Evidence for Myoblast-extrinsic Regulation of Slow Myosin Heavy Chain Expression During Muscle Fiber Formation in Embryonic Development

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Abstract. Vertebrate muscles are composed of an array of diverse fast and slow fiber types with different contractile properties. Differences among fibers in fast and slow MyHC expression could be due to extrinsic factors that act on the differentiated myofibers. Alternatively, the mononucleate myoblasts that fuse to form multinucleated muscle fibers could differ intrinsically due to lineage. To distinguish between these possibilities, we determined whether the changes in proportion of slow fibers were attributable to inherent differences in myoblasts. The proportion of fibers expressing slow myosin heavy chain (MyHC) was found to change markedly with time during embryonic and fetal human limb development. During the first trimester, a maximum of 75% of fibers expressed slow MyHC. Thereafter, new fibers formed which did not express this MyHC, so that the proportion of fibers expressing slow MyHC dropped to ~3% of the total by midgestation. Several weeks later, a subset of the new fibers began to express slow MyHC and from week 30 of gestation through adulthood, ~50% of fibers were slow. However, each myoblast clone (n = 2,119) derived from muscle tissues at six stages of human development (weeks 7, 9, 16, and 22 of gestation, 2 mo after birth and adult) expressed slow MyHC upon differentiation. We conclude from these results that the control of slow MyHC expression in vivo during muscle fiber formation in embryonic development is largely extrinsic to the myoblast. By contrast, human myoblast clones from the same samples differed in their expression of embryonic and neonatal MyHCs, in agreement with studies in other species, and this difference was shown to be stably heritable. Even after 25 population doublings in tissue culture, embryonic stage myoblasts did not give rise to myoblasts capable of expressing MyHCs typical of neonatal stages, indicating that stage-specific differences are not under the control of a division dependent mechanism, or intrinsic "clock." Taken together, these results suggest that, unlike embryonic and neonatal MyHCs, the expression of slow MyHC in vivo at different developmental stages during gestation is not the result of commitment to a distinct myoblast lineage, but is largely determined by the environment.

ADULT skeletal muscles of humans are heterogeneous tissues. Limb muscles have a characteristic proportion and spatial arrangement of type I (slow-twitch) and type IIA and type IIB (fast-twitch) fibers (for review, see Schmalbruch, 1985). This arrangement of fiber types is responsible for the unique contractile properties of the muscle as a whole. In adult muscle, fiber-type classifications have been established by correlation of physiological, biochemical and histochemical properties (for review, see Schmalbruch, 1985). By contrast, in embryonic muscle, the biochemical and histochemical properties of fibers do not correlate and fiber typing has been problematic (Guth and Samaha, 1972). Recently, this problem was overcome by the development of immunological reagents. Especially useful are mAbs to myosin heavy chains (MyHCs).[1] MyHCs are largely responsible for the ATPase activity, and therefore contraction rate, of individual fibers. In particular, antibodies have had a major impact on studies of fiber development, because they can distinguish developmentally-regulated neonatal and adult fast myosins in the presence of embryonic isoforms (Schiaffino et al., 1982; Webster et al., 1988*b*). As a result, immunohistochemistry now permits an analysis of the basis for fiber-type heterogeneity and pattern formation during embryogenesis.

Myosin is a multimeric complex of two heavy chains and four light chains. Both the heavy and light chains are encoded by multigene families. The heavy chains, which contain the ATPase activity of the myosin complex, are a diverse

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1. *Abbreviations used in this paper*: β-ME, β mercaptoethanol; ECL, enhanced chemoluminescence; FM, fusion medium; GM, growth medium; HS, horse serum; MyHCs, myosin heavy chains.

group comprising both developmental-stage specific and fiber-type specific isoforms (Whalen et al., 1981; Wydro et al., 1983; Leinwand et al., 1983; Saez and Leinwand, 1986; Weydert et al., 1987). The heavy chain composition of skeletal muscle fibers changes throughout development. The cellular and molecular basis for these changes in gene expression and how they result in the ultimate pattern of fiber types in individual muscles is unclear.

In particular, the cellular origin of specialized muscle fibers with different contractile properties remains unknown. Two hypotheses are that (*a*) myoblasts that form the fast and slow fibers are distinct and their intrinsic properties confer a specific pattern of gene expression on the multinucleated fibers they form upon cell fusion, or (*b*) myoblasts are similar and extrinsic factors induce a specific pattern of gene expression on the differentiated multinucleate muscle fibers they form. In support of the first hypothesis, analyses of cultured myoblasts isolated from the limb buds of chick (Miller and Stockdale, 1986*a*,*b*) and mouse (Vivarelli et al., 1988) embryos distinguished populations of myoblasts that differed in their expression of slow and fast MyHCs. These results led to the proposal that myoblasts are committed to fast and slow fiber-type lineages which are responsible for the development of fiber-type diversity.

Here, in support of the second hypothesis, we provide evidence that the expression of fast and slow MyHCs in newly formed secondary fibers is not due to the intrinsic potential of myoblasts but is determined by their environment. We show that there are marked differences in the proportion of fibers expressing slow MyHC in muscle tissue at different stages of human embryonic development. To determine whether these differences reflect properties of myoblast populations, we tested the intrinsic capacity of human myoblasts to express slow MyHC when isolated from muscle of six developmental stages, ranging from week 7 of human gestation to adult. The results show that, although myoblasts are heterogeneous with respect to the expression of stage-specific MyHC isoforms, these differences do not account for functionally distinct fast and slow fiber types. Thus, myoblasts may differ in their potential to express embryonic and neonatal MyHC isoforms, but all myoblasts at all stages are capable of expressing slow MyHC isoforms upon differentiation in vitro.

What regulates the expression of fast and slow MyHC expression in vivo? From mid-gestation on, the phenotype of preexisting fibers into which myoblasts fuse is likely to be the major determinant (Hughes and Blau, 1992). However, dominance of preexisting fibers cannot explain the observations at earlier stages. Before and at the time when the first fast fibers are forming (week 10 of gestation), all myoblasts are capable of expressing slow MyHC. Thus, we conclude that the lack of expression of slow MyHCs in the earliest-forming fast muscle fibers in vivo is largely determined by the environment.

Materials and Methods

Cell Culture

Cells were isolated from human skeletal muscle as described previously (Blau and Webster, 1981). All samples were from individuals with no known myopathies or other abnormalities affecting muscle development. Muscle samples were from the vastus lateralis muscle, with the exception of the youngest stages of development (weeks 7–9) for which the entire thigh was used. Muscles from six developmental time points were analyzed: embryonic (three samples each at weeks 7 and 9 of gestation), fetal (weeks 16 and 22 of gestation), neonatal (2 mo after birth), and adult (2.5 yr after birth). Muscle from individuals 2 yr or older is defined as adult because it is fully developed with respect to fiber type. At week 7 and week 9, tissue samples from three different individuals were analyzed in each case. Embryonic and fetal muscles were obtained subsequent to therapeutic abortion, postnatal muscles at autopsy, and adult muscles consequent to corrective orthopedic surgery. All samples were procured in accordance with the guidelines of the Stanford Human Subjects Committee.

For clonal analysis, cells were grown as previously described (Webster et al., 1988*a*). Briefly, cells were plated at ~1 cell/cm^2 in a 1:1 mixture of fresh and conditioned growth medium (GM: Ham's nutrient mixture F-10 with 15% FBS (Hyclone Laboratories, Inc., Logan, UT), 0.5% chick embryo extract (GIBCO) and 1% penicillin/streptomycin [GIBCO BRL, Gaithersburg, MD]). Conditioned medium was harvested from adult human muscle cultures and filtered through a 0.2 μm filter (Nalge Co., Rochester, NY) before use. Dishes were precoated with a solution of type I collagen (0.15% calf skin collagen, Sigma Chemical Co., St. Louis, MO). When the majority of colonies contained ~10^3 cells (~10 d after plating), cultures were switched to fusion medium (FM: DME with 5% horse serum [Hyclone Laboratories], 10^{-6} M bovine insulin [Sigma Chemical Co.] and 10^{-6} M dexamethasone [Sigma Chemical Co.]) and thereafter fed every 2 d with FM until analysis. For some experiments, 0.5% chick embryo extract was included in the FM, but this did not change the results.

Monoclonal Antibodies

Mice were immunized by subcutaneous injection of partially purified fetal (week 15 of gestation; antibodies F1.652, F1.188), neonatal (day 5 postnatal; antibody N3.36) or adult (21 year; antibody A4.951, previously referred to as 4A.951) human skeletal muscle myosin preparations and hybridomas produced 3 d later, as previously described (Silberstein and Blau, 1986). Clones were screened for production of antibodies that reacted with MyHC in ELISA, specifically labeled subpopulations of human skeletal muscle fibers in immunofluorescence assays on tissue sections (Webster et al., 1988*b*) and reacted with a 220-kD MyHC band on Western blots of the immunogen (data not shown). In addition, the antibodies were shown to react with specific MyHCs separated on high resolution gels and with peptides encoded by specific cDNAs[2] (Hughes et al., 1993). All antibodies are available from American Type Culture Collection (Rockville, MD).

Extraction of MyHCs from Human Muscle Tissues

MyHCs were extracted from human muscle tissue essentially as described in Whalen et al. (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 mg/ml trypsin inhibitor, 0.1 mM pepstatin A and 0.1 mM leupeptin). After 20–40 min of extraction on ice, debris was removed by centrifugation at 13,000 *g* for 30 min 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 min and resuspended in 0.5 M NaCl/10 mM $NaPO_4$, pH 7.0 with protease inhibitors and allowed to dissolve overnight on ice. Samples were then boiled for three min in Laemmli SDS sample buffer and stored in 10-ml aliquots at −80°C for up to 3 mo.

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 h at 15°C, 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 Laboratories, Richmond, CA) and 1.5% bis-acrylamide (Bio-Rad Laboratories). Portions

2. Cho, M., S. M. Hughes, I. Karsch-Mizrachi, L. A. Leinwand, and H. M. Blau, submitted for publication.

of the gel were either stained (BioRad Silver Stain Plus Kit, Bio-Rad Laboratories) or transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA).

For Western blot analysis, proteins were transferred in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS, Sigma Chemical Co.), 5% methanol for 16–18 h at 4°C at constant current (50 mA). Membranes were blocked for 2–4 h in blocking buffer (PBS, 0.02% sodium azide, 5% horse serum (HS), 5% nonfat dry milk) and air dried at room temperature before 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 h 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 Laboratories (Malvern, PA); diluted 1:1,000 in PBS, 0.02% sodium azide, 5% HS) for 1 h 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 Corp., Arlington Heights, IL) for one minute and exposure to x-ray film (XAR-5, Kodak) until bands were visible (1 s–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.

Immunofluorescence

For immunohistochemistry, fresh tissue was mounted in OCT mounting medium (Baxter Scientific Products, McGaw Park, IL) and frozen in melting isopentane (Sigma Chemical Co.). Transverse 8-μm cryostat sections were placed on gelatin-coated slides and stored desiccated at −20°C until staining. Cultured cells were fixed and stained with mAbs as described previously (Silberstein et al., 1986). For these studies, the following 1:1 mixtures of IgG- and IgM-containing supernatants were used: A4.951 (IgG) and F1.188 (IgM) or F1.652 (IgG) and N3.36 (IgM). Secondary antibodies were conjugated either to fluorescein (goat anti-mouse IgG, Cappel Laboratories) or to rhodamine (goat anti-mouse IgM, Cappel Laboratories). Cells were examined with a Leitz epi-fluorescence Orthoplan microscope using fluorescein-specific (excitation 470–490 nm) and rhodamine-specific (excitation 530–560 nm) optical filters and a 25× objective. Photographs were taken with Kodak Tri-X film using a Leitz Vario-Orthomat E camera.

Results

Myoblasts from All Stages Express Slow MyHCs upon Differentiation

Slow MyHC is Recognized by Antibody A4.951. We determined the pattern of slow MyHC expression in primary and secondary human muscle fibers in vivo with a mAb, A4.951. The specificity of this antibody has been extensively characterized (Hughes et al., 1993). Briefly, A4.951 exclusively recognizes a 220-kD protein in Western blots of whole-cell lysates of cultured human myotubes, indicating that it is specific for the heavy chain of myosin. Furthermore, A4.951 recognizes a MyHC which migrates at the type I position in Western blots of high-resolution SDS-polyacrylamide gels in which types I, IIa, and IIb MyHCs of adult skeletal muscle can be resolved (Klitgaard et al., 1990). A similar band of mobility specific to type I is evident on blots of extracts of muscle at all stages analyzed: tissues from weeks 14 (Hughes et al., 1993) and 22 of gestation, adult muscle, and in extracts of cultured human myotubes (Fig. 1). This is true also when MyHCs of earlier stages were analyzed by Western blot of denaturing gels (Hughes et al., 1993). In addition, A4.951 has been shown to recognize the head domain of human b-cardiac/slow MyHC expressed from a cDNA (Hughes et al., 1993). Finally, expression of A4.951-reactive myosin has been correlated with slow ATPase activity in serial frozen sections of adult muscle tissue (Webster et al., 1988*b*). Therefore, by numerous criteria, A4.951 recognizes a slow MyHC present in human muscle development.

F1.188 (Control) Recognizes Prenatal MyHCs in All Fibers. Antibody F1.188 reacts with MyHC in muscle tissue and in cultured muscle cells. It reacts uniformly with all fibers in frozen sections of human muscle tissue, including fast and slow, primary and secondary, and cultured muscle cells from samples at all stages of prenatal development (data not shown). F1.188 recognizes either an epitope common to all MyHCs or a MyHC isoform that is prevalent in all fibers at all stages of prenatal development. Thus, F1.188 serves as a useful control, as a marker of the presence of MyHC at any given point in gestation or in cultured cells.

Slow MyHC is Expressed in Two Phases During Human Embryogenesis. An analysis of slow MyHC in tissue sections revealed that the proportions of slow fibers change markedly during human embryonic development (Fig. 2). At 8 wk of gestation, widely-separated clusters of small-diameter fibers with central nuclei are interspersed with mononuclear cells and connective tissue (Fidzianska, 1980*a* and Fig. 2 *a*). Myofibers can be identified by their expression of embryonic MyHC, as detected by antibody F1.652 (see Fig. 6 *a*). Although all myofibers at this stage of development express embryonic MyHC, none react with A4.951 (Fig. 2 *h*).

By 10 wk of gestation, A4.951+ slow MyHC is first expressed, in the vast majority of fibers (Fig. 2 *i*). A4.951+ MyHC is expressed most strongly in the largest diameter pri-

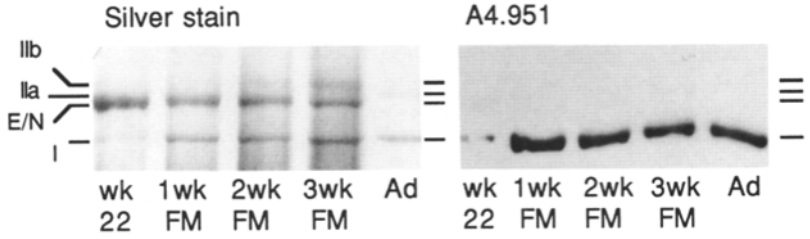

Figure 1. A4.951 recognizes slow MyHC in human muscle tissue and cultures. Extracts of human thigh muscle at week 22 (*wk 22*) of gestation, adult human muscle cultures differentiated in culture for 1 wk (*1 wk*) 2 wk (*2 wk*) or three wk (*3 wk*) and adult human diaphragm tissue (*Ad*) were electrophoresed through high-resolution glycerol/SDS 5.5% polyacrylamide gels. One set of samples was silver stained (*left*) and two identical sets were transferred to PVDF membrane. Individual panels of membrane were reacted with antibodies as described in Materials and Methods. The positions of types I, IIa, and IIb MyHC are indicated, as defined by Klitgaard et al. (1990). A4.951 recognizes slow MyHC in extracts of both muscle tissue and cultures.

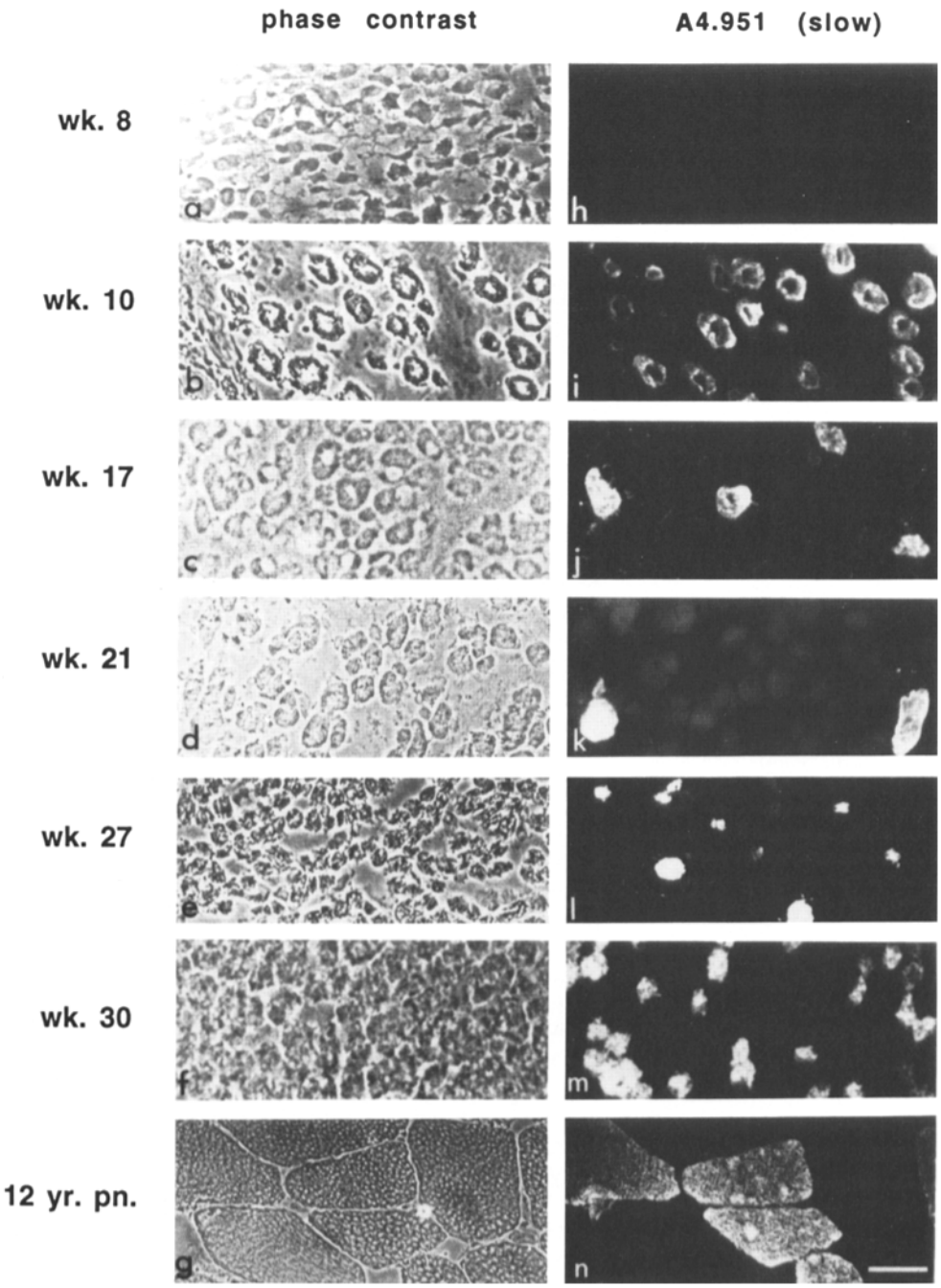

Figure 2. The proportion of slow MyHC-expressing fibers changes markedly during development. Transverse sections of human limb muscle at weeks 8 (*a*, *h*), 10 (*b*, *i*), 17 (*c*, *j*), 21 (*d*, *k*) 27 (*e*, *l*) and 30 (*f*, *m*) of gestation, and 12 yr postnatal (*g*, *n*) were stained with antibody A4.951 (*h–n*) followed by an FITC-conjugated goat anti-mouse IgG secondary antibody. Phase contrast micrographs (*a–g*) are the same fields shown in immunofluorescence (*h–n*), *a–e* are also shown in Fig. 6. Bar, 100 μm.

mary fibers, which are labeled with a characteristic ringlike pattern when viewed in cross-section because of the location of the nucleus in the center of the fiber. Some fibers react weakly with A4.951, and are likely to be immature primary fibers because of their intermediate size and clearly visible central nuclei. The fibers that do not react with A4.951 are very small in diameter, and the location of the nuclei is unclear, presumably because of their small size (Fig. 2 *b*), although their identity as muscle fibers is confirmed by their expression of embryonic MyHC (Fig. 6 *b*) and MyHC detected by antibody A4.1519.[2]

Between weeks 10 and 21 of gestation, the proportion of fibers that reacts with A4.951 progressively declines (Fig. 2 *i–l*, Table I). Although ~75% of fibers present appear to express A4.951+ MyHC at week 10 of gestation, by week 17, the percentage of fibers expressing this isoform has decreased to ~10%. By week 21, the maturation of primary fibers has progressed, as reflected by the absence of central nuclei (Fig. 2 *k*) and the loss of expression of embryonic MyHC (see Fig. 6 *d*). At this stage (midgestation), A4.951+ fibers comprise only 3% of total fibers, and are clearly larger than A4.951 fibers, indicating that at this stage, like earlier stages of development, slow MyHC expression is restricted to primary fibers.

The decrease in the proportion of slow primary fibers between week 10 and 21 of gestation can be accounted for by the dramatic increase in the numbers of secondary fibers during these stages (Fidzianska, 1980*a*), which express fast

*Table I. Percentage of Fibers Expressing Slow MyHC in Developing Human Muscle**

Gestational Stage‡	Mean number total fibers/field§	Mean number A4.951-positive (slow) fibers/field§	Percent A4.951-positive (slow) fibers‖
wk			
8	134 ± 21	0 ± 0	0% ± 0%
10	168 ± 8	126 ± 9	75% ± 3.3%
12	258 ± 12	118 ± 18	48% ± 3.1%
17	253 ± 11	24 ± 6	9.5% ± 1.8%
21	261 ± 17	8 ± 4	3.1% ± 1.1%
27	651 ± 25	23 ± 8	3.5% ± 0.72%
30	319 ± 12	157 ± 8	49% ± 2.8%
2 postnatal	280 ± 9	137 ± 7	49% ± 3.0%
adult	19 ± 2	11 ± 2	58% ± 11%

*Transverse sections of human muscle were reacted with antibody A4.951 and reactivity detected by immunofluorescence as described in the legend to Fig. 3.
‡ Three to five samples were analyzed at each developmental stage.
§ Three to five separate microscopic fields (10^{-5} m2 each) were analyzed for each data point, with error calculated as SEM. Number of fibers have been normalized to unit area of 10^{-5} m2. The increase in fiber number between weeks 8 and 27 of gestation reflects an increase in fiber formation and density. The decrease after week 27 reflects the growth of fibers and accompanying increase in diameter.
‖ Error is calculated as the standard error of the proportion.

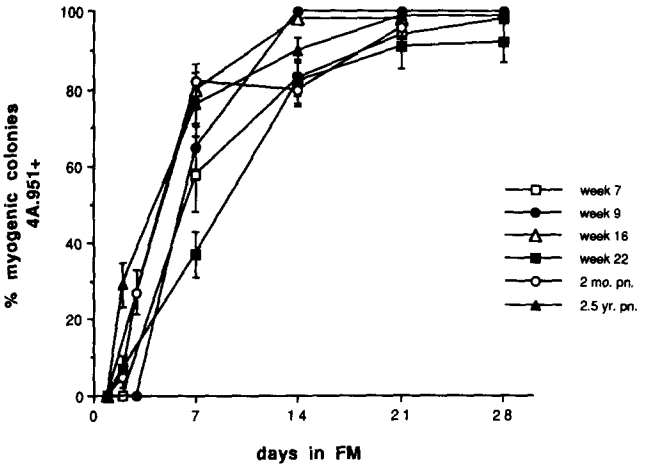

Figure 3. Time course of slow MyHC expression in cultured human muscle cell colonies. Colonies were derived from muscle tissues of embryonic week 7 (n = 165), week 9 (n = 393), week 16 (n = 270), week 22 (n = 407), 2 mo postnatal (n = 432), or 2.5 yr postnatal (n = 452) stages of human development, where n = number of myogenic colonies scored from each developmental stage. The presence of myogenic colonies and the percent of those colonies expressing slow MyHC was determined by double-immunofluorescence staining with the antibody F1.188, that recognizes all prenatal MyHCs and A4.951, that recognizes slow MyHC. A colony was scored positive for a given antibody if five or more myotubes were recognized by A4.951 in that colony. Error bars were calculated as the standard error of the proportion.

MyHC. Over this period of human muscle development, secondary fibers can be distinguished from the primary fibers by their smaller diameters. We found that, at week 27 of gestation, the mean fiber diameter of the large-diameter fibers is approximately twice that of the smaller-diameter fiber population (see Fig. 2 *l*), and that cross-sectional diameter distinguishes two populations of fibers with virtually nonoverlapping size distributions, a distinction that persists until approximately week 30 of gestation (Webster and Blau, unpublished results; Wohlfart, 1937).

By week 27, A4.951+ MyHC is induced in a subset of secondary fibers (Fig. 2 *e* and *l*). At this stage, primary fibers, which continue to express slow MyHC, are still distinguishable by their greater diameters (Fig. 2 *l*). Over the next 3 wk, the percentage of total fibers expressing slow MyHC increases rapidly to ~50% of all fibers in the vastus lateralis. This is due to a conversion of some secondary fast fibers into slow fibers. The proportion of fast and slow fibers remains constant from that point onward and is typical of adult muscle (Fig. 2, *f–g*, *m–n*, Table I). Thus, the proportion of slow MyHC-expressing fibers in human muscle changes from 75% during the first trimester to a minimum of 3% at midgestation and stabilizes around 50% at 30 wk of gestation and beyond into adulthood.

Slow MyHC is Expressed in All Colonies of Cultured Myogenic Cells Irrespective of the Developmental Stage of the Source. To determine whether myoblasts are specialized for slow or fast fates, we examined whether the proportions of slow MyHC-expressing myogenic clones in vitro correlated with the proportions of slow expressing fibers in vivo. We hypothesized that the presence of slow MyHC in primary but not secondary fibers shortly after their formation was due to an intrinsic difference in myoblasts to express slow MyHC. To address this possibility, myoblasts were isolated from muscle tissues at a range of developmental stages, from week 7 of gestation to 2.5 yr after birth, including key periods of both primary and secondary fiber formation, when the proportions of slow fibers differ markedly (Table I). Primary dissociates were grown at clonal density and each clone allowed to differentiate to form multinucleated myotubes. The expression of MyHCs was defined in the resulting myotubes at several time points after the cultures were placed in FM to differentiate.

We identified those clones which were myogenic by double immunofluorescence. Antibody F1.188 recognizes an epitope present in all fiber types in prenatal muscle, and thus, is a control for the presence of MyHC-expressing cells. Identification of myogenic clones was necessary because the proportion of total clones in culture that are myogenic differs in cultures from different stages of development (see below).

Fig. 3 shows that cells isolated from tissues of early and late developmental stages (weeks 7, 16, and 22 of gestation and 2.5 years after birth) contain cells which are capable of expressing slow MyHC in culture. In fact, by 4 wk in FM, virtually all individually scored myogenic colonies (n = 2119, total from six developmental stages) expressed the slow MyHC recognized by A4.951 (Fig. 3). These results indicate that the progeny of all clonable human myoblasts have the potential to express slow MyHC in culture. Thus, even at week 22, a time in development when the majority of myoblasts would have contributed to the 97% of fibers that did not synthesize detectable slow MyHC, these myoblasts readily give rise to fibers that express slow MyHC in culture.

Expression of Slow MyHC Does Not Require Continuous Presence of Serum Factors. The results presented above suggested that slow MyHC expression in muscle cells in vivo is mediated by muscle-extrinsic factors. Humoral factors are

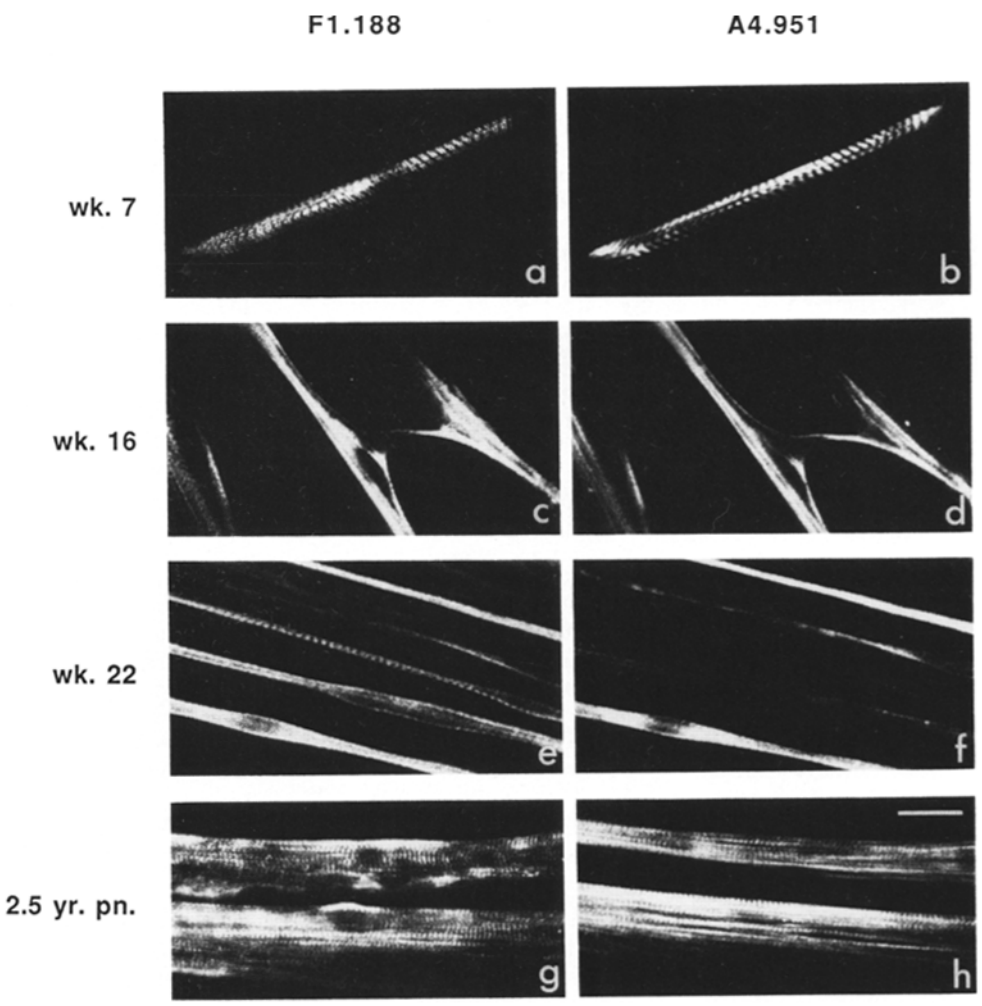

Figure 4. Slow MyHC is expressed in cultured myogenic colonies regardless of developmental stage of the source. Muscle tissues at embryonic week 7 (*a*, *b*), 16 (*c*, *d*) and 22 (*e*, *f*), or postnatal year 2.5 (*g*, *h*) were dissociated and mononucleated cells grown in GM for 10 d, then allowed to differentiate in FM for 4 wk. The simultaneous presence of F1.188- (*a*, *c*, *e*, *g*) and A4.951-reactive (*b*, *d*, *f*, *h*) myosins were assayed by double-immunofluorescence 4 wk after switching to FM. All colonies derived from each developmental stage eventually expressed moderate to high levels of slow MyHC. Bar, 30 μm.

known to affect the phenotype of muscle fibers after birth in vivo and in culture (Gustafson et al., 1985; Izumo et al., 1986; Narusawa et al., 1987; Weydert et al., 1987). Innervation is also known to influence MyHC expression in mature fibers in vivo (for review see Pette and Staron, 1990) and could, in theory, alter myoblast phenotypes in vivo. However, because the expression of slow MyHC that we observed occurred in tissue culture, it clearly did not require the continuous presence of nerves. To determine the extent to which the expression of slow MyHC required soluble factors in the culture medium that might have been contributed by serum, mass cultures of cells derived either from muscle at week 9 of gestation or from adult muscle were induced to differentiate in a defined medium containing DME with insulin (10^{-6} M) and dexamethasone (10^{-6} M). Although these cells were exposed for FBS and chick embryo extract during growth as nondifferentiating myoblasts, during the time course of the experiment examining differentiation potential (~2 wk), the cells were not exposed to neural signals, and were maintained under well-defined serum-free conditions. No difference was observed in the ability of both early embryonic and adult-derived cultures to express slow MyHC under these conditions. These results suggest that factors from serum were not responsible for the induction of slow MyHC during differentiation in tissue culture.

Although Myoblasts from All Stages Express Slow MyHC, They Differ in Their Expression of Developmental Stage-specific MyHCs

Although cells from different stages did not differ in their expression of slow MyHC, they differed with respect to at least three other parameters. First, myotubes formed from myogenic cells of muscle at week 7 of gestation were generally short and small in diameter (Fig. 4 *a*), whereas myotubes formed from muscle cells at later stages of gestation were much longer and greater in diameter (see Fig. 4 *g*), in agreement with previous studies (Bonner and Hauschka, 1974; Hauschka, 1974). Second, the proportion of myogenic cells, as determined by reactivity of myotube colonies with antibody F1.188, differed at different stages of development. Striations in MyHC+ cells of week 7 muscle cultures (Fig. 4 *a*) indicate that these small, labeled cells are indeed myotubes. The percentage of myogenic colonies after 4 wk of differentiation in culture increased from 24% (n = 442) at week 7 of gestation; to 88.7% (n = 122) at week 22; to 90.3% (n = 557) at 2.5 yr postnatal. Third, myogenic cells from different stages differed in the potential to express a developmentally regulated neonatal fast MyHC isoform, as described in detail below.

The potential to express slow MyHC did not distinguish

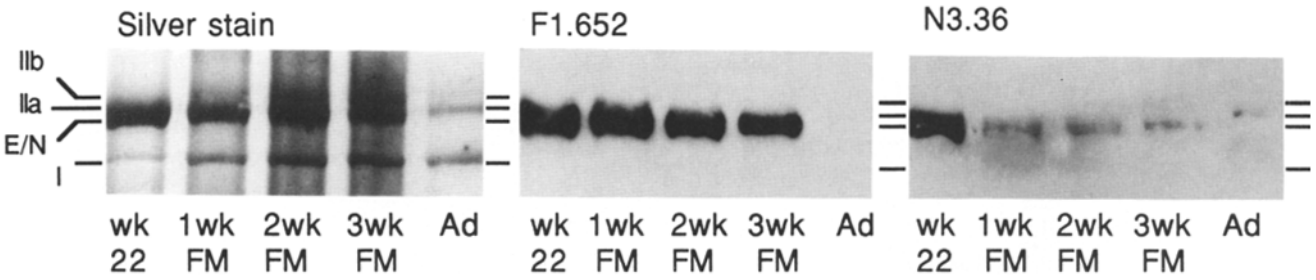

Figure 5. F1.652 recognizes embryonic MyHC and N3.36 recognizes neonatal MyHCs in human muscle tissue and cultures. Extracts of human thigh muscle at week 22 (*wk 22*) of gestation, adult human muscle cultures differentiated in culture for 1 wk (*1 wk*) 2 wk (*2 wk*) or 3 wk (*3 wk*) and adult human diaphragm tissue (*Ad*)) were electrophoresed through high-resolution glycerol/SDS 5.5% polyacrylamide gels. One set of samples was silver stained (*left*) and two identical sets were transferred to PVDF membrane. Individual panels of membrane were reacted with antibodies as described in Materials and Methods. The positions of types I, IIa, and IIb MyHC are indicated, as defined by Klitgaard et al. (1990). In MyHC extracts of prenatal muscle and of muscle cultures, antibodies F1.652 and N3.36 both recognize a band of distinct mobility (*E/N*), which has been shown to contain embryonic and neonatal MyHC isoforms[2]; we have determined that, in these extracts, N3.36 recognizes a MyHC that is distinct from embryonic MyHC because, unlike F1.652, it does not recognize MyHC as early as week 14 of gestation.

myoblasts of different stages, however, it remained possible that other characteristics which distinguish developing muscle fibers are determined at the level of the myoblast, as suggested by studies in other species (Bonner and Hauschka, 1974; Cossu et al., 1985; Mouly et al., 1987; Smith and Miller, 1992; Song et al., 1992). For example, two types of muscle fibers, designated "primary" and "secondary" fibers, differ in time of appearance, size and morphology. Both are produced by the fusion of mononucleated myoblasts into differentiated multinucleated fibers. Primary fibers form in clusters, interconnected by gap junctions, and begin to separate by the formation of basal laminae. Secondary fibers are thought to arise from replicating myoblasts adjacent to primary fibers which eventually become distinct by forming their own basal laminae (Kelly and Zacks, 1969; Ontell and Dunn, 1978). Until late gestation in humans, primary fibers can be readily distinguished from secondary fibers by their relatively large size (Wohlfart, 1937; Fidzianska, 1980*a*) and by their expression of slow MyHC (Rubinstein and Kelly, 1981; Phillips and Bennett, 1984; Crow and Stockdale, 1986; Condon et al., 1990*a*).

These differences between primary and secondary fibers suggested that the human myoblasts that give rise to them might differ with respect to the potential to express proteins which are specific to primary or secondary fibers. Therefore, to characterize the pattern of developmental stage-specific MyHC expression we examined stages when primary and secondary fibers are being formed. mAbs were used that could distinguish MyHC isoforms early in muscle development, even in the presence of other MyHC isoforms. These reagents were raised against human MyHCs partially purified from either fetal, neonatal or adult human muscle, as described previously (Silberstein et al., 1986; Webster et al., 1988*b*). The reactivity of these antibodies with isoforms of MyHC was extensively characterized by Western blot analysis (Cho et al., submitted): first, each of the antibodies was tested in Western blots of whole-cell lysates of cultured human myotubes and rat muscle tissue and found to react exclusively with a protein of relative mol wt of ~200 kD, the size of MyHC. Second, each antibody showed a distinct pattern of reactivity in Western blots of purified human MyHCs that were separated on high-resolution glycerol/SDS-polyacrylamide gels (Klitgaard et al., 1990; La Framboise et al., 1990). On these gels, four bands are resolved, type I (slow), embryonic/neonatal (E/N), type IIa (fast) and type IIb (fast). Third, the MyHC isoform specificity of each antibody was determined by immunoblotting of antibodies with proteins encoded by MyHC cDNAs or with cell- or tissue-lysates which contain one predominant MyHC isoform.[2]

F1.652 Recognizes Embryonic MyHC in Primary and Secondary Fibers. mAb F1.652 is specific for embryonic MyHC by several criteria. First, F1.652 reacts exclusively with ~220-kD protein in Western blots of whole-cell lysates of human myotubes differentiated in tissue culture (Silberstein et al., 1986) which shows that it is specific for the heavy chain of myosin. Second, F1.652 reacts only with partially purified extracts from embryonic stages of human muscle tissue in Western blots. For these blots, extracts were electrophoresed through high-resolution SDS-polyacrylamide gels under conditions which resolve types I, IIa, and IIb MyHCs from adult muscle (Klitgaard et al., 1990). In such blots, F1.652 reacts with a distinct MyHC both in extracts of skeletal muscle at weeks 14[2] and 22 of gestation, and in extracts of cultured human myotubes, but not with any MyHCs (I, IIa, or IIb) in extracts of adult muscle (Fig. 5). The F1.652+ MyHC in prenatal muscle migrates faster than adult type IIa but slower than type I MyHC under our conditions. Third, F1.652 reacts with the protein expressed from the human embryonic MyHC cDNA, pSMHCE (Karsch-Mizrachi et al., 1989). We previously designated the MyHC recognized by antibody F1.652 as fetal (Silberstein and Blau, 1986), because human muscle at 15 wk of gestation was used as the source of the MyHC antigen. However, because the gene encoding the epitope recognized by this antibody is most homologous to the embryonic MyHC gene in the rat (Karsch-Mizrachi et al., 1989; Strehler et al., 1986), and is expressed not only at fetal but also at embryonic stages of human development, we now refer to this isoform as embryonic MyHC.

Embryonic MyHC is the first detectable MyHC in newly-formed primary and secondary fibers (Fig. 6 *a*). Its expression persists in most fibers throughout embryonic development, ceasing shortly after birth (Webster et al., 1988*b*). Embryonic MyHC is clearly detectable in primary fibers as

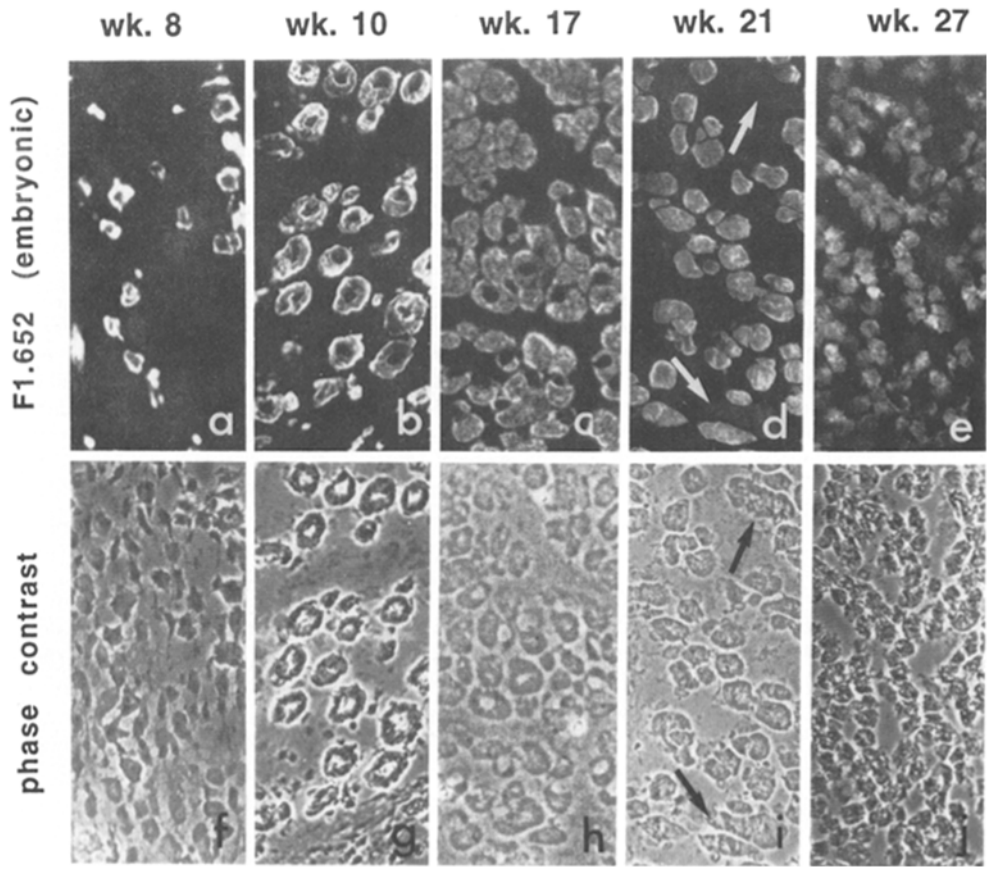

Figure 6. Embryonic MyHC is expressed in all primary and secondary fibers during gestation. Transverse sections of human limb muscle at weeks 8 (*a*, *f*), 10 (*b*, *g*), 17 (*c*, *h*), 21 (*d*, *i*), and 27 (*e*, *j*) of gestation were reacted with antibody F1.652 (*a–e*) followed by an FITC-conjugated goat anti-mouse IgG second antibody. Phase contrast micrographs (*f–j*) are the same fields shown in immunofluorescence (*a–e*) and in Fig. 2. Arrows indicate the same primary fibers in (*d*) and (*i*) which no longer express embryonic MyHC by week 21. Bar, 100 μm.

early as week 6 (data not shown) and week 8 of gestation (Fig. 6 *a*) and thereafter in all muscle fibers (both primary and secondary), a pattern that continues throughout the first half of human gestation (Fig. 6, *a–c*). Embryonic MyHC expression declines progressively first in large primary fibers in midgestation (Fig. 6 *d*) and the overall staining intensity with F1.652 begins to decline in late gestation (Webster et al., 1988*b*). Embryonic MyHC is no longer detectable in any muscle fibers by 2 wk after birth (Webster et al., 1988*b*). These data corroborate the results obtained from a time course of tissue samples analyzed by Western blotting. The F1.652 antibody therefore serves as a marker of early MyHC expression in both primary and secondary fibers.

N3.36 Recognizes Neonatal MyHC Present in Secondary but Not Primary Fibers. mAb N3.36 recognizes neonatal MyHC in developing human muscle (Fig. 5). First, it reacts exclusively with a 220-kD protein in whole-cell lysates of cultured human myotubes (Silberstein et al., 1986). Second, analysis of N3.36 reactivity in Western blots of high-resolution gels showed that it is specific for a neonatal-specific MyHC which is of mobility intermediate between adult type I and IIa MyHCs. Western blots of tissue samples on denaturing gels show reactivity of N3.36 with neonatal and adult stages, but not fetal, whereas F1.652 is specific for fetal stages (Silberstein et al., 1986). However, N3.36 recognizes a MyHC that is distinct from embryonic MyHC because, unlike F1.652, it does not recognize MyHC as early as week 14 of gestation.[2] Third, the reactivity of N3.36 with polypeptides encoded by MyHC cDNAs revealed that the antibody recognizes an MyHC encoded by the neonatal (perinatal) MyHC cDNA[2] (pSMHCP; Feghali and Leinwand, 1989; Karsch-Mizrachi et al., 1990). A probe to the 5′ half of neonatal MyHC cDNA detects mRNA by RNase protection in muscle at midgestation (week 21) but not in the adult. This timecourse of mRNA expression parallels the timecourse observed for N3.36-reactive proteins, with the exception that in adult muscle, N3.36 reacts with a bend migrating at the type IIa position. These data suggest that N3.36 recognizes an epitope present on at least two MyHC isoforms: a neonatal MyHC in developing muscle which is first expressed in midgestation and corresponds to the perinatal cDNA, and an adult MyHC which is expressed only in adult muscle. It should be noted that, as shown in Fig. 5, only the neonatal isoform is recognized by N3.36 in extracts of cultured human muscle cells. For the purposes of the studies described here which use cultured cells, N3.36 can be considered as neonatal-specific and F1.652 as embryonic-specific.

N3.36 recognizes MyHCs only in secondary, fast fibers (Fig. 7, *a–c*). Secondary fibers form around week 9 of human gestation and can be distinguished from primary fibers by their smaller size until week 30 of gestation (Wohlfart, 1937; Fig. 7, *a–c*); thereafter, primary and secondary fibers are essentially equivalent in size. In addition, reactivity of primary fibers with a mAb to slow MyHC, A4.951, distinguishes them from newly-formed secondary fibers (Fig. 7, *d–f*). These fibers are not recognized by antibody N3.36. Neonatal MyHC is not apparent by immunofluorescence analysis of cryostat sections at week 12 of gestation, but is prevalent by week 21 (Fig. 7). Thus, embryonic MyHC precedes neona-

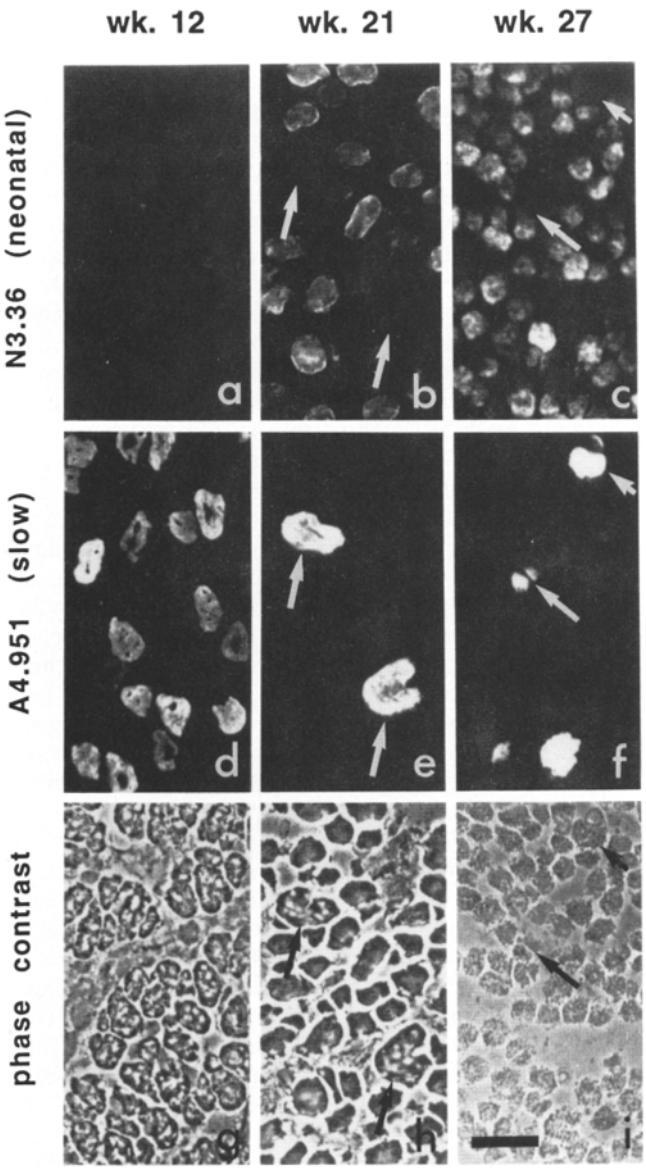

Figure 7. Neonatal MyHC is expressed only in secondary fibers. Transverse sections of human limb muscle at weeks 12 (*a*, *d*, *g*), 21 (*b*, *e*, *h*), or 27 (*c*, *f*, *i*) of gestation were analyzed by double-immunofluorescence: reaction with both antibody N3.36 (*a–c*) to neonatal MyHC followed by a rhodamine-conjugated goat anti-mouse IgM second antibody and with antibody A4.951 (*d–f*) to slow MyHC followed by an FITC-conjugated goat anti-mouse IgG second antibody. Phase contrast micrographs of the same fields shown in *a–c* and *d–f* are shown in *g–i*. Arrows in *b*, *e*, and *h* indicate the same primary fibers, which express slow but not neonatal MyHC. Short arrows in *c*, *f*, and *i* indicate a primary fiber, whereas the long arrows indicate a secondary fiber. Both fibers express slow MyHC but not neonatal MyHC. Bar, 100 μm.

tal MyHC in secondary fibers. By week 27 of gestation, neonatal MyHC is present in all fast fibers, but absent in fibers which contain slow MyHC (Fig. 7 *c*). In adult muscle, N3.36 reacts very weakly with fibers that contain fast ATPase activity (Silberstein et al., 1986; Webster et al., 1988*b*). These results are consistent with those obtained by Western blotting and suggest that a neonatal MyHC is expressed only in secondary, fast fibers of developing muscle. Thus, N3.36 serves as a useful marker of MyHC specific to secondary, but not primary, fibers.

In summary, antibodies F1.652 and N3.36 recognize embryonic and neonatal MyHCs that are developmentally regulated and differentially expressed in primary and secondary fibers. By contrast, F1.188 serves as a control to indicate the presence of sarcomeric MyHC in muscle cultures and tissues. Thus, F1.652 serves as a marker of early fibers, whereas N3.36 is specific to later stages of fiber formation.

Evidence for Heterogeneity: Myoblasts from Early and Late Embryos Express Different Developmentally Regulated MyHCs. We determined whether the myoblasts present when primary and secondary fibers are forming differ in their potential to express developmentally regulated MyHCs. Myoblasts were isolated from muscle tissues at 7 wk (early primary fiber formation) and 9 wk (early secondary fiber formation) of gestation and compared with myoblasts from muscle at four later stages of development (16 and 22 wk of gestation, 2 mo and 2.5 yr after birth). The cells were plated at low density and the progeny of each cell were followed as distinct clones. Individual clones were allowed to differentiate to form multinucleated myotubes for up to 4 wk in FM. Between 150 and 450 individual colonies were analyzed for each of the six developmental stages. Colonies were identified as myogenic by their synthesis of MyHC using antibody F1.188 and assessed for their expression of developmentally regulated embryonic or neonatal MyHC isoforms by double immunofluorescence with antibodies F1.652 or N3.36.

A marked difference in MyHC expression was observed between myoblasts isolated from the two earliest stages of muscle development (Fig. 8). All myogenic clones at week 7 (total of 165 clones) expressed embryonic MyHC after 4 wk of differentiation in culture, but none expressed the neonatal isoform. By contrast, all myogenic clones from week 9 (total of 393 clones) or later stages (total of 1,109 clones) expressed both the neonatal and embryonic MyHCs. These results were consistent for clones isolated from muscle tissues of three different individuals at week 7 and week 9, respectively (total of 938 clones). Because the results for myoblasts from tissue at the four later stages were similar, only data for myoblasts from the latest, 2.5 yr. postnatal muscle, are shown. They do not differ from those obtained from week 9 clones, except that the lag period before the onset of neonatal MyHC expression is shortened. These results suggest that myoblasts differ in their intrinsic potential to induce expression of a developmentally-regulated gene.

Week 7 and week 9 clones also differed in their ability to turn off gene expression. Week 7 myotubes did not induce neonatal MyHC expression, and continued to express embryonic MyHC, even after 4 wk in FM (Fig. 9, *a* and *b*). By contrast, a reciprocal relationship was observed in cells from week 9: those myotubes that initiated expression of neonatal MyHC ceased to express the embryonic isoform (Fig. 9, *c–h*). This difference was apparent when the myotubes within clones were analyzed. A clone could be positive for both MyHCs, but some myotubes were N3.36+ whereas other myotubes were positive for F1.652; at late time points in culture, these MyHCs were generally not co-expressed. That week 7 myotubes continue to express embryonic MyHC and do not induce neonatal MyHC is not due to their failure to thrive; these myotubes persist for as many weeks as my-

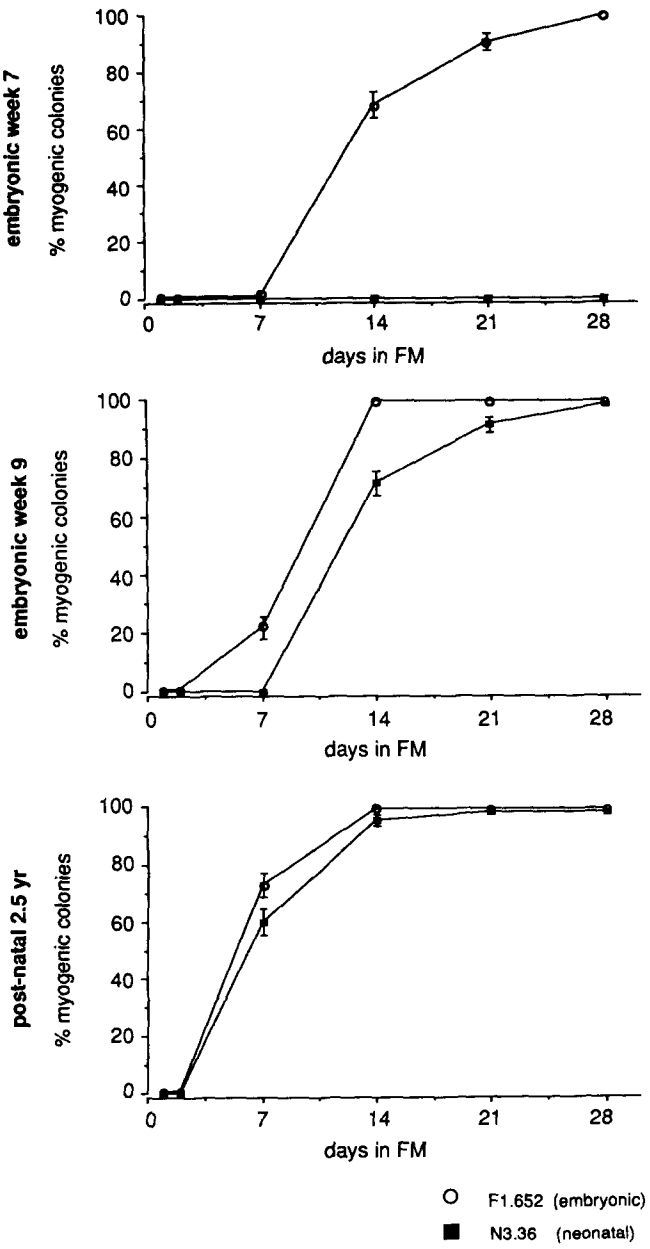

Figure 8. Time course of embryonic and neonatal MyHC expression in cultured human muscle cell colonies. Colonies were derived from myoblasts in muscle tissues at embryonic week 7 (n = 165), week 9 (n = 393), or 2.5 yr postnatal (n = 452) where n = number of colonies at each stage scored as myogenic, based on reactivity with antibody F1.188. Myoblasts were plated at clonal density in growth medium (GM). Colonies were grown to ~1,000 cells each, and then switched to a low-serum fusion medium (*FM*) to induce differentiation. The percent of myogenic colonies that expressed embryonic (F1.652) or neonatal (N3.36) MyHC was determined by double-immunofluorescence. A colony was scored as positive for a given antibody if five or more myotubes exhibited a signal above background. Error bars were calculated as the standard error of the proportion. The results at each time point (weeks 7 and 9 of gestation) are representative of three muscle samples from three different individuals.

otubes from older stages and are capable of inducing expression of other MyHCs, for instance, slow MyHC. Thus, myoblasts differ in their intrinsic potential not only to express but also to repress a developmentally regulated gene upon differentiation.

The switch from embryonic to neonatal MyHC expression in week 9 cultures represents a developmental progression in MyHC isoform expression, as illustrated by the following three observations. First, the overall intensity of staining with the F1.652 antibody at late time points was reduced relative to that in colonies at early time points. Second, an analysis of the proportion of individual myotubes within a colony expressing embryonic or neonatal MyHC revealed a gradual replacement of the embryonic by the neonatal isoform. Accordingly, most (90%) of myotubes within a myogenic colony from week 9 expressed only embryonic MyHC after 1 wk in FM, a mixture of embryonic and neonatal MyHCs at 2–3 wk, and only neonatal MyHC by 4 wk (data not shown). However, a few myotubes in each colony could be seen that express embryonic MyHC even after 28 d in culture. These were presumably the last to form, because they were generally smaller in size and located at the periphery of the colony. This heterogeneity among myotubes within a given clone explains why, in Fig. 8, clones derived from week 9 or later stages appear to continue to express embryonic MyHC in culture; in this analysis, clones were scored as positive for a specific MyHC if they contained five or more myotubes that expressed detectable levels of that MyHC by immunofluorescence. By contrast, in previous studies with adult-derived C2C12 mouse muscle clones, all myotubes eventually replaced embryonic with neonatal MyHC (Silberstein et al., 1986). In these experiments, cytosine arabinoside was used to inhibit myoblast proliferation and de novo myotube formation at the periphery of colonies. The use of an inhibitor of DNA synthesis was precluded here, because it is toxic to embryonic muscle cells. Thus, week 9, but not week 7, cultures sequentially express two developmentally distinct MyHCs, eventually replacing embryonic with neonatal MyHCs.

We conclude from these studies that human muscle colonies from week 7 differ from their later counterparts in at least two aspects of gene regulation: early cells express embryonic MyHC continuously and do not switch on expression of neonatal MyHC.

Extrinsic Factors: No Effect on Intrinsic Potential of Myoblasts in Culture. To rule out the possibility that the developmental differences in isoform expression arose because the myoblasts from very early embryos were more sensitive to culture at the low density required for clonal analysis, we plated cells at high density. In these experiments, cells plated at 1,000 cells/cm^2, and grown to confluence were compared with those plated at a clonal density of 1 cell/cm^2. For clones of both week 7 and week 9 samples, differentiation of myotubes and detection of MyHC by antibody F1.188 was apparent three to 4 d earlier in the high density cultures than in low density cultures. In addition, the progression from embryonic to neonatal MyHC was accelerated twofold in the week 9 samples. By contrast, in spite of their precocious differentiation, week 7 samples strongly expressed embryonic MyHC for the 20 d duration of the cultures in FM and neonatal MyHC was not induced. Thus, the inability of week

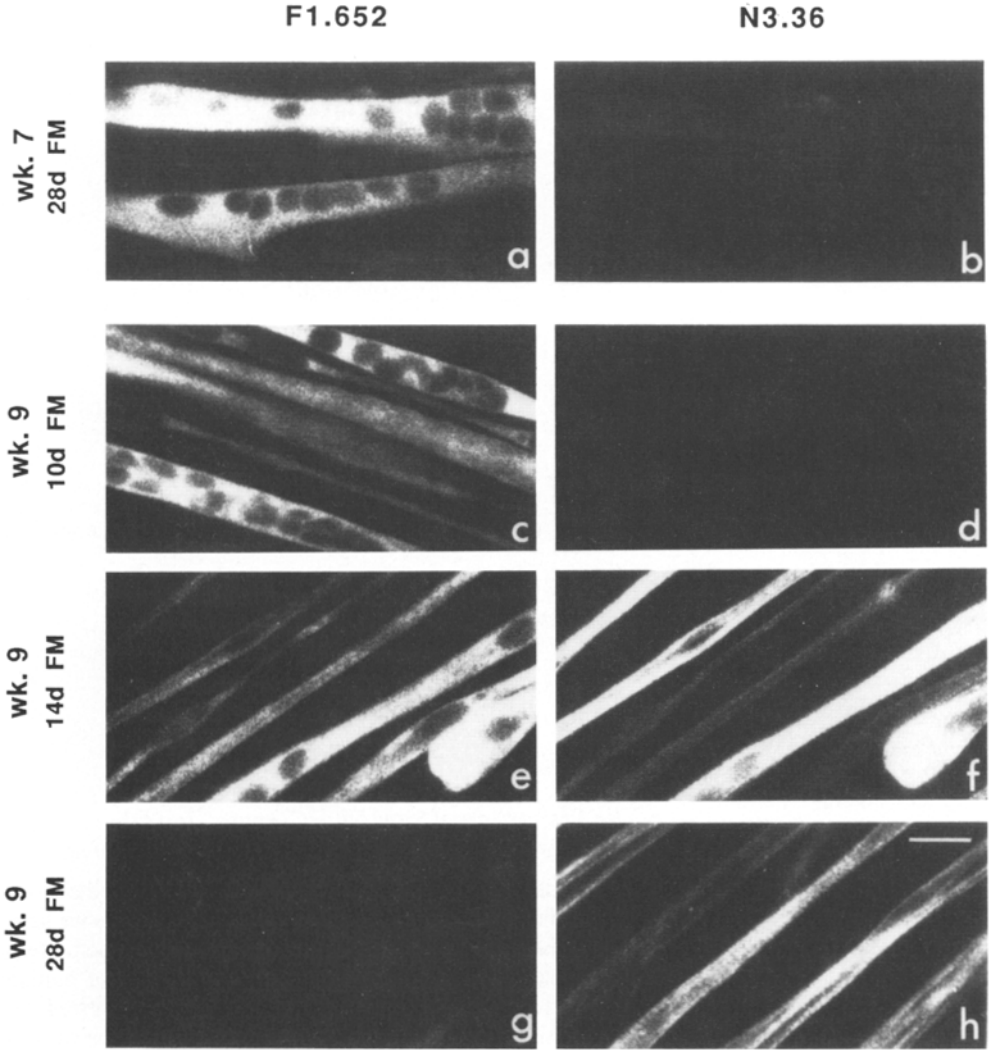

Figure 9. Developmental progression from embryonic to neonatal MyHC expression occurs only in late-stage human muscle cells. Colonies were derived from tissues of embryonic week 7 (*a*, *b*), and week 9 (*c–h*) as described in the legend to Fig. 4. They were analyzed simultaneously for expression of embryonic MyHC (F1.652-reactive: *a*, *c*, *e*, *g*) and neonatal MyHC (N3.36-reactive: *b*, *d*, *f*, *h*) by double-immunofluorescence at 10 (*c*, *d*), 14 (*e*, *f*), or 28 (*a*, *b*, *g*, *h*) days after switching to FM. Left and right panels are the same fields in each case. Bar, 30 μm. The myotubes in *g* have lost F1.652 reactivity and are typical of most mature myotubes at the center of each week 9 myogenic colony. By contrast, the edges of each colony contained many (at least five) less mature myotubes which still reacted with F1.652. As a result, the data in Fig. 5 do not show the decline in expression of embryonic MyHC with time shown here. Striations are not visible in the myotubes stained with antibodies to embryonic MyHC, because this isoform is overwhelmingly predominant in these cells and the resulting fluorescent staining of this MyHC is very intense. (See Fig. 4 for visualization of striations in similar cultures, using antibodies to slow MyHC.)

7 cultures to express neonatal MyHC was not altered by accelerated differentiation at higher plating density (Table II).

To determine whether factors in the culture medium were responsible for inducing the changes in isoform expression in week 9 cultures, cells were differentiated in a defined medium. For this purpose, tissues were dissociated and plated at high density, grown to confluence in GM, and then differentiated either as usual in FM containing chick embryo extract and horse serum or in a serum-free medium consisting of DME with insulin (10^{-6} M) and dexamethasone (10^{-6} M) for ~2 wk. Differentiation and expression of MyHC occurred several days earlier in defined medium than in complete medium, indicating that undefined humoral factors in the media are not the basis for neonatal MyHC induction in week 9 samples (Table II). Week 7 cells could not be analyzed because they detached from the culture dishes within one week of culture in serum-free medium.

The possibility remained that the inability of myogenic cells in week 7 cultures to express neonatal MyHC resulted from an inhibitory factor in those cultures provided, for example, by the nonmyogenic cells. The proportion of clones that were myogenic in week 7 cultures was ~30% compared to 50% in week 9 cultures, determined by morphological evidence of myotube formation and detection of MyHC with antibody F1.188. We designed experiments to test whether the nonmyogenic clones contributed factors that suppressed the expression of neonatal MyHC in myogenic clones. For this purpose, cells from week 7 were mixed with cells from week 9 either at a ratio of 10:1 or 5:1 and plated at clonal density. Colonies were grown in GM, switched to FM, and scored for expression of neonatal MyHC after 21 and 28 d in FM. The proportion of myogenic clones that expressed neonatal MyHC at these two ratios was 41% (43/106) and 48% (43/90), respectively. Because in these mixed cultures, week 9 myogenic clones should comprise 14 and 30% of the total clones, respectively, it appears that the lack of expression of neonatal MyHC is not due to an inhibition by the presence of week 7 nonmyogenic cells in the cultures. To show definitively that a positive factor contributed by week 9 cultures induced week 7 cultures to express neonatal MyHC

Table II. Extrinsic Factors and Cell Division Do Not Induce Expression of Neonatal MyHC in Embryonic Week 7 Muscle Cultures

Extrinsic factors	Week 7 Sample	Week 7 % N3.36 positive	Week 9 Sample	Week 9 % N3.36 positive
Clonal cultures, differentiated in FM* (1 cell/cm²)	sample 1‡: 165 sample 2: 87 sample 3: 64 total: 316 colonies	0%	sample 1: 393 sample 2: 189 sample 3: 40 total: 622 colonies	100%
High density cultures differentiated in FM* (10^3 cells/cm²)		0%		100%
High density cultures, in defined medium‖ (10^3 cells/cm²)		ND§		100%
Cell divisions: serial subcloning	Five serially-passaged subclones (25 doublings each)	0%	Three serially-passaged subclones (25 doublings each)	100%

* Fusion medium (DME, 5% horse serum, 10^{-6} M insulin, 10^{-6} M dexamethasone).
‡ Data for Fig. 4 were obtained from sample 1 of each stage; as shown here, similar results were obtained from clonal cultures derived from two additional samples from week 7 and week 9 each (samples 2 and 3 from each stage).
§ Not determined.
‖ Same as in * but without horse serum.

will require additional experimentation in which cells are labeled so that the week 7 and week 9 myoblasts can be distinguished and the plating efficiency of the two cell types determined to be equal.

Thus, the pattern of MyHCs expressed by myoblasts that differentiate in culture mimics that of the stage of development in the intact organism from which they were derived, irrespective of extrinsic cues provided by media components, cell density or culture composition.

Expression Patterns of MyHC Are Stably Heritable. Two possible explanations for the differences between myoblasts from week 7 and week 9 muscle are (*a*) two independent myoblast populations arise at different times, or (*b*) the early population gives rise to the later one. If week 7 myoblasts gave rise to week 9 myoblasts, this could be in response to either an environmental cue or to a cell-intrinsic division-dependent maturation. To test the possibility that commitment to a particular pattern of MyHC expression was under control of an intrinsic mechanism, myoblasts from week 7 and from week 9 muscle were serially subcloned in culture until they had undergone a minimum of 25 population doublings in GM. The cells were then induced to differentiate by exposure to FM. As the cell cycle for human embryonic myoblasts is ~24 h, this period is nearly equivalent to 4 wk, twice the difference in gestational age at the time of myoblast isolation. The five serially passaged clones analyzed from week 7, like the parental clones from which they were derived, expressed embryonic, but no neonatal MyHC (Table II). The three serially passaged clones from week 9 muscle, on the other hand, all expressed neonatal MyHC. From these results it seems likely that the expression of neonatal MyHC is not under the control of a division-dependent mechanism, or intrinsic "clock".

Discussion

The Proportion of Slow MyHC-expressing Fibers Changes Markedly During Embryonic Development

During development, tissues arise that are composed of multiple cell types distributed in complex patterns. Here we have addressed one possible mechanism for achieving the pattern of diverse muscle cells, or fibers, characteristic of human skeletal muscle of the limb. Specifically, we have tested the hypothesis that the heterogeneity of muscle fibers specialized for fast or slow contractions is intrinsically determined at the level of the myoblasts, as suggested by Miller and Stockdale (1986*a*,*b*).

During human gestation, the percentage of total fibers in muscle tissue that are slow fibers changes markedly from ~75 to 3 to 50%. This is due to the progression of events underlying muscle development. The early, or primary population of fibers is homogenous with respect to both onset and continued expression of slow MyHC. At a time when the vast majority primary fibers express slow MyHC, a population of secondary fibers arises, which does not express slow MyHC. Indeed, secondary fibers fail to express slow MyHC for the entire second trimester of gestation (for >12 wk). At the beginning of the third trimester, slow MyHC begins to be detected in a small subset of secondary fibers. From this time onward, the increase in the number of slow fibers is extremely rapid, reaching adult proportions in only three weeks. Because this increase cannot be accounted for by de novo formation of fibers, it appears that a fiber-type transition is occurring in a preexisting set of secondary fibers. These results in human muscle are in agreement with those in rat (Narusawa et al., 1987) which show that slow

MyHC expression in mammals occurs in two phases: the first phase occurring early in primary fiber development and the second phase occurring late in secondary fiber development.

The Intrinsic Potential of Myoblasts to Express Slow MyHC Is Suppressed In Vivo

The failure of secondary fibers to express slow MyHC for months after their formation suggested to us that these fibers might differ inherently from their primary counterparts. Because fast and slow myoblast lineages had been reported by others (Stockdale et al., 1988), we tested the possibility that the myoblasts that form primary and secondary fibers differ intrinsically in their commitment to a program of slow MyHC expression.

The results were striking: virtually all myoblasts, irrespective of the stage of development from which they were derived, gave rise to clones that expressed slow MyHC upon differentiation in culture. This was true even for myoblasts from muscle at midgestation, a time when only 3% of fibers expressed slow MyHC. These results suggest that culture conditions are permissive for an inherent ability of myoblasts from all developmental stages to express slow MyHC. By contrast, in vivo, this expression must be suppressed. Humoral factors found in media are not likely to be responsible for the expression of slow MyHC in the cultures. Elimination of serum from the medium did not affect the phenotype of myoblasts cultured from either embryonic or adult tissues. Instead, the lack of expression of slow MyHC in vivo is likely to reflect a specific suppression in secondary fibers.

Reconciliation of Apparent Species Differences in Slow MyHC Expression

Our results appear to differ markedly from those previously reported by others, however, these differences can be readily reconciled and explained by a single unifying principle, as described below. First, human myoblasts differ from avian myoblasts in tissue culture in that an early population of human myoblasts is homogeneous with respect to the potential to express slow MyHC, whereas chicken myoblasts are heterogeneous (Miller and Stockdale, 1986*a*,*b*). This distinction is also reflected in the primary fibers of intact muscles of the organism. Avian muscles contain both slow twitch (type I) and slow tonic (type III) fibers. Slow tonic fibers retain multiple innervation and exhibit graded contractions, in contrast to the monoinnervation and all-or-nothing contractile response of both fast and slow twitch fibers in mammals (for review see Schmalbruch, 1985). Indeed, in zebra finch muscles, an antibody that recognizes the SM2 slow myosin of chicken reacts only with fibers that have multiple terminals (Bleisch et al., 1989). Thus, it may be that the expression of the slow isoform SM2 by early but not late chicken myoblasts represents additional fiber-type heterogeneity typical of avian but not mammalian muscle.

Second, in spite of the differences among species in the types of slow fibers they contain, similar rules appear to govern fast and slow fiber type development. At first, it appears that the existence of multiple slow MyHC isoforms in the chicken precludes a direct comparison of clonal analyses in chicken and human. Early results from chicken muscle cells (Miller and Stockdale, 1986*a*,*b*) showed that the slow myosin isoform SM2 was expressed by early myoblasts but not late myoblasts and that a similar expression pattern was found in muscle fibers from corresponding stages in vivo. As a result, it was proposed that distinct lineages of myoblasts gave rise to fast and slow fiber types. However, more recent studies of chicken myoblasts (Miller and Stockdale, 1989) that used a different mAb to analyze expression of another slow myosin isoform of chicken muscle, SM1, are in good agreement with our results. These studies show that SM1 is expressed by both early and late myoblasts in culture and in the corresponding fibers in vivo and therefore that slow MyHC per se does not distinguish early and late myoblast populations. Experiments in mouse muscle that demonstrate expression of slow MyHC in early- but not late-type myoblasts (Vivarelli et al., 1988) could also be explained by the presence of multiple slow isoforms in this species. Indeed, results in our laboratory suggest that this is the case (Hughes et al., 1993). A comparison of A4.951 with two other antibodies specific to slow MyHC (A4.840 and N2.261) in rodent and in human muscle revealed that although A4.951 is expressed at all stages of development, two other slow MyHCs are sequentially expressed in the same fibers at later stages of development. Taken together, these results indicate that myoblasts may be heterogeneous with respect to expression of developmentally-regulated MyHC isoforms, but that these differences do not necessarily give rise to functional distinctions specific to fast and slow fiber types.

Neuronal Factors May Be Permissive for Fiber-type Determination

Our studies suggest that slow MyHC expression is actively repressed in vivo. This interpretation is also supported by data obtained from chicken muscle. Clonal analysis of early chick myoblasts in culture showed that, of the colonies that expressed slow MyHCs, none expressed the SM1 isoform without also expressing SM2 (Miller and Stockdale, 1989). In contrast, previous studies in vivo detected the SM1 isoform in the absence of SM2 in primary fibers (Stockdale et al., 1988). These results are consistent with our interpretation that a specific suppression of slow myosin is occurring in vivo, in this case SM2, a suppression similar to that which we observed in secondary fibers in human muscle.

Innervation has long been known to play a role in fiber-type determination, but whether this role is permissive or instructive has not always been clear. There is strong evidence from experiments in rodent and avian muscle that early primary fiber type determination is not neuronally controlled (Phillips and Bennett, 1984; Crow and Stockdale, 1986; Phillips et al., 1986; Sohal and Sickles, 1986; Narusawa et al., 1987; Weydert et al., 1987; Condon et al., 1990*b*). However, innervation is thought to be responsible for regulating slow MyHC expression in secondary fibers (Crow and Stockdale, 1986). Indeed, the appearance of slow MyHC in secondary fibers of most muscles of the rat hindlimb has been recently shown to be dependent on innervation (Condon et al., 1990*b*).

This apparent nerve-dependence of slow MyHC expression in vivo might seem to conflict with our findings that expression of slow MyHC is intrinsic to myoblasts from all stages of development. However, nerves could play a permissive rather than an instructive role in the regulation of slow

MyHC. According to this view, innervation might serve to relieve fibers from factors in vivo which partially suppress the program of MyHC expression intrinsic to the myoblast. The following observation lends support to our hypothesis that neural signals modulate the expression of slow MyHC but that positional information from the environment dictates the specific response of a myofiber to those signals. Investigation of the role of nerves in the regulation of slow MyHC in neonatal and embryonic rats showed that the response to denervation varied with the position of a fiber within the leg (Narusawa et al., 1987; Condon et al., 1990*b*). In general, deep regions of muscle lost expression of slow MyHC whereas other regions retained or increased slow MyHC expression. Our finding that 100% of the human myoblasts tested as clones in culture had the potential to express slow MyHC, taken together with the denervation studies, suggest that myoblast-extrinsic factors which are non-neuronal yet region-specific play a role in determining the pattern of fiber type in the developing muscle.

At later stages of development (mid- to late-gestation), the fast/slow phenotype of preexisting multinucleated fibers is likely to dominate over the developmental potential of the myoblasts which fuse into them (Hughes and Blau, 1992). Thus, the heterokaryon environment of the fibers is probably the most influential extrinsic influence at this stage. However, fusion of myoblasts into preexisting fibers cannot explain the phenotype of the earliest-forming fibers. The earliest-forming secondary fibers are fast, yet begin to be generated at a stage (week 10 of gestation) when all myoblasts have the intrinsic potential to express slow MyHC. Thus, neither fusion into preexisting fibers nor intrinsic potential alone can explain the control of expression of MyHCs in the early stages of fiber development. Instead, environmental cues could be provided by neighboring slow primary fibers, nonmyogenic cells, nerves or hormones.

Early and Late Myoblasts Express Different MyHCs

Although we found that myoblasts at all stages of development tested could express slow MyHC, the same myoblasts differed in their ability to express a protein specific to secondary fibers, neonatal MyHC. Each myogenic clone from week 9 muscle, a stage when secondary fiber formation is just beginning, was capable of sequentially expressing first the embryonic MyHC and then the neonatal MyHC. An analysis of the myotubes within clones revealed that this sequence reflects a progressive replacement of the early by the late isoform. In contrast, each myogenic clone of the total of 316 clones analyzed from week 7 muscle tissue of three different individuals (Table I), at a stage when primary fibers are forming, was capable of embryonic MyHC expression but not neonatal MyHC, a pattern which persisted for 4 wk of cultivation in differentiation medium. Thus, the populations of cells at these two stages of development clearly differ intrinsically.

Previous studies by others suggested that myoblasts might be heterogeneous at different stages of development. Hauschka and co-workers (Hauschka, 1974) defined developmentally distinct populations of chicken and human myoblasts that differed in their morphology and medium requirements (Bonner and Hauschka, 1974; White et al., 1975). Muscle colonies from early-stage embryos differentiated to form small myotubes and required conditioned medium, whereas colonies from later stage embryos formed large myotubes containing hundreds of nuclei in the absence of conditioned medium. Cossu et al. (1985) showed differences in response to factors added to media: Human myoblasts from gestational weeks 6–8 were prevented from differentiating by exposure to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), whereas myoblasts from week 10 and later stages gradually acquired resistance to TPA during development. Here we show that myoblasts from two different developmental stages differ biochemically and that these stages correlate with periods when primary and secondary fibers are forming. Our results demonstrate that the myoblasts that give rise to primary and secondary fibers differ in their potential to express developmentally regulated genes encoding distinct MyHCs. This is in agreement with results obtained with chick, quail (Mouly et al., 1987), mouse (Smith and Miller, 1992) and rat (Song et al., 1991) myoblasts which indicate that early and late myoblasts differ in their expression of several muscle-specific proteins once they differentiate. By contrast, as shown here, all myoblasts appear to be able to express a "fiber type"-specific, slow MyHC after differentiation.

Control of Switch in Potential to Express Neonatal MyHC

We designed experiments to examine the prerequisites for the isoform switch observed in week 9 cultures. To determine whether extrinsic factors in the differentiation medium containing chick embryo extract and serum were responsible for the shift in MyHC expression, we analyzed the clones after differentiation in a defined medium. These experiments showed that week 9 myogenic clones were able to express neonatal MyHC even when the only humoral factors provided were insulin and dexamethasone. To determine whether cell density was critical to the expression of neonatal MyHC, cultures were grown at high density. It seemed possible that week 7 cultures did not undergo the developmentally regulated isoform transition simply because the clones were smaller and sparser than those derived from week 9 tissues. When plated as mass cultures and grown to confluence, week 7 cultures exhibited accelerated differentiation relative to clonal cultures. However, in spite of the precocious appearance of MyHC, the embryonic isoform persisted and was not replaced by the neonatal isoform after 20 d in differentiation medium. Thus, the expression of the neonatal isoform was not influenced by undefined serum components or cell density.

Paracrine factors could be responsible for preventing or promoting maturation. To explore this possibility, we analyzed cocultures of week 7 and week 9 clones. We reasoned that the lack of maturation and expression of neonatal MyHC by the early stage myogenic clones could be due to the secretion and accumulation of inhibitory factors in the medium, as the proportion of nonmyogenic clones in week 7 cultures was higher (50%) than in week 9 clonal cultures (30%). Accordingly, we examined whether week 9 myoblasts could be prevented from maturing by the presence of nonmyogenic cells, presumably fibroblasts. However, when week 9 myogenic clones were plated under conditions where they were greatly outnumbered by week 7 nonmyogenic clones, they still underwent their program of embryonic to neonatal MyHC isoform transition. These experiments suggest that inhibitory factors are not responsible for the lack of matura-

tion, but do not rule out the possibility that a dose-dependent maturation-promoting factor is required. To address these possibilities definitively, independent methods must be developed for labeling and distinguishing individual clones in week 7 and week 9 cocultures.

Generation of Two Myoblast Types

The complete change in the ability to express neonatal MyHC that occurs in the clonable myogenic population between week 7 and week 9 of human gestation is striking. This finding suggests that a relatively synchronous change occurred in the entire pool of undifferentiated muscle cells during this two week period. Such a change might either be signaled by an extrinsic environmental cue or be part of an intrinsic program inherent to each myoblast. Both mechanisms have been elegantly demonstrated to contribute to the generation of oligodendrocytes and type-2 astrocytes from 0-2A progenitor cells during the development of the rat optic nerve. The differentiation of the bipotential glial progenitor into oligodendrocytes is timed by a biological clock intrinsic to the progenitor cell (Raff et al., 1985; Temple and Raff, 1986). However, an extrinsic factor (Richardson et al., 1988) is necessary to drive this clock. In contrast, the decision to differentiate into type 2 astrocytes is not intrinsic to the progenitor cell, but is apparently controlled by an inducing factor, ciliary neurotrophic factor (Hughes et al., 1988). Cell-intrinsic mechanisms also were found to play a role in the generation of cell diversity in erythroid cells. Fetal and adult erythropoietic stem cells differ in their potential to express developmental stage-specific hemoglobins (Papayannopoulou et al., 1977, 1979). Experiments in which stem cells of fetal sheep were transplanted to an adult animal demonstrated that the pattern of hemoglobin production was determined by the gestational ages of the fetal donor cells, suggesting that the control of fetal to adult globin switching was cell-autonomous (Wood et al., 1985).

We tested the hypothesis that a cell-intrinsic mechanism controlled the switch from early- to late-type myoblasts. To this end, early myoblasts were propagated for at least 25 doublings in culture over a period of more than 3 wk, a period twofold longer than that during which the myoblast population changed in vivo, yet a progression to neonatal MyHC was still not observed in FM. Thus, it appears unlikely that a division-dependent clock controls the conversion of the week 7 to the week 9 myoblast phenotype; instead environmental cues may be critical.

One possible extrinsic cue that could convert the early to late myoblasts is innervation, which begins during secondary myogenesis (Fidzianska, 1980*b*). However, morphogenic factors produced locally in the tissue or blood-borne factors could also play a role. An alternate possibility which cannot be ruled out is that week 7 myoblasts do not convert to week 9 type myoblasts, but are replaced by them. In this case, at week 7 the late-stage myoblasts would comprise <1% of the total clonable population, whereas by week 9 it would have grown to comprise 99%. A possible mechanism for this replacement might involve differential responsiveness between the early and late myoblast populations to growth and differentiation factors present in the organism at these stages. The disappearance of week 7 myoblasts may be due to apoptosis, a mechanism for specific elimination of cells which has been shown to occur in a diversity of tissues (Raff, 1992). We, like White et al. (1975 and Cossu et al. (1985), observed that week 7 myotubes do not survive under serum-free conditions, indicating that expression of neonatal MyHC may not be the only feature distinguishing week 7 and week 9 cells. It should now be possible using retroviral marking techniques (Hughes and Blau, 1990) to distinguish among these possibilities and determine the cellular origin of primary and secondary fibers in vivo.

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