Slow and Fast Myosin Heavy Chain Content Defines Three Types of Myotubes in Early Muscle Cell Cultures

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ABSTRACT We prepared monoclonal antibodies specific for fast or slow classes of myosin heavy chain isoforms in the chicken and used them to probe myosin expression in cultures of myotubes derived from embryonic chicken myoblasts. Myosin heavy chain expression was assayed by gel electrophoresis and immunoblotting of extracted myosin and by immunostaining of cultures of myotubes. Myotubes that formed from embryonic day 5–6 pectoral myoblasts synthesized both a fast and a slow class of myosin heavy chain, which were electrophoretically and immunologically distinct, but only the fast class of myosin heavy chain was synthesized by myotubes that formed in cultures of embryonic day 8 or older myoblasts. Furthermore, three types of myotubes formed in cultures of embryonic day 5–6 myoblasts: one that contained only a fast myosin heavy chain, a second that contained only a slow myosin heavy chain, and a third that contained both a fast and a slow heavy chain. Myotubes that formed in cultures of embryonic day 8 or older myoblasts, however, were of a single type that synthesized only a fast class of myosin heavy chain. Regardless of whether myoblasts from embryonic day 6 pectoral muscle were cultured alone or mixed with an equal number of myoblasts from embryonic day 12 muscle, the number of myotubes that formed and contained a slow class of myosin was the same. These results demonstrate that the slow class of myosin heavy chain can be synthesized by myotubes formed in cell culture, and that three types of myotubes form in culture from pectoral muscle myoblasts that are isolated early in development, but only one type of myotube forms from older myoblasts; and they suggest that muscle fiber formation probably depends upon different populations of myoblasts that co-exist and remain distinct during myogenesis.

There are two major sets of biochemical differences in developing muscles and in muscle fibers. One set is found between physiologically fast and slow muscles. Fibers in these two muscle types contain different isoforms of myosin and other muscle-specific proteins, although both fibers and muscles are found with mixed properties. A second set is due to the appearance of isoforms specific to different developmental ages within a single muscle. For example, a sequence of myosin heavy chain and myosin light chain isoform expression occurs during the formation of all muscles (reviewed in reference 1). The developmental basis for the formation of different fiber types and sequential expression of isoforms in putative fast and slow fibers is not known, but fiber diversification has been postulated to result from either a single cell lineage or multiple cell lineages (2).

The sequential appearance of different isoforms at different stages of avian muscle development is found for a number of proteins, including the fast myosin heavy chain (3–9), myosin light chain (10–14), troponin (15), C-protein (16), tropomyosin (14, 17), and creatine kinase (18). Similar isoform transitions are found in mammals (19–22). One particularly well-studied isoform transition is that of the fast myosin heavy chains of the avian breast muscle. Three fast isoforms that appear sequentially during mid-embryonic to adult development of this muscle have been described (6, 9). The first isoform appears on or before embryonic day (ED)[1] 10 and is expressed through ED 18, and the second is expressed from ED 16 to several weeks after hatching. The first two isoforms

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[1] *Abbreviations used in this paper:* ALD, anterior latissimus dorsi; ED, embryonic day; McAb, monoclonal antibody; PM, pectoral muscle.

are shared by several muscles. The third isoform, which is apparently unique to adult pectoralis major, appears several weeks after hatching. Isoform transitions in the mid-embryonic to adult pectoral muscle (PM) are, therefore, well demonstrated. Biochemical differentiation of the PM during very early development (ED 4–6) is, however, comparatively unstudied.

As a step toward understanding differentiation in the early PM, we have studied myosin heavy chain expression in cultures of early PM cells. Myoblasts were isolated from the PM of embryos of different ages and allowed to form myotubes in culture. Myosin heavy chain expression was assessed using monoclonal antibodies to fast and slow classes of isoforms as probes in immunoblotting and immunocytochemical procedures. We found that, unlike the myotubes formed in cultures of ED 8 or older donors, cultures of pectoral myoblasts from ED 5–6 formed biochemically distinct populations of myotubes. Many of these myotubes contained the slow class of myosin heavy chain—an unexpected finding for cultured myotubes. The results show that cultured myoblasts can form myotubes that synthesize slow myosin heavy chain and maintain three different phenotypes in a single culture, and that these properties depend upon the age of the embryo from which the myoblasts are derived.

MATERIALS AND METHODS

Cell Culture: PM cells were isolated and cultured as described by O'Neill and Stockdale (23). The PM was identified and dissected from stage 27–28 (ED 5), stage 29 (ED 6), ED 8, and ED 12 embryos, minced, dissociated with trypsin, and plated at 2.5×10^4 cells/cm^2 on gelatin-coated dishes. Whereas it was easy to dissect the PM in ED 8 or older embryos, the PM in ED 5–6 embryos was recognized as a linear thickening on the forming chest wall and care was taken to avoid the inclusion of tissue from the trunk muscles, ribs, and upper wing during the dissection. Culture medium, 0.3 ml/cm^2, consisted of Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 2.5% chick embryo extract, penicillin, streptomycin, and fungizone. When used, conditioned medium was prepared from ED 12 PM cultures as described (24). Cultures of these cells contained multinucleated cells after 2–3 d. The number of contaminating cells could be reduced without changing the results by preplating the trypsin-treated cells for 30 min before initial culture and by treating the cultures with cytosine arabinoside at 10 μg/ml for 48 h beginning on the third day of culture.

Myosin Preparations: Myosin was purified from adult white Leghorn chicken by repeated cycles of high salt solubilization and low salt precipitation (25), and stored as described below. The predominantly fast PM and predominantly slow anterior latissimus dorsi (ALD) muscle were used as sources of myosin. Myosin was extracted from cultured myotubes after the cells were removed from culture dishes with trypsin and collected by centrifugation. The cell pellet was extracted on ice for 15 min with a buffer consisting of 0.6 M NaCl, 10 mM sodium phosphate, 1 mM sodium pyrophosphate, 0.5 mM MgCl$_2$, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5% Triton X-100, pH 6.8. The cell pellet was extracted with 1 ml of this buffer per 150 cm^2 of culture area. The extracted cells were centrifuged at 250 g for 10 min, and the supernatant was dialyzed in 500 ml of 5 mM KCl, 0.5 mM sodium phosphate, and 0.25 mM EGTA, pH 6.8, overnight at 4°C. Myosin was collected from the dialysate by centrifugation at 15,000 g for 2 min. The pellet was dissolved in 80 mM sodium pyrophosphate, 2 mM MgCl$_2$, and 2 mM EGTA at one-tenth of the extraction buffer volume, mixed with an equal volume of glycerol, and stored at −20°C until used. Protein was quantitated with the dye-binding assay of Bradford (26).

Monoclonal Antibodies: Monoclonal antibodies (McAb's) to chicken myosin heavy chains were prepared as described previously (13). BALB/c mice were immunized with myosin purified from adult PM (the source of McAb F59) or ED 19 upper leg muscle (the source of McAb's S58 and S46), and the spleen cells were hybridized with the myeloma cell line P3-NS1/1 Ag 4-1. Immunization, hybridoma formation, and selection were performed as described by Oi and Herzenberg (27). Hybridoma culture supernatants were initially screened with a solid-phase binding assay using purified myosin heavy chain fractions adsorbed to polyvinylchloride plates as the antigen (13). Hybridomas with positive supernatants were recloned by limiting dilution.

Immunocytochemistry and Microscopy: Fixed myotube cultures and cryostat sections (8-μm thick) of frozen, unfixed muscle were used to determine the myosin heavy chain content of individual cells. Cryostat sections were prepared as described (13) but the sections were not fixed. Endogenous peroxidase activity was blocked by a 10-min incubation of the sections in methanol and 3% H$_2$O$_2$ at room temperature. Myotube cultures were fixed for 5 min with 3.7% formaldehyde in phosphate-buffered saline (PBS) and for 5 min more in 100% ethanol. The procedure for myosin visualization was identical for sections and cultures. The samples were incubated in 2% bovine serum albumin (BSA), 2% horse serum in PBS for 30 min and then with a 1:10 dilution of the hybridoma supernatant in PBS-BSA-horse serum for 1 h. The cells were washed with several changes of PBS and incubated with biotinylated horse anti-mouse IgG at 5 μg/ml. Bound antibody was visualized with either an avidin-biotin horseradish peroxidase or an avidin-biotin-glucose oxidase complex as described by the manufacturer (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA).

For double-label immunofluorescence, McAb's F59 and S46 were purified from ascites fluid on DEAE Affi-gel blue columns (Bio-Rad Laboratories, Richmond, CA) as described (28). The purified McAb's were biotinylated by reaction with N-hydroxysuccinimidobiotin as described (29). Cultures were fixed as above and incubated overnight with biotinylated McAb S46 at 1 μg/ml in PBS-BSA-horse serum at 4°C. As was the case between each change of reagents, the cultures were washed with four changes of PBS. The cultures were then incubated with fluorescein-conjugated avidin (Vector Laboratories; Inc.) for 3 h at 25 μg/ml at room temperature, biotin for 30 min at 25 μg/ml, biotinylated McAb F59 for 1 h at 1 μg/ml, and rhodamine-conjugated avidin (Vector Laboratories, Inc.) for 1 h at 10 μg/ml. Control experiments showed that the biotin blocking step was sufficient to saturate biotin binding sites on the fluorescein-conjugated avidin, and also that the order in which the biotinylated S46 and F59 were added did not affect the results. The cultures were mounted for microscopy in PBS at pH 8.6 supplemented with 90% glycerol and 2.5% 1,4-diazabicyclo (2, 2, 2) octane to retard fluorescence bleaching (30). Observations were made with a Zeiss photomicroscope equipped for epifluorescence, and Kodak Tri-X film was used for photography.

Immunoelectrophoresis and Myosin Quantitation: SDS PAGE was performed as described by Laemmli (31) on 5% gels. Proteins were electrophoretically transferred to nitrocellulose as described by Burnette (32). Myosin heavy chain on the nitrocellulose transfer was detected by incubating the transfers for 1–2 h with hybridoma supernatants diluted 1:10 with 2% nonfat powdered milk (33) in PBS. The horseradish peroxidase- or glucose oxidase-linked systems described above were used to visualize McAb binding to myosin heavy chain.

A dot blot assay (34) was used to quantitate myosin isoforms in cultured myotubes. Myosin was extracted from cultures as above and diluted with 0.2 M NaCl, 5 mM HEPES, 10 mM 2-mercaptoethanol, and 1% SDS (dot blot buffer). Fast myosin heavy chain from the PM and slow myosin heavy chain from the ALD were extracted as above and subjected to SDS PAGE on 5% gels. The areas of the gel that contained myosin heavy chain were cut out, the myosin heavy chain was electroeluted, and protein was quantitated. These purified heavy chain preparations were diluted with dot blot buffer and used as standards in the assays. 20-μl samples were applied to nitrocellulose as serial 1:3 dilutions starting with 2 μg of protein in 20 μl. The blots were air dried, incubated with hybridoma supernatants, and visualized with the horseradish peroxidase system as described above. Myosin was quantitated by comparing the size and intensity of dots produced by the standards with those of the samples. Similar results were obtained using a ^{125}I-labeled rabbit anti-mouse secondary antibody, but the peroxidase-linked system was used routinely.

RESULTS

McAb's to Myosin Heavy Chains

McAb's to myosin heavy chains were prepared much as McAb's to myosin light chains were prepared previously (13). Mice were injected with purified heavy chain, hybridomas were formed, and hybridoma supernatants were screened in a solid-phase assay for binding to myosin heavy chain. Hybridomas with positive supernatants were cloned and expanded, and the binding specificity of the McAb's was further investigated.

Several McAb's were found that bound specifically to either the fast or slow isoform of the myosin heavy chain. The properties of three of these McAb's are illustrated in Fig. 1. Cryostat sections of adult PM and ALD were stained with
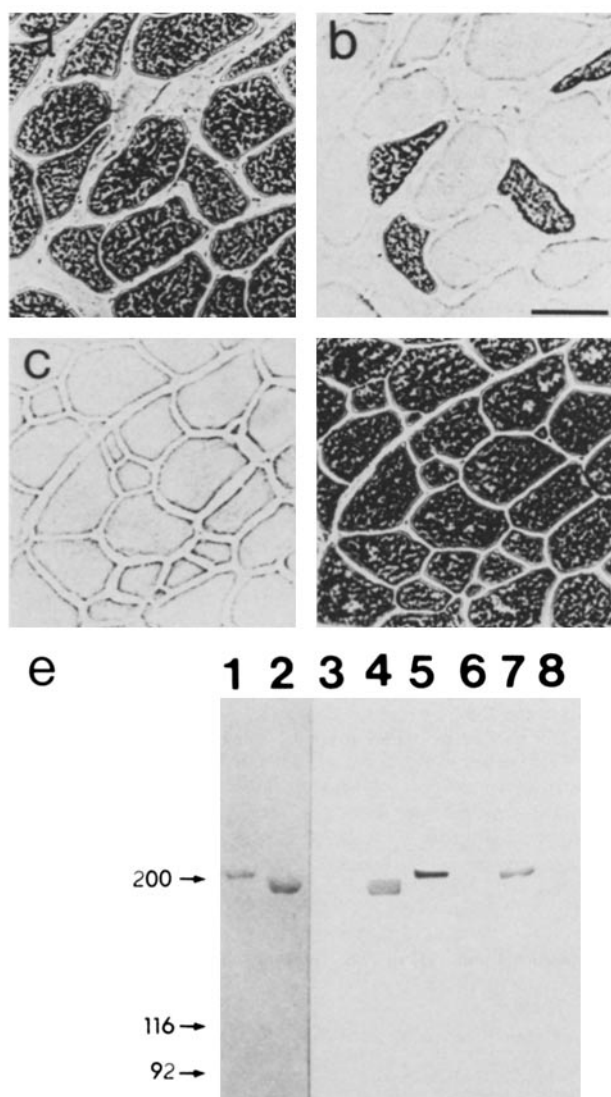
FIGURE 1 Properties of McAb's specific for fast and slow classes of myosin heavy chain. Serial sections of the predominantly slow adult ALD (a and b) and predominantly fast adult PM (c and d) were incubated with either McAb S58 (a and c) or McAb F59 (b and d). McAb binding was visualized with a horseradish peroxidase–linked detection system as described in Materials and Methods. Bar, 40 μm. × 250. In e, samples of myosin heavy chain purified from adult ALD (lanes 1, 3, 5, and 7) and adult PM (lanes 2, 4, 6, and 8) were analyzed by SDS PAGE on 5% gels and by immunoblotting. Lanes 1 and 2 were stained with Coomassie Blue. After transfer to nitrocellulose, lanes 3 and 4 were incubated with McAb F59, lanes 5 and 6 were incubated with McAb S58, and lanes 7 and 8 were incubated with McAb S46. McAb binding was visualized as above. A 1-μg sample of myosin was analyzed in each lane. When the lanes were overloaded a small amount of the fast class of heavy chain was detectable in ALD extracts. The positions of standards of molecular weight 200,000, 116,250, and 92,500 are indicated.

these antibodies. McAb S58 labeled muscle fibers in the predominantly slow ALD muscle (Fig. 1a), but not in the predominantly fast PM (Fig. 1c). McAb S46 gave identical results (not shown). In contrast, McAb F59 labeled the PM but not the ALD fibers, except for a small subset of ALD fibers that did react with F59.

Immunoblots confirmed that McAb F59 bound specifically to the fast isoform of myosin heavy chain and McAb's S58

and S46 bound specifically to the slow isoform (Fig. 1e). Myosin was purified from PM and ALD muscle and subjected SDS PAGE and immunoblotting. The fast and slow isoforms of myosin heavy chains were separable in 5% polyacrylamide gels (4). The immunoblots showed that McAb F59 bound to the fast isoform of myosin heavy chain found in adult PM muscle, and McAb's S58 and S46 bound to the slow isoform from adult ALD. Thus, the results from solid-phase binding assays, immunohistochemistry, and immunoblotting showed that these McAb's were specific to fast and slow isoforms of myosin heavy chain.

Because myosin heavy chain isoforms change during development of muscles (3–9), the McAb's were tested by immunoblotting on 5% gels and immunohistochemistry for reaction with myosin heavy chain extracted from muscles at different stages of development. McAb F59 reacted with fast myosin heavy chain taken from the PM of ED 8, 12, 16, 2-wk post-hatching, and adult animals. Similarly, McAb's S58 and S46 reacted with slow myosin heavy chain from ED 8 thigh muscle and ED 16, 2-wk post-hatching, and adult ALD (35, 36, and Miller, J. B., and F. E. Stockdale, unpublished observations). These McAb's reacted, therefore, with fast or slow myosin heavy chain epitopes that were expressed from mid-embryonic development through the adult. The McAb's described here, therefore, recognize myosin heavy chains that belong to either a fast or slow class, both of which are found at all developmental ages studied (35, 36).

It is not yet known how many isoforms of the myosin heavy chain are expressed in striated muscle during the life of the chicken. This study and others (1, 4, 6, 35, 36), however, suggest that expression of myosin heavy chain isoforms occurs within the fast and slow classes distinguished by reactivity with McAb's F59, S58, or S46, by relative mobility on 5% SDS gels, and by immunohistochemical localization in embryonic and adult tissue. Reactivity with a specific McAb and characterization by electrophoresis can, therefore, confirm if a myosin heavy chain is in these fast or slow classes but cannot tell which isoform within the fast or slow class is present. In the discussion that follows, designation of a myosin heavy chain as fast or slow should be understood to mean specifically that the myosin has a particular electrophoretic mobility and immunological reactivity that could be shared by other isoforms within the class.

Myosin Heavy Chain Isoforms in PM Cultures

The class of myosin heavy chain isoforms found in cultures of PM cells depended on the age of the donor of the myoblasts. Myoblasts were isolated from ED 5, 6, 8, and 12 PM and cultured for 6 d to allow myotubes to form. Myosin was then extracted from the cultures and compared by immunoblotting with myosin extracted from adult PM or adult ALD. As expected from earlier work (7, 37), only a fast isoform of myosin heavy chain, as demonstrated by reaction with McAb F59, was found in cultures of ED 8 or older PM myotubes (Fig. 2). Surprisingly, however, both slow and fast isoforms, as demonstrated by reaction with McAb's S58 and F59, were synthesized in cultures of ED 5–6 PM myotubes. The slow and fast isoforms found in the cultured myotubes had relative molecular weights in 5% SDS PAGE that were indistinguishable from those of the slow and fast myosin heavy chain isoforms found in adult muscle.

The amount of slow myosin heavy chain found in ED 5–6

PM cultures was much less than the amount of the fast isoform. Myosin was extracted from cultures 6 d after plating myoblasts from 6-d PM and quantitated with a dot blot assay using electrophoretically purified fast and slow adult myosin as standards. In one measurement, the cultures contained 0.5 μg slow myosin heavy chain and 3 μg fast heavy chain per 100-mm dish. In four further such measurements, the amount of myosin with slow heavy chain epitopes was 10–20% of the amount of fast myosin heavy chain in ED 5–6 cultures. This assay did not detect slow myosin heavy chain in cultures of ED 8 or older PM.

Immunocytochemistry of Myotubes in Culture

Myoblasts from ED 5–6 PM formed a heterogeneous population of myotubes in culture, but only a single population of myotubes was found when myoblasts were cultured from ED 8 or older muscle. Myoblasts were cultured for 6 d to allow myotube formation. The cultures were fixed and incu-
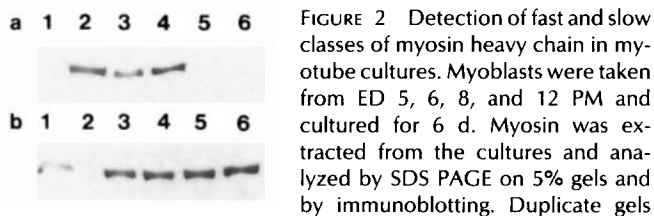
FIGURE 2   Detection of fast and slow classes of myosin heavy chain in myotube cultures. Myoblasts were taken from ED 5, 6, 8, and 12 PM and cultured for 6 d. Myosin was extracted from the cultures and analyzed by SDS PAGE on 5% gels and by immunoblotting. Duplicate gels were run and nitrocellulose transfers were reacted with McAb S58 (a) or F59 (b), and McAb binding was visualized with a glucose oxidase–linked system. As references, fast myosin heavy chain from adult PM was used in lane 1, and slow myosin heavy chain from adult ALD was used in lane 2. Myosin heavy chain from cultures of ED5 (lane 3), ED 6 (lane 4), ED 8 (lane 5), and ED 12 (lane 6) PM were compared with the reference heavy chains. Approximately 1 μg of myosin was analyzed in each lane, but 4 μg myosin was used in lanes 3–6 of a.

bated either with McAb F59 or S58, and the antibody binding sites were visualized with a horseradish peroxidase–linked detection system (Fig. 3). Between 4 and 8% of the myotubes formed in cultures from ED 5 or 6 breast muscle myoblasts reacted with McAb's specific for the slow class of myosin heavy chain, and a McAb specific for the fast class of myosin heavy chain bound to >90% of the identifiable myotubes in parallel cultures (see below). Only myotubes that contained fast heavy chain were detected in cultures from ED 8 or 12 breast muscle. Thus, just as slow myosin heavy chain amounted to a small percentage of the fast isoform, the number of myotubes that contained slow myosin was a small percentage of the number that contained fast myosin.

To determine the stability and characteristics of the myotubes that contained fast or slow isoforms we further investigated the novel observations that a slow myosin heavy chain was synthesized and that biochemically distinct myotubes coexisted in cultures of ED 5–6 PM. First, as shown in Table I, we found that the numbers of myotubes containing slow or fast myosin increased severalfold during the initial 3–4 d of culture and then remained constant for 7 d more. Multinucleated myotubes appeared from day 2 onward and increased to 65–75% of the myotubes on day 6 (see below). Myotubes that contained the two heavy chain classes, therefore, developed in culture and did not simply result from fibers that were differentiated in the animal and had survived in culture. The results in Table I also demonstrate that the cultures developed similarly in fresh and conditioned medium and that the myotubes that reacted with McAb's specific to the slow class of myosin heavy chain amounted to a relatively constant 4–8% of the myotubes that reacted with McAb's to the fast class. When cultures were reacted simultaneously with both S58 and F59, all multinucleated cells were stained.

Myotubes in cultures of ED 5–6 PM that contained the slow class of heavy chain were not markedly different in size
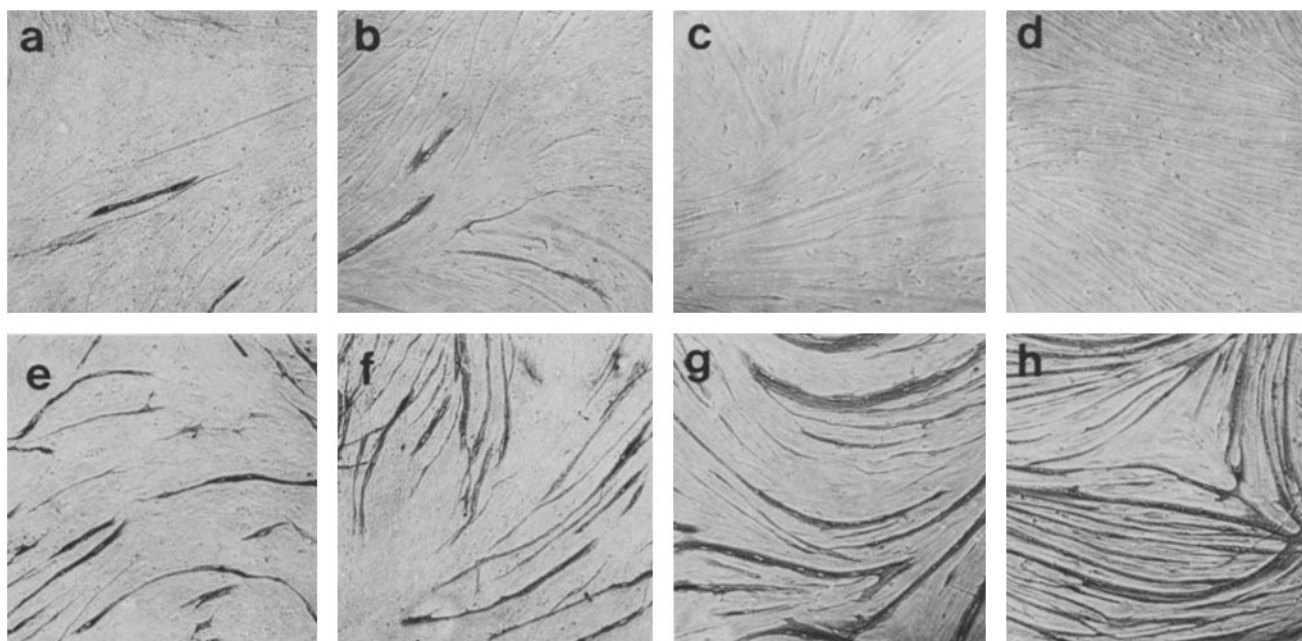
FIGURE 3   Localization of fast and slow classes of myosin heavy chain in cultured myotubes. Myoblasts were taken from ED 5 (a and e), ED 6 (b and f), ED 8 (c and g), and ED 12 (d and h) pectoral muscle and cultured for 6 d to allow myotube formation. The cultures were fixed and incubated with either McAb S58 (a–d) or McAb F59 (e–h). McAb binding was visualized with the horseradish peroxidase–linked system described in Materials and Methods. 1 cm = 200 μm. × 50.

Table I. *Effects of Medium and Time in Culture on the Number of Muscle Fibers Stained with McAb's Specific to the Fast and Slow Classes of Myosin Heavy Chain*

| | | | No. of stained fibers/ cm² | |
| | | | --- | --- |
Source of myo- blasts	Days in culture*	McAb probe	In fresh medium	In condi- tioned me- dium
ED 6 PM	1	S58	8	7
		F59	203	156
	2	S58	10	12
		F59	333	292
	3	S58	23	30
		F59	695	582
	4	S46	48	45
		S58	58	51
		F59	882	1,070
	6	S46	52	42
		S58	60	48
		F59	1,033	1,134
	8	S46	42	38
		S58	73	50
		F59	1,101	1,008
	11	S46	36	54
		S58	49	48
		F59	560	844

* Parallel cultures of myoblasts were plated at an initial density of 2.5 × 10⁴ cells/cm² in either fresh medium or conditioned medium. Cultures were fixed on the indicated day, McAb binding was visualized with a horseradish peroxidase–linked system, and stained cells were counted. Cultures were treated with cytosine arabinoside on days 3–5. Cells stained on day 1 were 95% mononucleated and 5% binucleated. No myotubes with more than two nuclei were observed until day 2.
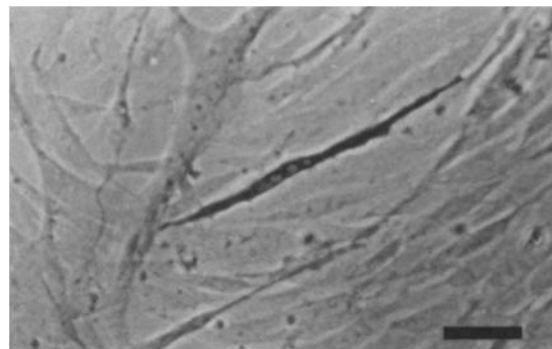

Figure 4   Myotubes containing the slow class of myosin heavy chain in mixed cultures of myoblasts from donors of different age. Myoblasts were taken from ED 6 and 12 PM, mixed, plated at 10⁴ of each cell type/cm², and cultured for 6 d to allow myotube formation. The cultures were fixed and incubated with McAb S58. Antibody binding was visualized as in Fig. 3. Bar, 100 μm. × 100.

or shape from myotubes with the fast class of heavy chain. The nuclei within myotubes were enumerated in 6-d-old cultures stained with the two antibodies. The average number of nuclei was 2.6 (range 1–9, $n = 135$) in myotubes containing slow heavy chain and 3.4 (range 1–16, $n = 177$) in myotubes containing fast heavy chain. This difference was entirely due to a small number of fast heavy chain–containing myotubes with >10 nuclei. Other than this tendency for the larger myotubes to contain fast heavy chain, the different types of myotubes appeared identical. Myotubes in ED 5–6 PM cultures that stained with either the fast or slow class of heavy chain–specific antibody were often mononucleated (25 to 35%) or binucleated (30 to 40%). In contrast, myotubes formed in cultures from ED 9 or older PM cultures were longer and contained more nuclei. The myotubes in a 6-d-old culture of ED 12 myoblasts, for instance, had an average of 23.4 nuclei (range 3–81, $n = 51$), and mononucleated or binucleated cells that contained myosin were rare (see Fig. 3). Thus, the appearance in the PM of myoblasts that formed long, highly multinucleated myotubes in culture occurred at roughly the same time as the disappearance of myoblasts that formed myotubes containing the slow class of myosin heavy chain.

Myoblasts from different sources are known to fuse with each other (38), and muscle-specific protein expression is often altered in artificially induced cell hybrids (39–41). Therefore, we investigated whether myotubes that contained the slow class of myosin heavy chain would still appear if myoblasts taken from ED 6 were mixed and cultured with myoblasts from ED 12 PM. In fact, myotubes that had slow

heavy chain did appear in such mixed cultures (Fig. 4). A constant number (10⁴ cells/cm²) of cells from ED 6 PM was mixed with either an equal number or one-tenth the number of ED 12 PM cells, and the mixed cells were cultured for 6 d. The number of myotubes that contained slow heavy chain was no different in mixed cultures than in unmixed control cultures, which suggested that the two groups of myoblasts do not fuse with each other. An average of 28.4 myotubes (range 23–36, $n = 7$) that contained slow myosin heavy chain was found in each 18-mm dish initially seeded with ED 6 PM myoblasts. In the mixed cultures, an average of 30.2 (range 27–34, $n = 14$) myotubes that contained slow myosin heavy chain was found. No myotubes that contained slow myosin heavy chain were found in unmixed ED 12 PM cultures. The only major difference between the mixed and the pure ED 6 PM cultures was the presence of the very long myotubes characteristic of ED 12 PM cultures. The generally small myotubes (from one to five nuclei) that contained the slow class of heavy chain were often side by side with these larger, unstained myotubes.

To determine if fast and slow classes of myosin heavy chains were expressed in separate myotubes or if mixed myotubes appeared, we observed ED 6 cultures with double-label immunofluorescence. These observations showed that three classes of myotubes co-existed in cultures of ED 6 PM. Because all of our McAb's were derived from mice, these observations were performed with biotinylated McAb's F59 and S46 and fluorescein- or rhodamine-labeled avidin as described in Materials and Methods. As shown in Fig. 5, myotubes were observed that labeled with McAb's specific only to the slow class of heavy chain (a and b), only to the fast class of heavy chain (e and f), or to both classes of heavy chain (c and d). Both McAb's revealed striations in some myotubes, which indicated that both isoforms could be assembled into sarcomeres. As expected, a large majority (~95%) of the myotubes contained only fast heavy chain. Of the ~5% of the myotubes that contained slow heavy chain, those that contained only slow heavy chain amounted to 10, 26, and 50% of the total slow heavy chain–containing myotubes in three separate experiments ($n = 50$ in each case), whereas mixed myotubes that contained both classes of heavy chain comprised the remainder of the slow heavy chain–containing myotubes.
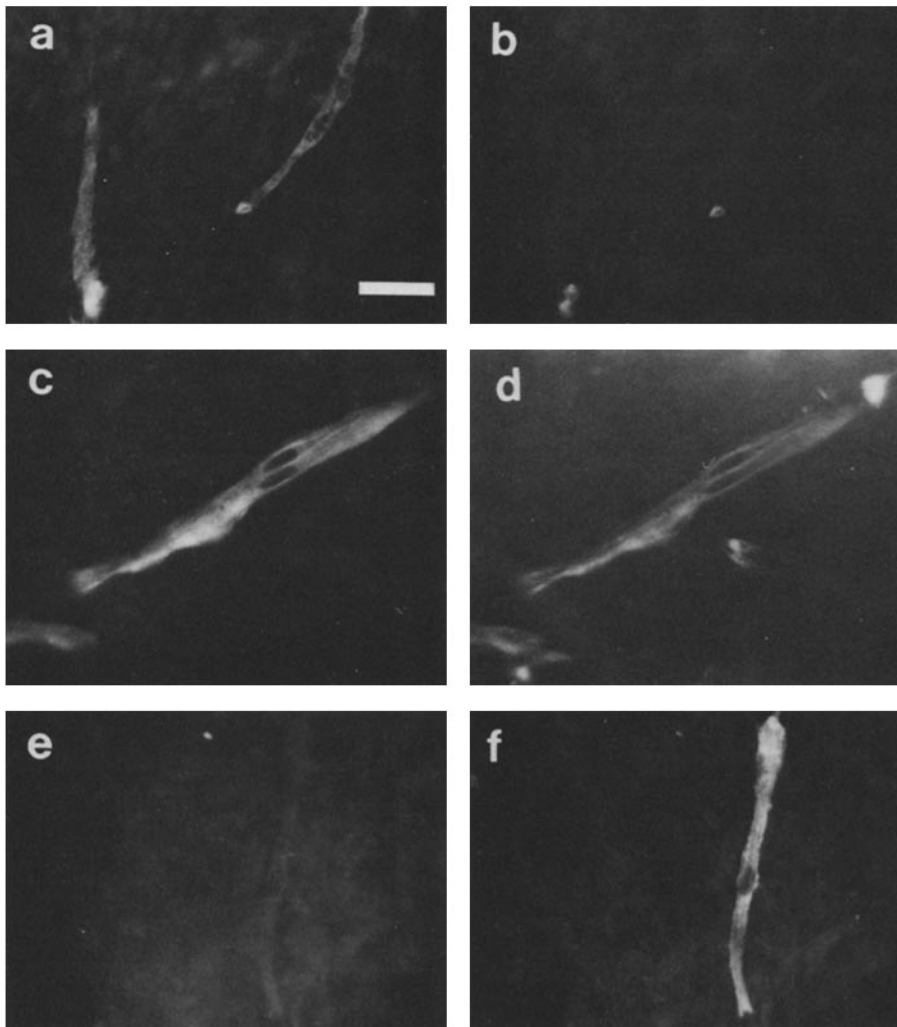
FIGURE 5 Double immunofluorescent detection of the fast and slow classes of myosin heavy chain in cultured myotubes. Myoblasts from ED 6 PM were cultured for 6 d to allow myotube formation. The cultures were fixed and incubated sequentially with biotinylated McAb S58, fluorescein-conjugated avidin, biotin, biotinylated McAb F59, and rhodamine-conjugated avidin, as described in Materials and Methods. Fluorescein fluorescence, representing the presence of the slow class of myosin heavy chain, is shown in a, c, and e. Rhodamine fluorescence of the same fields, representing the presence of the fast class of myosin heavy chain, is shown in b, d, and f. Bar, 50 μm. × 200.

DISCUSSION

Several unexpected results arose from our investigation of myosin heavy chain expression in cultured myotubes. The first was that myosin heavy chain of the slow class was synthesized in cultures of ED 5–6 PM cells. In previous studies of cultured myotubes formed from later myoblasts, only the fast "embryonic" isoform of myosin heavy chain was detected (7, 37). An important observation in these studies is that a slow class of myosin heavy chain was expressed in myotubes in cultures for at least 11 d. This result shows that innervation is not necessarily required for the initiation of synthesis of the slow class of myosin heavy chain, and that polyinnervation is not required for continued synthesis. Also, a sizable portion of the cultured myotubes synthesized both a fast and a slow class of myosin. In this respect, the cultures are like muscles developing in vivo, where it appears that mixed fibers form in the absence of innervation (1, 36, 42, 43).

Another unexpected finding of this work was that three biochemically distinct populations of myotubes co-existed in cultures of ED 5–6 pectoral cells. To the extent that differences among myotubes reflect differences among the myoblasts that form them, this result suggests that three distinct populations of myoblasts exist in the early developing PM. If so, at least two of the three early myoblast populations must disappear after mid-embryonic development, because a slow class of heavy chain was not found in myotubes from cultures derived from ED 8 or older pectoral myoblasts. Alternatively, all three early populations of myoblasts could disappear to be replaced by a new population. The dramatic morphological difference between the small myotubes formed from ED 5–6 myoblasts and the large myotubes formed from ED 9 or older myoblasts supports such an idea.

It is generally thought that three sequential periods of fast myosin heavy chain expression occur during the development of avian PM (6, 9). Thus, three different fast myosin heavy chain isoforms, termed embryonic, neonatal, and adult, are synthesized during these periods. The results presented here and our recent finding that early embryonic muscles in vivo contain slow as well as fast myosin heavy chain (35, 36) indicate that the "embryonic" isoform would be more appropriately called the fetal form because it is now clear that a fourth period of heavy chain expression precedes the other three. It is during this "embryonic period" (before 7–8 d of development), before completion of morphogenesis, that slow and fast heavy chain isoforms are co-expressed in developing muscle and in cultured myotubes made from these muscles. Whether the isoforms expressed in vivo in this embryonic period are structurally identical to the later isoforms or if the isoforms expressed in cultures of early and late myoblasts are the same remains to be determined.

Recent findings of Sweeney et al. (44) are consistent with

the idea of an embryonic period of heavy chain expression. By immunohistochemistry alone, they find that myosin with cardiac myosin heavy chain epitopes is expressed in skeletal muscle during the embryonic period but disappears by late embryonic or fetal development. However, this cardiac-like heavy chain did not react with antibodies to either adult fast or slow myosin heavy chain. This observation is difficult to reconcile with the recent observation that the cardiac heavy chain, at least in mammals, appears by molecular genetic analysis to be identical to the adult slow heavy chain (45, 46) and with our finding that a monoclonal antibody that reacted with slow myosin heavy chain of the adult also reacted with slow myosin heavy chain in the embryo. Experiments are needed to investigate the biochemical structures of the fast, slow, and cardiac heavy chains expressed in early skeletal muscle development.

Myoblasts in the developing limb have been classified as "early" or "late" by Hauschka, Bonner, and colleagues (47–49). This classification is based on the finding that myoblasts from early (e.g., ED 5–6) and late (e.g., ED 10–12) embryonic limbs form muscle colonies of different morphology and have different medium requirements for differentiation. When cloned, the early population of myoblasts forms small myotubes much like those we saw in ED 5–6 pectoral cultures. It is highly likely that the fast, slow, and mixed myosin containing myotubes that we found in ED 5–6 PM cultures were all formed from the early myoblast type defined by White et al. (48). Because myogenesis of early myoblasts in mass cell culture has not been as well studied as in clonal cultures, it is not yet clear how to interpret the large number of mononucleated "myotubes" that we found in such cultures. It is well known that myoblasts can differentiate without fusing. Because only 5–10% of the cells taken from ED 5–6 muscle are myogenic, the intermyoblast contacts required for fusion may be limited. Thus, small myotubes would be more numerous in ED 5–6 cultures than in cultures of cells taken from ED 12 muscles where >90% of the cells are myogenic.

Besides showing that the early myoblasts form a heterogeneous population of myotubes, our results suggest that early and late myoblasts form myotubes independently of each other. This conclusion follows from the experiment in which the number of myotubes containing the slow class of myosin heavy chain was the same in co-cultures of early and late myoblasts as in cultures of early myoblasts alone. This observation implies that there are recognition events between myoblasts that may permit the selective formation of distinctive myotube types.

We postulate that the three types of myotubes that form in cell cultures from early developing muscle are those that give rise to the primary fibers of developing muscle in vivo. Early embryonic muscle contains rudimentary myotubes called primary muscle fibers around which later secondary muscle fibers form (50, 51). The primary generation of fibers are of at least two types in the bird, fast and fast/slow (35, 36), which correspond to the two primary fiber types recognized by ATPase staining (43, 52, 53). As in cell culture, different primary fiber types form in the embryo independently of innervation (52, 53). We think that fiber development in cell culture mimics that in vivo, which indicates that the different primary muscle fiber types in the early embryo emerge as different muscle fiber types rather than as a single type upon which environmental factors such as innervation impose differences.

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