6 (a–c), 8 (d–f), 11 (g–i), 14 (j–l), 22 (m–o), and 33 (p–r) of gestation and 55 years postnatal (s–u) were reacted with antibody A4.840 (center column)
this possibility, the MyHC isoforms expressed during development were analyzed on denaturing gels by Western blotting, conditions that remove noneovalent attached molecules (Fig. 10). We extracted MyHCs from hindlimb muscle of rats at Embryonic Day 20 (E20), postnatal days 1 (P1), 7 (P7), and 27 (P27), and adult stages. The time course of expression detected by the antibodies in Western blots paralleled the time course in tissue sections. The A4.840 antibody reacted with purified MyHC at all stages; N2.261 \(^+\) MyHC was not detectable until P7 and A4.951 \(^+\) MyHC was not apparent until P27. The reactive bands of molecular weights lower than 200 kDa are likely to be degradation products of MyHC because the lanes contained purified MyHC and because each of the antibodies was shown to react exclusively with a single 200-kDa band in whole-cell lysates of muscle cells prepared under conditions much more favorable to the isolation of intact MyHCs than those used here for purification (Fig. 1). These data rule out the possibility that the differences in antibody reactivity are due to MyHC-associated proteins such as the myosin light chains. Although this experiment does not rule out the possibility that developmentally regulated post-translational modifications of MyHCs lead to the creation of the N2.261 and A4.951 epitopes, this possibility seems unlikely, since no such developmentally regulated modifications have been reported although a large number of MyHCs have been studied. Instead, these data strongly suggest that at least three slow MyHC isoforms are differentially expressed, both spatially and temporally, during rat slow fiber development.

One significant feature of our results is the difference in timing between slow MyHC isoform expression in human and rat. Both species first express a A4.840 \(^+\) N2.261 \(^+\) A4.951 \(^+\) MyHC at the initiation of primary muscle fiber formation. Both gain expression of a N2.261 \(^+\) MyHC in all A4.840 \(^+\) fibers shortly after secondary fiber formation commences. However, whereas in human A4.951 \(^+\) MyHC is first expressed in slow primary fibers from around the time of the start of secondary fiber formation and prior to N2.261 expression, in the rat A4.951 \(^+\) MyHC appears after N2.261 is expressed and all fiber formation is complete. Moreover, in the adult human, so far as our limited sampling of muscles would suggest, A4.840 and A4.951 are always expressed in the same cells. By contrast, in the rat, cells can readily be found that express A4.840 in the absence of detectable A4.951.

Two explanations could account for these findings. First, humans and rats could have a similar MyHC expression pattern but a MyHC isoform that appears early in muscle development may contain the A4.951 epitope in humans but not rats. Alternatively, the expression pattern of the MyHC isoforms differs for functional reasons, perhaps related to the size of the organism or the force of muscle contraction. Resolution of this issue will await cloning of further MyHC coding regions from each species.

The N2.261-Epitope Appears on a Fast IIA Fiber MyHC

When N2.261 first appears it reacts with slow fibers in both human and rat (Figs. 7i and 8K) and binds to a slow MyHC in high-resolution gels (Fig. 2). However, the N2.261 epitope is not restricted to slow fibers throughout development. By human Fetal Week 33, expression has appeared in most, but not all, A4.840 \(^-\) A4.951 \(^-\) fast fibers. This pattern of expression is maintained into the adult (Fig. 7r) at which time N2.261 reacts with a second MyHC isoform that comigrates with IIA MyHC on SDS-PAGE (Fig. 2). These results suggest that N2.261 detects an epitope common to two distinct MyHC isoforms.

A similar phenomenon is observed in the rat, and this allows a more detailed analysis. During the first postnatal week an epitope reacting with N2.261 appears in a population of fibers with the spatial distribution and anti-MyHC antibody profile of slow fibers (Figs. 5K, 80, and 8S). Later, during the second postnatal week, N2.261-reactivity begins to appear in A4.840 \(^-\) fibers in locations overlapping, but more extensive than, the slow fiber-containing areas (compare Figs. 8M and 8O). The N2.261 \(^+\) A4.840 \(^-\) fibers can be identified as type IIA fast fibers by their distribution, frequency, and reactivity with three antibodies, A4.74, N1.551, and A4.1519, that react with IIA MyHC on Western blots (Fig. 2; Cho et al., 1993a). Consistent with this view, Figs. 8P and 8T show that A4.74 detects the A4.840 \(^-\) A4.951 \(^-\) fibers in the soleus. In other muscles, such as plantaris, A4.74 \(^+\) A4.1519 \(^+\) N1.551 \(^+\) N2.261 \(^+\) fast IIA fibers occur in similar regions to the A4.840 \(^+\) A4.951 \(^+\) N2.261 \(^+\) slow type I fibers but extend more superficially (Figs. 5Q, 8R, 8S, and 8T; Cho et al., 1993a). Similar regional differences exist in many other muscles, including lateral and medial gastrocnemius, extensor digitorum longus, and tibialis anterior (data not shown), and suggest that the N2.261 epitope is present in an adult fast IIA MyHC isoform recognized by A4.74.

Taken together, the most parsimonious explanation of these results is that the N2.261 epitope resides on

and A4.951 (right column) simultaneously, followed by FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgM secondary antibodies. The same fields were visualized by phase-contrast (left column) and fluorescence microscopy (center and right columns). Bar, 25 \(\mu\text{m}\).
Fig. 7. N2.261 recognizes a third slow MyHC expressed in early development and also a fast MyHC in late development. Transverse sections of human limb muscle at weeks 8 (a–c), 11 (d–f), 14 (g–i), 22 (j–l), and 33 (m–o) of gestation and 55 years postnatal (p–r) were reacted with antibody A4.840 (center column) and N2.261 (right column) simultaneously, followed by FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgM secondary antibodies. The same fields were visualized by phase-contrast (left column) and fluorescence microscopy (center and right columns). N2.261-reactivity is first detected in most, but not all, A4.840+ fibers at week 14 of gestation (l; arrow indicates a A4.840+ fiber which is not labeled by N2.261). By week 22 and beyond, N2.261 labels all A4.840+ fibers (k, l). By week 33, N2.261 also reacts strongly with A4.840− (fast) fibers; one such N2.261+/A4.840− fiber is indicated by the arrows n and o. This pattern of reactivity is also seen in adult muscle (q, r). Bar, 25 μm.
Fig. 8. Three slow MyHC isoforms are differentially expressed in the rat. Transverse serial sections of whole embryonic E17 (A–D), E21 (E–H) and neonatal (I–L) hindlimbs or the posterior muscle groups of P27 (M–P) or adult (Q–T) hindlimbs were reacted with antibodies A4.840 (A, E, I, M, Q), A4.951 (B, F, J, N, R), N2.261 (C, G, K, O, S), or A4.74 (D, H, L, P, T) and visualized with ABC peroxidase. All panels at each age were photographed and printed identically. A, E, I, M, and Q have less nonspecific background on connective tissue because an anti-IgM second layer was used (compare I, J, K, and L). Arrows in M, N, O, and P indicate regions in which N2.261* fibers are more numerous than A4.840* fibers. Arrows in Q, R, S, and T indicate fibers with different MyHC contents: i, A4.840* A4.951* N2.261* A4.74*; ii, A4.840* A4.951* N2.261* A4.74*; iii, A4.840* A4.951* N2.261* A4.74*; iv, A4.840* A4.951* N2.261* A4.74*. B, bone; EDL, extensor digitorum longus; LG, lateral gastrocnemius; MG, medial gastrocnemius; PL, peroneus longus; PLN, plantaris; rLG, red strip of LG; SOL, soleus; TA, tibialis anterior. Bar, 500 μm.

both a slow MyHC isoform and on a second fast MyHC present in type IIa fibers.

Parallel Fast and Slow MyHC Isoform Transitions

The maturation of fast fiber types is well known to be characterized by transitions in fast MyHC isoform expression (Whalen et al., 1981; Lyons et al., 1983; Weydert et al., 1987; Schiaffino et al., 1989; LaFramboise et al., 1991). These findings are confirmed in this report for fast IIa fibers in Figs. 8L and 8P and in detail elsewhere (Cho et al., 1993a). In addition we show here that such transitions are also characteristic of the MyHC isoforms in slow fiber types. Indeed, the maturation of the MyHC phenotype of type I slow and type IIa fast fibers follows a similar time course. A4.74* MyHC appears in fast IIa fibers at the same stage of development that A4.951* MyHC appears in slow fibers (Figs. 8N and 8P). A more detailed analysis of the time course than shown in Fig. 8 reveals a precise correlation in the appearance of A4.74* and A4.951* (data not shown). Taken together with evidence that adult type IIb fast MyHC also commences expression in the third postnatal week (LaFramboise et al., 1991), these data suggest that a similar
developmental event may be responsible for concordant isoform transitions in all fiber types of the rat.

**DISCUSSION**

The results presented in this report provide evidence that at least three slow MyHC isoforms are expressed in a distinct order in developing human and rat skeletal muscle. A4.840-, A4.951-, and N2.261-reactive epitopes are all expressed on slow MyHC based on reactivity with (a) the slow MyHC band on high resolution SDS-PAGE, (b) the expression products of the β-cardiac/slow MyHC gene, and (c) the slow muscle fibers in tissue sections. However, these antibodies each recognize MyHCs that first appear at different stages of development, indicating that three slow MyHC isoforms exist. Primary amino acid sequence differences are likely to be responsible for the different isoforms detected. Explanations based on post-translational modifications or protein conformation are unlikely, because expression in bacteria or *Dictyostelium* and the denaturing conditions of Western analysis do not prevent detection of the epitopes.

At late stages of development, however, A4.840, A4.951, and N2.261 epitopes could exist on the same MyHC molecule. Three pieces of evidence support this hypothesis. First, A4.840 reacted with a C-terminal fragment of β-cardiac/slow MyHC expressed from a cDNA, whereas A4.951 and N2.261 reacted with an N-terminal fragment of β-cardiac/slow MyHC. Although these cDNAs are not from the same species, they are likely to be homologs. Second, in situ hybridization showed that the mRNA encoded by the β-cardiac/slow MyHC gene is expressed in the same cells that contain MyHC expressing all three epitopes in human skeletal muscle at midgestation. Third, all three antibodies reacted with MyHC from the cardiac tissue of the hypothyroid rat, which is reported to express only the β-cardiac/slow gene (Izumo et al., 1986). These observations suggest that all three epitopes may well be on the same molecule in adult cardiac tissue.

Multiple slow MyHC isoforms appear to be generated from independent genes or derived by alternative splic-
ing, depending on the species. Multiple fast MyHC genes which are developmentally regulated have also been identified in mammals and chickens (Leinwand et al., 1983; Strehler et al., 1986; Karsch-Mizrachi et al., 1989, 1990; Bober et al., 1990; Stedman et al., 1990). In contrast, alternative splicing gives rise to multiple MyHC isoforms in Drosophila (Rozek and Davidson, 1987; Kronert et al., 1991). It has also been suggested that alternative splicing could have given rise to two different β-cardiac MyHC cDNAs in human muscle (Jandreski et al., 1987). The comparison of a cDNA isolated from cardiac tissue (pHM3; Jandreski et al., 1987) and one isolated from skeletal muscle (pSMHCZ; Saez and Leinwand, 1986) showed that, although 482 nucleotides specifying the C-terminal coding region and 3′-untranslated region were identical between the two clones, the 5′-ends diverged. Because of the identity in the 3′-untranslated regions, it was suggested that the two cDNAs were derived from the same gene (Jandreski et al., 1987). It remains to be determined whether the antibodies described here recognize products generated by alternative splicing from the same gene or the products of two different genes.

Significance of Multiple Slow MyHC Isoforms

The role of developmentally distinct isoforms is unclear. MyHCs are highly conserved over the vast majority of their length. In particular, the size of the molecule shows very little variation, presumably due to the requirement that all skeletal MyHCs must be capable of packing into the same regular thick filament array. Several domains of the MyHC molecule are more variable in sequence than others (Warrick and Spudich, 1987), notably parts of the head, the head/rod junction, and the “hinge” region halfway down the rod, the point at which the MyHC rod can presumably bend out from the thick filament to allow interaction with the thin filament. One hypothesis is that the complex regulatory patterns necessary for proper tissue-specific expression and responsiveness to changing environmental signals require multiple functionally redundant genes which differ significantly only in their regulatory regions. A second hypothesis for MyHC diversity is that sequential expression of specific isoforms is required for the correct construction of the sarcomeric structure of the thick filament (Taylor and Bandman, 1989; Epstein and Fischman, 1991; Lowey et al., 1991). This possibility is supported by the reappearance of embryonic and neonatal MyHC isoforms in situations involving repair of muscle tissue, such as regeneration following dystrophic degeneration (Bandman, 1985; Webster et al., 1988). A third hypothesis for the observed changes in MyHC epitopes detected with our antibodies at different stages is that they impart critical contractile functions to MyHC isoforms. This hypothesis is suggested by the differences among MyHCs generated by the alternatively spliced MyHC isoforms of Drosophila (Rozek and Davidson, 1986; Kronert et al., 1991). Isoform variation occurs in the portion of the rod encoded by alternative exons and generates MyHCs with greatly differing functional properties (Collier et al., 1990). This observation suggests that in mammals, MyHC sequences in small areas of the rod or head may also be responsible for altering the ATPase rate. How this is achieved is a matter for speculation but one possibility is that an interaction of the hinge of one MyHC with the head of another MyHC further along the thick filament may be involved in regulating the ATPase cycle. Currently none of these hypotheses can be ruled out but it is appealing to consider that the series of slow MyHC isoforms that we have discovered, whether generated by alternative splicing or through the expression of distinct genes, may be involved in regulating changes in the contractile properties of slow fibers as the animal develops.

We are grateful to Drs. Lawrence A. Rinsky and Ronald L. Ariagno for muscle samples, to Drs. Bruce Paterson and James Spudich for growing Dictyostelium cultures, to Dr. Regina Sohn for expressed MyHCs, and to William LaFranoise for advice regarding high-resolution gels. This work was supported by National Institutes of Health predoctoral fellowships to M.C. (GM07149 and HD07249), a Lucille P. Markey Visiting Fellowship to S.M.H., and grants to H.M.B. from the National Institutes of Health (HD 18179) and the Muscular Dystrophy Association.

REFERENCES


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


Hughes et al. Mammalian Slow Myosin Heavy Chains