Fast myosin heavy chains expressed in secondary mammalian muscle fibers at the time of their inception

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SUMMARY

Mammalian skeletal muscle is generated by two waves of fiber formation, resulting in primary and secondary fibers. These fibers mature to give rise to several classes of adult muscle fibers with distinct contractile properties. Here we describe fast myosin heavy chain (MyHC) isoforms that are expressed in nascent secondary, but not primary, fibers in the early development of rat and human muscle. These fast MyHCs are distinct from previously described embryonic and neonatal fast MyHCs. To identify these MyHCs, monoclonal antibodies were used whose specificity was determined in western blots of MyHCs on denaturing gels and reactivity with muscle tissue at various stages of development. To facilitate a comparison of our results with those of others obtained using different antibodies or species, we have identified cDNAs that encode the epitopes recognized by our antibodies wherever possible. The results suggest that epitopes characteristic of adult fast MyHCs are expressed very early in muscle fiber development and distinguish newly formed secondary fibers from primary fibers. This marker of secondary fibers, which is detectable at the time of their inception, should prove useful in future studies of the derivation of primary and secondary fibers in mammalian muscle development.

Key words: skeletal muscle, fast myosin heavy chain, secondary fiber

INTRODUCTION

Mammalian skeletal muscles express diverse myosin heavy chain (MyHC) isoforms at different stages of development. These MyHCs are encoded by a family of highly conserved genes that are clustered on a single chromosome (Leinwand et al., 1983; Weydert et al., 1985; Buckingham et al., 1986). This group of MyHCs is designated as fast MyHCs because, like the MyHCs of fast fibers in adult muscle, they have a high calcium-dependent ATPase activity in histochemical assays performed at alkaline pH (Fitzsimons and Hoh, 1981). Some of these isoforms are expressed only at certain stages of development, such as embryonic MyHC (Whalen et al., 1981; Strehler et al., 1986) and neonatal (perinatal) MyHC (Periasamy et al., 1984; Feghali and Leinwand, 1989). Thus, although each member of this group of MyHCs is a fast type MyHC, both antibodies and cDNA clones distinguish developmentally regulated fast MyHCs expressed in the embryo or fetus from those expressed in mature muscle (Whalen et al., 1981; Periasamy et al., 1984; Weydert et al., 1987; Narusawa et al., 1987; Feghali and Leinwand, 1989). The functions of the diverse fast MyHCs, like the slow MyHCs (Hughes et al., 1993) that are expressed only at specific times in the developing mammal, are not known.

In addition to the temporally regulated embryonic and neonatal isoforms, diverse fast MyHC isoforms that are spatially restricted have been reported in adult muscle. For example, IIm and IIeom isoforms are found only in muscles of the head and neck derived from the first branchial arch or in extraocular eye muscles, respectively (reviewed by Pette and Staron, 1990). The restricted expression and ‘superfast’ ATPase activities characteristic of these muscles suggest that these MyHCs are likely to have evolved for highly specialized movements. In limb and trunk muscles of the rat, three fast MyHCs have been identified: Ila, IIb and IIx (Bar and Pette, 1988; LaFramboise et al., 1990b). In similar muscles of the human, only Ila and IIb isoforms have been described in the literature until now. Different adult fast MyHCs are sometimes found co-expressed in the same fiber in various combinations (Staron and Pette, 1987a,b; Klitgaard et al., 1990), probably giving rise to the continuum of contraction speeds typical of individual fibers of adult muscle.

Here we describe fast MyHCs that are expressed early in secondary fiber development but are distinct from embryonic and neonatal fast MyHCs. Distinctions between these isoforms were determined by their differential reactivity to monoclonal antibodies to MyHC. The specificity of these antibodies is extensively characterized. Furthermore, to permit a correlation
with studies using other antibodies or species, we identified the 
epitopes recognized by the antibodies on myosin rod segments 
expressed in vitro. Studies of rat muscles corroborated studies 
of human muscle and allowed an analysis of MyHC expression 
in several muscles with a variety of functional properties, over 
a full range of developmental stages. These antibodies provide 
molecular markers for distinguishing secondary fibers from 
primary fibers in the earliest stages of secondary fiber devel-

MATERIALS AND METHODS

Muscle tissue
Human muscle tissue was obtained from autopsy material through 
the Stanford Department of Pathology. Surgical specimens were obtained 
from 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 plug date, desig-
nated as embryonic day zero (E0). Pups were usually born on the night of E21/22.

Monoclonal antibodies
Mice were immunized by subcutaneous injection of fetal (week 15 of 
gestation; antibody F1.652 (IgG1)), neonatal (day 5 postnatal; anti-
bodies N3.36 (IgM), N1.551 (IgM)) or adult (21 year; antibodies 
A4.1077 (previously designated 4A.1077; IgG1) and A4.1519 (pre-
viously designated 4A.1519; IgM)) human skeletal muscle myosin 
preparations (Silberstein et al., 1986; Webster et al., 1988). Hybridio-
ama was produced three 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 immunoflu-
orscence 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 (see Fig. 
1). All hybridoma lines used in these studies were subcloned by 
limiting dilution to a minimum of three times.

Two antibodies appear to be species specific: A4.1077 does not 
react with rat MyHC, and therefore was used only in studies of human 
muscle; N1.551 reacts poorly with sections of human muscle tissue, 
and therefore was used only in studies of rat. All of these monoclonal 
antibodies were generated in our laboratory and 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 Dictyostelium 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-HCl, pH 7.4, 1 mM EDTA, and lysed in a buffer con-
taining 50 mM Tris-HCl, pH 7.5, 20 mM sodium pyrophosphate, 20 
mM sodium sulfite, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 20 
µg/ml trypsin inhibitor, 0.1 µM pepstatin A, 0.1 µM leupeptin and 
0.5% Triton X-100. Lysates were immediately diluted 1:1 in boiling 
Laemmli SDS-sample buffer (Laemmli, 1970) and loaded onto poly-
acrylamide 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 Escherichia 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 hypothyroid rats 
and from 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 mini-gel 
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 by Butler-Browne and Whalen (1984). Briefly, 50-200 
mg of frozen muscle tissue was weighed and minced with scissors for 
4-5 minutes 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 µM leupeptin). After 
20-40 minutes of extraction on ice, debris was removed by centrifu-
gation at 13,000 g for 30 minutes at 4°C. Supernatants were diluted 10-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 13,000 g for 30 minutes and resuspended in 0.5 M 
NaCl, 10 mM NaHPO4, pH 7.0, with protease inhibitors and allowed 
to dissolve overnight on ice. Samples were then boiled for 3 minutes 
in Laemmli SDS-sample buffer and stored in 10 µl samples at ~80°C 
for up to three months.

Whole-cell lysates
Whole-cell lysates were made from cultured human muscle cells. 
Primary cells comprising >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). Differentiated myotubes were 
obtained by changing the medium to a differentiation medium (DM; 
DMEM with 5% horse serum (HS) (Hyclone), b-mercaptoethanol (β-ME) and protease inhibitors (20 µg/ml 
trypsin inhibitor, 0.1 µM pepstatin A, and 0.1 µM leupeptin). After 
20-40 minutes of extraction on ice, debris was removed by centrifu-
gation at 13,000 g for 30 minutes at 4°C. Supernatants were diluted 10-fold in a low salt buffer with 0.1% β-ME and protease inhibitors, 
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in Laemmli SDS-sample buffer and stored in 10 µl samples at ~80°C 
for up to three months.

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. (1990b) but with modifications for 
optimal resolution of human isoforms. Samples were electrophoresed 
through a 3% polyacrylamide stacking gel and 5.5% polyacry-
lamide/35% glycerol separating gel for 22-24 hours at 15°C at 
constant voltage (80 V through stacking gel, 160 V through separat-
ing 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-
Sympex, Rockford, IL) 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 hours at 4°C at constant current (50 mA),
Membranes were blocked for 2-4 hours in blocking buffer (PBS, 5% HS, 5% non-fat 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 hours 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 hour 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 minute and exposure to X-ray film (XAR-5, Kodak) until bands were visible (1 second, 1 minute). 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.

### 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 pre-incubated with PBS, 5% HS, 0.02% sodium azide for 30 minutes to prevent non-specific binding. Slides were then incubated with a 1:1 mixture of IgG and IgM anti-MyHC monoclonal antibodies (tissue culture supernatants) for 1 hour, then washed three times for 10 minutes 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 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 minutes and then mounted under a coverslip in Gelvatol (Monsanto). Slides were viewed and photographed under epi-illumination using a Zeiss Axiophot fluorescence microscope.

The procedures for 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 pre-incubated with 0.1 mg/ml goat anti-mouse IgG (heavy and light) Fab (Cappel), 5% HS in PBS for 30 minutes to prevent non-specific binding. Sections were then incubated with undiluted tissue culture supernatant containing the anti-MyHC monoclonal antibody for 1 hour, then washed as described above. Sections were incubated in PBS, 5% HS for 1 hour 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 minutes. After three 10-minute washes in PBS alone, avidin-biotin complex (Vectastain ABC Elite kit, Vector) was applied to the sections for 1 hour. Slides were washed for 20 minutes in two changes of PBS, 0.2% Tween-20 and then in PBS alone for two 10-minute periods. Horseradish peroxidase reactivity was developed for approximately 1 minute with 0.6 mg/ml diaminobenzidine, 50 mM Tris-HCl, pH 7.2, 0.03% CoCl2 and 0.05% H2O2, and mixed immediately prior to staining.

RESULTS

Monoclonal antibodies A4.1519 and A4.1077 detect fast MyHCs and are distinct from antibodies to embryonic and neonatal fast MyHCs

We determined that monoclonal antibodies A4.1519 and A4.1077 are specific for epitopes on fast MyHCs (defined here as all non-slow isoforms) that are expressed in skeletal muscle but are distinct from embryonic and neonatal fast MyHCs. First, we determined that the antibodies were specific for MyHCs in western blots of whole-cell lysates of cultured human myotubes and rat muscle tissue. Both antibodies reacted exclusively with a protein of molecular mass of approximately 200 kDa, the size of MyHC (Fig. 1). Antibody F1.652, which recognizes embryonic MyHC (Silberstein et al., 1986; Karsch-Mizrachi et al., 1989; Cho et al., 1993), and N3.36, which recognizes neonatal MyHC (Silberstein et al., 1986; Cho et al., 1993), also detect a protein of this relative molecular mass (Fig. 1).

Second, we determined that the epitopes of A4.1519 and A4.1077 were distinct from those of F1.652 and N3.36 by immunoblotting of proteins expressed from cloned MyHC cDNAs or of cell or tissue lysates that contain one predominant MyHC isoform. Fig. 2 (lane 4, uppermost band) shows that F1.652 reacts with a MyHC synthesized by cultured muscle cells, in which only embryonic MyHC is detectable (Mahdavi et al., 1987). The lower molecular mass bands that react with the antibody are likely to be degradation products of MyHC, because this antibody reacts exclusively with a single 200 kDa band in whole-cell lysates of muscle cells (Fig. 1).

Antibody N3.36 recognizes an epitope encoded by a human neonatal (perinatal) MyHC cDNA (Karsch-Mizrachi et al., 1990; Dan-Goor et al., 1990) (Fig. 2, lane 8). This cDNA encodes the N-terminal half (heavy meromyosin subfragment) of the molecule. The C-terminal half of the neonatal MyHC, encoded by pSMHCP (Feghali and Leinwand, 1989) (Fig. 2, lane 6) is not recognized by N3.36. No other cDNAs analyzed encoded isoforms that were recognized by N3.36 (Fig. 2).

In contrast, A4.1519 detected only the protein expressed from a 3′ fragment of a fast MyHC cDNA (Fig. 2), pSMHCA (Saez and Leinwand, 1986). The A4.1519 epitope is contained within a region encoding amino acids 1661-1758 of the protein, in the light meromyosin (LMM) region (Sohn et al., unpublished data). pSMHCA is most homologous to the rat MyHC IIx cDNA, based on homology in the 3′ untranslated regions and over the regions encoding the eight C-terminal amino acids (DiNardi et al., 1993). Therefore, pSMHCA has been tentatively identified as a fragment of human IIx MyHC. Whether homology to the rat IIx clone extends over the A4.1519 epitope region, and thus whether A4.1519 would be expected to recognize the rat IIx MyHC protein, is not known. A4.1077 did not recognize any of the expressed proteins. It is likely that the A4.1077 epitope lies outside the region of MyHC encoded by the cDNAs we analyzed.

Third, we confirmed that the epitopes of A4.1519 and A4.1077 were distinct from those of antibodies to embryonic and neonatal MyHCs by the reactivity of the antibodies with purified human MyHCs that were separated on high-resolution glycerol/SDS-polyacrylamide gels (Fig. 3, Table 1). On these
gels, four bands are resolved, whose order of migration from most to least rapid is type I (slow), embryonic/neonatal (E/N), type IIa (fast) and type IIb (fast) (Klitgaard et al., 1990; La Framboise et al., 1990a). None of the antibodies reacted with the band migrating at the type I (slow) position. F1.652 detects only the band that migrates at the embryonic/neonatal position in extracts of muscle at weeks 14 and 22 of gestation, but does not detect any MyHC from adult muscle. The time course of F1.652+ MyHC expression in this western blot correlates with the time course of embryonic mRNA expression: in northern blots, the unique 3’ untranslated region of the cDNA encoding the human embryonic MyHC hybridizes to human mRNA
from skeletal muscle at week 22 of gestation but not mRNA from adult cardiac or skeletal muscle (Karsch-Mizrachi et al., 1989).

N3.36 reacts strongly with an isoform in week 22 muscle that migrates at the embryonic/neonatal position but, unlike F1.652, does not detect any MyHC in week 14 muscle. The time course of N3.36+ MyHC expression correlates with the time course of neonatal MyHC mRNA expression as determined by RNase protection (Feghali and Leinwand, 1989) and the reactivity of the antibody in tissue sections of human muscle (for a detailed analysis, see Cho et al., 1993). Both mRNA (Feghali and Leinwand, 1989) and protein (Fig. 3) are expressed in skeletal muscle around mid-gestation (week 21-22) and are present at substantially lower levels in the adult. In adult human muscle, N3.36 weakly labels both IIA and IIB fast fibers (Webster et al., 1988; Cho et al., 1993) and in western blots reacts weakly with an isoform that migrates at the IIA position. Taken together, these results suggest that N3.36 recognizes at least two different isoforms of MyHC in human muscle: a neonatal isoform, which is expressed only during the last half of gestation, and one or more adult isoforms, which are detected only in postnatal muscle.

In contrast, in western blots, A4.1519 and A4.1077 do not detect the embryonic/neonatal MyHC in week 14 or week 22 muscle, but do detect an MyHC in adult muscle that migrates at the IIA position. Taken together, these results show that A4.1519 and A4.1077 do not recognize slow MyHCs and are distinct from antibodies to embryonic and neonatal MyHCs.

Nascent secondary fibers are recognized by A4.1519 and A4.1077 in adult muscle

At all developmental stages analyzed, from week 11 of gestation to adult, A4.1077 and A4.1519 reacted with the same human fibers (data not shown); therefore, only results obtained with antibody A4.1077 are shown here. A4.1077+ (fast MyHC+) fibers were first observed as early as week 11 of human gestation (Fig. 4d), when secondary fibers were first forming. These fibers were very small in diameter and were found adjacent only to primary fibers, which were identified by their large size and their reactivity with an antibody to slow MyHC, A4.840 (Fig. 4c). The expression of A4.1077+ MyHC in only a small percentage of the total muscle in early gestation is likely to explain why it was not detected in western blots of muscle extracts from week 14 or 22 of gestation (Fig. 3). By week 24 of gestation, both A4.1519 and A4.1077 reacted with more mature secondary fibers of intermediate diameter (Fig. 4h). At week 26 (Fig. 4j) and in the adult (Fig. 4i), all fast fibers in the vastus lateralis were labeled. At no stage of development did the fibers that reacted with A4.1519 and A4.1077 also react with the antibody to slow MyHC, A4.840 (Fig. 4, left column).

The observation that A4.1519+ A4.1077+ MyHC is found only in very small secondary fibers, and not in larger, more mature secondary fibers between weeks 10 and 24 of gestation suggests that this MyHC is transiently expressed in early secondary fiber development. The A4.1519+ A4.1077+ epitopes reappear in mature secondary fibers by week 24 of gestation. However, it is not known whether these epitopes are on the same isoform as that expressed early in development.

A4.1519 appears to recognize a human IIX MyHC and at least one other fast MyHC in adult human muscle

In western blots of proteins expressed from MyHC cDNAs, A4.1519 recognizes the protein encoded by a cDNA clone, pSMHCA; the 3’ UTR and 5’ coding regions of this cDNA are most homologous to the gene encoding rat IIX MyHC (DiNardi et al., 1993). In situ hybridizations with muscle tissue sections, the probe containing the 3’ UTR of pSMHCA recognizes a subset of fast, A4.1519+ fibers (S. Schiaffino, unpublished observations). These results are consistent with the idea that A4.1519 recognizes IIX MyHC, and at least one other fast MyHC. Based on immunohistochemistry, this ‘other’ fast MyHC is most likely to be Ila because human and rat IIA fibers are labelled with A4.1519. Moreover, western blots of purified human MyHCs show only a single band of A4.1519+ MyHC and suggest that human Ila and IIX MyHC comigrate on this sort of gel, as reported for rat MyHCs (Schiaffino et al., 1989).

In the rat lower hindlimb, A4.1519 and N1.551 recognize fast MyHCs distinct from embryonic (F1.652+) and neonatal (N3.36+) fast isoforms

In the rat, A4.1519 also recognizes a MyHC early in development that is distinct from F1.652+ (embryonic) MyHC and N3.36+ (neonatal fast) MyHC. A4.1519 + MyHC appears at day 17 of gestation, as does F1.652+ MyHC. However, by postnatal day 27 (P27), F1.652+ MyHC is lost from all but the slow soleus and red strip of the lateral gastrocnemius muscles (Fig. 5A,E,I,M); this is in agreement with the findings of Laframboise et al. (1990a). Thereafter, F1.652+ MyHC is lost from all fibers except rare regions of fibers at myotendinous junctions (data not shown) until old age when it reappears in a minority of fibers in the soleus (Fig. 5Q) or until denervation or damage elicits regeneration (Webster et al., 1988). In contrast, A4.1519+ MyHC continues to be expressed in both fast and slow muscles through adulthood. A4.1519 is also
clearly distinct from N3.36 in that N3.36 does not recognize fibers until the time of birth (Fig. 5H, L). At that time, only secondary fibers are recognized; in adult muscle, N3.36+ MyHC is expressed in an exactly reciprocal pattern to A4.951+ slow MyHC (Fig. 5P,T; and see Webster et al., 1988). In contrast, A4.840+ N3.36+ fibers can be detected during

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**Fig. 4.** A4.1077 is a marker of newly formed secondary fibers in early development. Transverse sections of human limb muscle at weeks 8 (a,b), 11 (c,d), 14 (e,f), 22 (g,h) and 33 (i,j) of gestation and 55 years postnatal (k,l) were reacted with antibody A4.840 (left column) and A4.1077 (right column) simultaneously, followed by FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-mouse IgM secondary antibodies. Each panel in the left column (A4.840, rhodamine) shows the same field as the corresponding panel in the right column (A4.1077, FITC) as visualized by fluorescence microscopy. Arrows indicate: c,d, A4.840−A4.1077+ secondary fibers; g,h; i,j, A4.840+A4.1077− fibers. Bar, 25 µm.
postnatal development and in the adult animal. This time course is in good agreement with the findings of others that RNA encoding perinatal MyHC is present in late-gestation and newborn mouse muscle (Cox and Buckingham, 1992). Due to the location of these fibers in muscles in which the proportion of fast and slow fibers is changing, it seems likely that dual expression of both N3.36 and A4.840 MyHCs may reflect a transition state from fast to slow or slow to fast.

In the rat, A4.1519 does not appear to specifically recognize nascent secondary fibers as it does in the human. Although it does not react with embryonic MyHC isoforms on gels, A4.1519 reacts with all fibers at early stages of development, A4.1077 does not recognize rat muscle at any stage of development that we have examined. However, nascent secondary fibers are recognized at E17 and E21 of gestation by a different MyHC-specific antibody, N1.551; this is in agreement with the findings of Condon et al. (1990). These results suggest that A4.1519 and N1.551 recognize MyHCs early in rat development that are distinct from F1.652+ (embryonic) and N3.36+ (neonatal fast) MyHCs. The data also suggest that the MyHC recognized by A4.1519 in developing rat muscle is a functionally distinct molecule from that detected in human nascent secondary fibers.

**DISCUSSION**

**Nascent secondary fibers are recognized by A4.1519 or A4.1077 in human muscle, and by N1.551 in rat muscle**

Although A4.1519 and A4.1077 detect MyHCs that are characteristic of fast fibers of adult muscle, they also identify non-slow fibers very early in human muscle development. N1.551 recognizes the equivalent fibers in the rat. All three of the antibodies recognize a MyHC present in nascent secondary fibers. Based on their pattern of labelling of muscle fibers in vivo, these antibodies are most likely to recognize the same MyHC as the antibodies NOQ 7.5.28 (Draeger et al., 1987) and WBMHCf (EcoB-Prince et al., 1989). Both NOQ 7.5.28 and WBMHCf detect very small fibers at week 17 of gestation, which were considered to represent 'tertiary' fibers because of the time in development when these fibers were first generated. However, very early time points were not analyzed in these studies. Our results argue against the existence of 'tertiary' fibers because we detected A4.1519+ and A4.1077+ fibers as early as week 10-11 of human gestation, the time when secondary fibers begin to form (Fidzianska, 1980). At this stage, the morphology of A4.1519+ and A4.1077+ fibers, which were found closely juxtaposed to and nearly engulfed by primary fibers, was typical of nascent secondary fibers (Kelly and Zacks, 1969; Fidzianska, 1980). Thus, we conclude that these antibodies are useful markers of an epitope on fast MyHC, which distinguishes secondary fibers at the earliest stages of their development.

**From the time of their inception, primary and secondary fibers have different developmental programs**

Morphological differences distinguish primary and secondary fibers (Rubinstein and Kelly, 1981). Here we show that differences in MyHC isoform expression also distinguish these two...
fiber populations as soon as they are formed. The expression of slow MyHCs in primary fibers and fast MyHCs in secondary fibers immediately upon formation suggests that the two populations of fibers differ in contraction speeds or sarcromere assembly. Interestingly, adjacent primary and secondary fibers are initially within the same basal lamina (Kelly and Zacks, 1969) and are likely to be electrically coupled via gap junctions (Schmalbruch, 1982). This type of coupling has been shown to cause whole muscles to contract as a unit in response to electrical stimulation (Dennis et al., 1981). Despite this coordinated electrical activity, we observed that newly formed primary and secondary fibers have different MyHC isoform compositions. This suggests that an intrinsic difference between primary and secondary fibers could over-ride effects of electrical activity, a difference that could reflect distinctions between the myoblast populations from which the two fiber populations are derived (Miller and Stockdale, 1986; Cho et al., 1993). This suggestion is consistent with (1) the appearance of distinct primary and secondary fibers in denervated rat embryos (Condon et al., 1990) and (2) the innervation of both primary and secondary fibers by the same, immature, motor neuron.

Alternatively, extrinsic signals in the microenvironment encountered by the secondary fiber may be subtly different from those encountered by the primary fiber, leading to the different patterns of MyHC expression in the two populations of fibers. For example, because nascent secondary fibers form in the mid-regions of primary fibers, and thus are not initially anchored to tendons (Duxson et al., 1989), primary and secondary fibers may be subject to different levels of tension. Furthermore, the formation of secondary fibers upon a scaffold of a primary fiber (Kelly and Zacks, 1969; Fidzianska, 1980) implies that the substrata on which these two populations form are not identical. Retroviral marking experiments (Hughes and Blau, 1990, 1992; Evans et al., 1994) suggest that single myoblast clones can contribute to both primary and secondary fibers shortly after their formation through adult stages of development. However, to determine whether the first myoblasts that give rise to a new fiber determine the subsequent phenotype of the fiber will require analysis at earlier stages of development.

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