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Cell Biology

Synthesis of adult myosin light chains by embryonic muscle cultures

(fast and slow fiber types/skeletal muscle/differentiation/clonal analysis)

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ABSTRACT **Myosin light chain synthesis has been analyzed in cultures of fast and slow muscles from chicken and quail embryos. Synthesis was assayed by [^{35}S]methionine incorporation and two-dimensional electrophoresis of total cell extracts. Our results show that differentiated cultures of embryonic anterior latissimus dorsi and pectoral muscles synthesize proteins that comigrate on two-dimensional gels with the five myosin light chains of adult fast (pectoral) and slow (anterior latissimus dorsi) muscle. Partial proteolytic digestion and peptide analyses further confirm the identity of these proteins as adult light chains. Cultures of dividing myoblasts do not synthesize any of these fiber type isozymes, and synthesis of the isozymes is initiated at myoblast fusion. Also, myogenic clones derived from single myoblasts differentiate to synthesize these five myosin light chains, indicating that individual myoblasts have the potential to express the synthesis of all fiber type light chain isozymes. We conclude that the primary events in muscle differentiation include the initiation of synthesis of the entire set of adult fast and slow myosin light chain isozymes. The developmental and physiological implications of these results for the establishment of fiber type specificity are discussed.**

Skeletal muscles are classified as either fast or slow fiber types on the basis of physiological and biochemical criteria including the specific subset of skeletal muscle contractile protein isozymes they contain (1–3). In adult muscles, the expression of these fiber type specificities is changeable because both the contraction characteristics and the isozymes of one fiber type can switch to the other in response to altered innervation patterns (4, 5). In developing muscles, however, the cellular and molecular mechanisms that control the genes responsible for fiber type specificity are not well understood, and there is controversy about the intrinsic potential of embryonic fibers for synthesis of fiber type-specific contractile proteins prior to innervation.

Because the myosin light chains are different in adult fast and slow muscles (2, 6), they have been used as markers to determine which fiber type-specific proteins are initially present in embryonic fibers. Some investigators propose that both fast and slow fiber type myosins are present initially in the same fibers and that the synthesis of subsets of the isozymes is repressed later in development, probably in response to innervation (7; 8). Other investigators conclude that all embryonic muscles are of the fast type initially and that slow muscles switch to synthesis of slow myosin light chains after innervation (9, 10). Still other work suggests that embryonic myosin contains a unique embryonic light chain isozyme in addition to fast myosin light chains (11). The conflicting conclusions of these earlier studies have left unresolved the question of when developing muscles first synthesize fiber type-specific myosin light chains.

Few studies have specifically examined the molecular mechanisms that control expression of the genes responsible for fiber type specificity during muscle development. It is generally accepted that cultured and embryonic muscles synthesize and accumulate two of the fast myosin light chains, LC_1f and LC_2f (9–13). However, there are quantitative differences between the amounts of mRNAs for these light chains as assayed by cell-free translation and the rates of synthesis of the light chain proteins in cultured cells. Furthermore, LC_3f has been detected by cell-free translation of cultured muscle mRNAs, but the synthesis of this light chain protein has not been detected in culture (12, 14). These results suggest some control of light chain expression at a posttranscriptional level. Most studies, however, have restricted their analyses to the light chains that accumulate posttranslationally in a form extracted by standard myosin purification procedures and identified by gel electrophoresis or by antibody probes (9–13). Therefore, the capacity of embryonic muscle for synthesis of the various fiber-type light chain isozymes has not been examined.

In this study we have attempted to define the potential of early embryonic muscle for fiber-type gene expression by examining the synthesis of fast and slow myosin light chain isozymes in cultures of embryonic avian muscles. Light chain synthesis was assayed by [^{35}S]methionine pulse labeling of cultures derived from embryonic anterior latissimus dorsi (ALD) (presumptive slow) and pectoral (presumptive fast) muscles of chicken and quail. The total cellular proteins from these cultures were then fractionated by two-dimensional electrophoresis, and the newly synthesized fast and slow light chain isozymes were identified by their comigration with purified light chain markers. We find that differentiated muscle cultures synthesize the five adult fast and slow myosin light chains present in adult muscles. Dividing myoblasts do not synthesize any of these isozymes, and their synthesis is activated at the time of myoblast fusion. In addition, myogenic clones derived from single cells synthesize the five fast and slow myosin light chains. These results show that synthesis in cultured embryonic muscle is not restricted to isozymes of a specific fiber type and that synthesis of all fast and slow light chains is a part of the primary events occurring at myoblast fusion. We conclude, therefore, that fiber type differentiation is not regulated by the sequential activation of light chain synthesis during development but is controlled through events that occur later in development, possibly by the selective turning off of subsets of light chain genes after innervation or by posttranslational mechanisms such as differential assembly into functional myosin molecules and filaments or selective degradation of specific isozymes.

METHODS

Culture Conditions. Secondary cultures of myoblasts were prepared as described (15) from pectoral muscle of day 10 Japanese quail embryos and from pectoral and ALD muscles of chicken embryos of various ages. Clones were identified as

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Abbreviation: ALD, anterior latissimus dorsi.

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arising from single myoblasts by marking single cells immediately after plating and monitoring their subsequent growth. In some experiments, cells were inoculated into minimal essential medium (5-15 MEM) and the medium was changed after 24 hr to defined medium completely lacking horse serum and embryo extract but containing Ham's F12 medium (16).

Mass cultures of well-fused myotubes on day 4 or 5 after plating were labeled with [^{35}S]methionine in complete medium (80 μCi/ml; 1 Ci = 3.7×10^{10} becquerels; Amersham; specific activity, >500 Ci/mmol). Plates were rinsed twice with saline G (15), extracted directly with lysis buffer for isoelectric focusing (17), homogenized, and frozen at −80°C. Two-dimensional gel patterns were similar in the absence and presence of the protease inhibitor phenylmethylsulfonyl fluoride, so it was not routinely used. Individual clones were labeled similarly by using sterile glass cylinders to confine the labeled medium and extraction buffers. Cultures of unfused myoblasts were labeled within 36 hr of plating. Incorporation was determined by precipitating aliquots of extracted cells with 15% trichloroacetic acid (15). Protein determinations were conducted according to Bensadouin and Weinstein (18) with ovalbumin as a standard.

Gel Electrophoresis. Actomyosin, myosin, and [^{35}S]methionine-labeled cellular extracts were fractionated by two-dimensional gel electrophoresis essentially as described by O'Farrell (17). An equilibrium isoelectric focusing gradient of pH 4–7 was used for separation in the first dimension. The second dimension gels were either 15% or 10–20% gradient $NaDodSO_4$/polyacrylamide gels as described by Laemmli (19) and Maizel (20). Gels were stained with Coomassie blue R-250 and were dried under heat and vacuum and exposed to Kodak x-ray film (type SB or XR) for autoradiography.

Incorporation of [^{35}S]methionine into specific proteins was quantitated by scanning densitometry of autoradiograms with a Photoscan P1000 system as described (21). In some cases, spots of specific proteins were excised from the dried gels and counted in 10 ml of toluene-based scintillation cocktail (15).

Purification of Light Chain Markers. Actomyosin was extracted from the pectoralis and ALD of adult quail and 12-day-old chickens by two cycles of extraction in high-salt buffer (0.6 M KCl/2 mM $MgCl_2$/5 mM phosphate, pH 7.3) and precipitation by dilution with 5 vol of cold distilled H_2O (22). Smooth muscle or nonmuscle myosin standards were prepared in a similar manner from embryonic gizzard muscle (23).

Limited Proteolysis of Light Chains. Samples containing a mixture of chicken actomyosin markers and labeled cellular proteins from cultured myotubes were analyzed by limited proteolysis peptide mapping (24). Areas of stained gels containing the myosin light chain markers were excised, placed in stacking gel wells of a 15% $NaDodSO_4$/polyacrylamide gel, and digested with 10 μg of *Staphylococcus aureus* V8 protease (Miles, 36-900-1). The gel was stained with Coomassie blue R-250 to determine migration patterns of the purified light chain marker fragments and then prepared for fluorography (25) to determine migration patterns of labeled light chain proteolytic fragments.

RESULTS

Light Chain Synthesis by Embryonic Fast and Slow Muscles in Culture. Cultures of well-fused myotubes derived from day 12 embryos were pulsed for 1 hr with [^{35}S]methionine, and total cell lysates were fractionated by two-dimensional isoelectric focusing/$NaDodSO_4$ electrophoresis. Fig. 1 shows an autoradiogram of the proteins synthesized by differentiated cultures of embryonic chicken pectoral muscle. ^{35}S-Labeled proteins that comigrated with all five of the light chains present

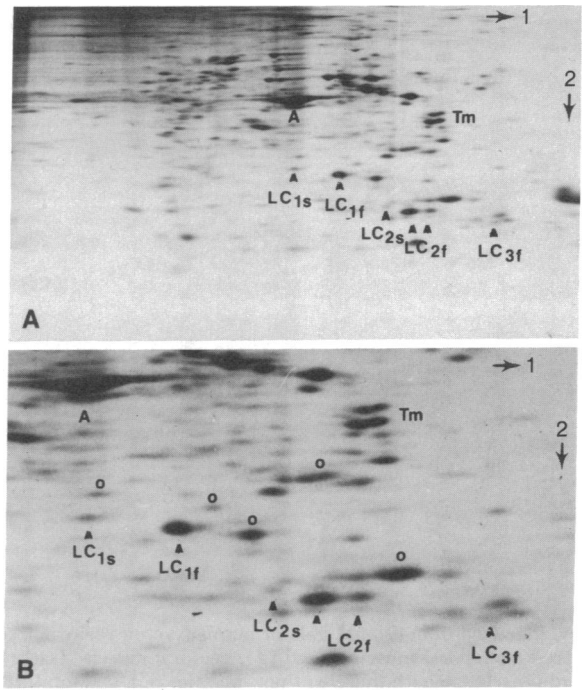

FIG. 1. (*A*) Two-dimensional gel (arrows show direction; 1, isoelectric focusing, first dimension; 2, $NaDodSO_4$/polyacrylamide gel electrophoresis, second dimension) autoradiogram of [^{35}S]methionine-labeled total cellular protein synthesized by day 12 embryonic chicken pectoral muscle cultures during a 1-hr pulse. The less-intense spot to the acidic (right) side of LC_2f is believed to be the phosphorylated form of this light chain (26). Trichloraacetic acid-precipitated label, 200,000 cpm; exposure, 10 days; film, Kodak SB. (*B*) Enlargement of the light chain region of the gel shown in *A*. Open circles indicate "landmark" spots which can be used for orientation in the light chain region enlargements of other experimental gels.

in adult pectoral and ALD muscles were detected. Comigration with adult isozymes was established by cofractionating labeled cell extracts with unlabeled actomyosin markers from adult chicken pectoral and ALD muscles and comparing the positions of stained markers with labeled proteins on autoradiograms of the same gel. The myosin light chains of chicken actomyosin from the fast (pectoral) and the slow (ALD) skeletal muscle and from gizzard smooth muscle migrated to distinct and reproducible positions in this gel system (Fig. 2). To demonstrate more precisely the comigration of the labeled light chain proteins synthesized by muscle cultures with stained adult marker light chains, a portion of each stained light chain spot was removed from the gel with a pasteur pipette tip. Autoradiograms of the altered gel indicated that a portion of each labeled protein corresponding to a light chain was simultaneously ablated (not shown). These results demonstrate that cultures of embryonic chicken pectoral myofibers synthesize all five of the myosin light chains of fast and slow adult muscle. These include LC_1s, LC_2s, and LC_3f, whose synthesis has not been previously detected in cultured embryonic muscle, in addition to LC_1f and LC_2f which earlier studies have identified (9–13). Cultures derived from day 12 embryonic ALD muscles and day 10 embryonic quail pectoral muscles were also found to synthesize the five fast and slow myosin light chains when assayed under these same conditions, indicating that synthesis of these five light chains also occurs in muscles cultured from different tissues and organisms.

We have further established that the myosin light chains synthesized by cultured cells are adult fiber-specific light chains by limited proteolysis peptide mapping. [^{35}S]Methionine-la-

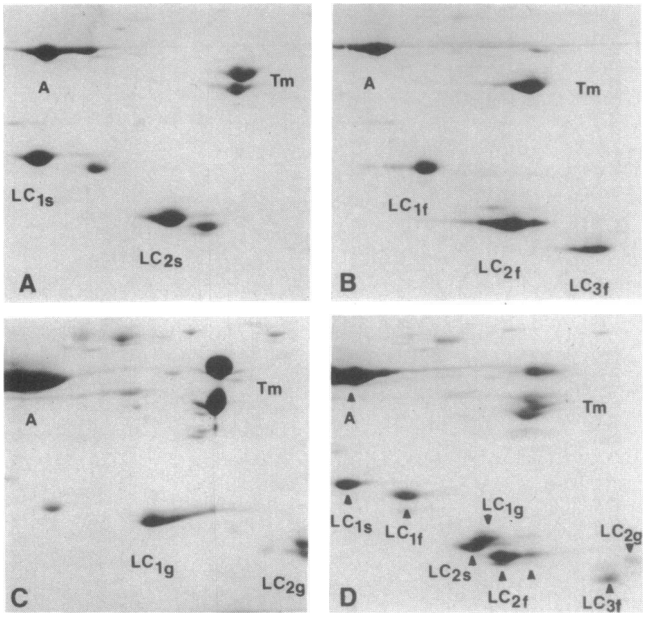

FIG. 2. Two-dimensional gels of stained actomyosin markers prepared from ALD muscle (slow) (*A*), pectoral muscle (fast) (*B*), gizzard muscle (smooth muscle or non-muscle) (*C*), and mixture of *A*, *B*, and *C* (*D*). Pectoral myosin further purified from an actomyosin pellet by ammonium sulfate fractionation and DEAE-cellulose chromatography contained light chains that migrated in the same position as those in preparations of actomyosin from pectoral muscle (not shown). Approximately 25–50 μg of protein was loaded on each gel. Molecular weights of the myosin light chains, determined from their mobility on $NaDodSO_4$ gels, are: LC_1s, 27,000; LC_1f, 25,000; LC_2s, 20,000; LC_2f, 19,000; and LC_3f, 17,000. The proteins that migrate to the right of LC_1s and LC_2s in *A* represent fast light chains contributed by the 10% contamination of this slow muscle by fast fibers (9).

beled proteins from cultured cells and unlabeled actomyosin standards were cofractionated on two-dimensional gels. The myosin light chain bands were excised and digested with *S. aureus* protease, and the peptides were resolved on a one-dimensional slab gel. The ^{35}S-labeled peptides for each light chain comigrated with the stained peptides, and a unique map was obtained for each protein (Fig. 3). Stained peptides of ^{35}S-labeled cellular proteins are not normally detected without addition of light chains from adult actomyosin preparations. On the basis of their precise comigration on two-dimensional gels and peptide digestion maps, it is concluded that the proteins synthesized by cultured cells are the myosin light chains of fast and slow adult muscle.

Myoblast Cultures Do Not Synthesize Adult Skeletal Myosin Light Chains. Synthesis of myosin light chains was examined in undifferentiated cultures of dividing quail myoblasts. Quail myoblasts were used for these studies because they are not contaminated by differentiated myofibers early in culture (27). Myoblast cultures that were determined to be free of myofibers by phase microscopy were labeled for 2 hr with

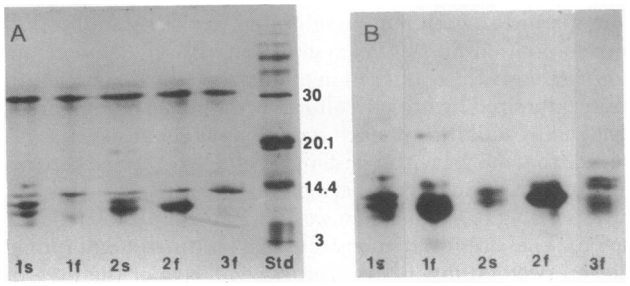

FIG. 3. Peptide maps of the myosin light chains produced by limited digestion with *S. aureus* V8 protease. (*A*) Coomassie blue-stained peptides of the individual adult myosin light chains. (*B*) Fluorogram of the same gel shown in *A*, showing ^{35}S-labeled peptides of the light chains synthesized by cultured muscle. Size in kilodaltons is shown by numbers in middle of figure. The stained peptides migrating at approximately 30 and 14 kilodaltons in all the light chain wells in *A* are attributable to *S. aureus* protease because they were present in a well containing only protease on the same gel (not shown). The protease sample was not boiled before application to the gel, and its subunits are therefore not completely dissociated. All light chain samples were run in parallel in different lanes on the same gel. However, to adjust for differences in radioactivity in each sample, lanes 1s and 3f in *B* are from a fluorogram that was exposed for 14 days; lanes 1f, 2s, and 2f in *B* were exposed for 64 hr.

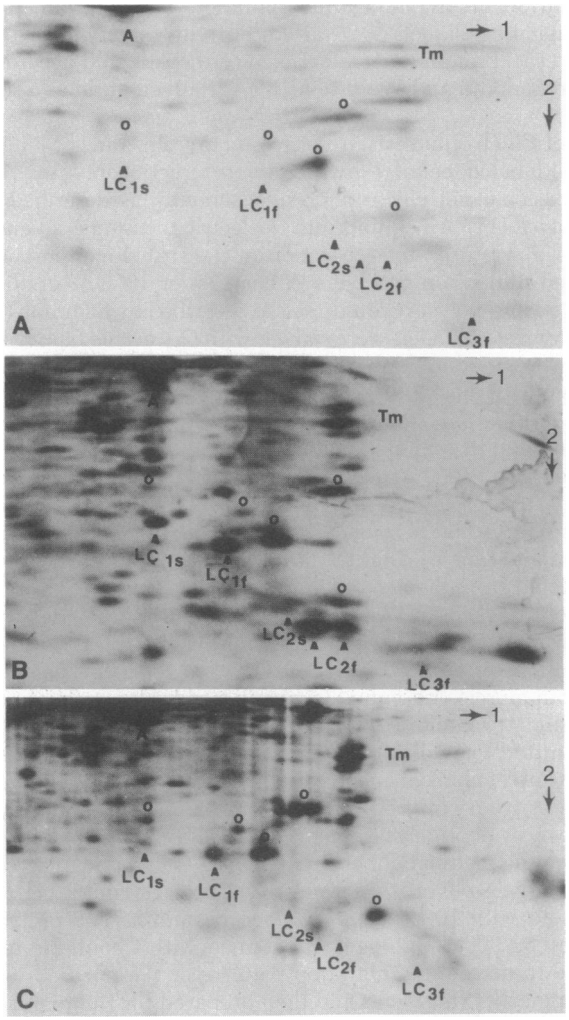

FIG. 4. Autoradiograms of [^{35}S]methionine-labeled total cellular proteins: (*A*) synthesized by cultures of dividing quail myoblasts during a 3-hr labeling period (400,000 precipitated cpm; exposure, 8 days; film, Kodak XR); (*B*) synthesized by a myogenic clone derived from day 10 embryonic quail pectoral muscle during a 3-hr labeling period. (400,000 cpm; exposure, 12.5 days; film, Kodak XR); (*C*) synthesized by cultures of day 12 embryonic chicken pectoral muscle, which were allowed to differentiate in defined medium (F12) and were labeled for 3 hr (500,000 cpm; exposure, 13 days; film, Kodak XR). The migration of actin, tropomyosin, and the "landmark" spots are indicated for orientation. The clone in *B* was grown in culture for 8 days prior to labeling and contained multinucleated fibers as well as unfused cells; data quantitating light chain synthesis by this clone are shown in Table 1 under clone 3.

Table 1. Percentage of light chain synthesis

	Quail pect., day 10	Chicken		Quail			Chicken pect., day 12, 15-min pulse
		Pect., day 12	ALD, day 12	Clone 1	Clone 2	Clone 3	
LC_1s	7.22 ± 2.18	7.35 ± 1.21	13.56	2.96	4.54	15.13	5.01
LC_1f	31.93 ± 1.01	32.17 ± 7.23	30.62	40.04	39.67	36.69	42.07
LC_2s	13.84 ± 0.13	6.60 ± 5.01	17.71	6.16	5.81	5.37	2.28
LC_2f	36.22 ± 2.57	49.67 ± 5.01	30.55	47.62	46.95	41.97	49.25
LC_3f	3.92 ± 2.99	4.23 ± 2.65	7.56	3.21	3.02	0.84	1.39

Optical density for each light chain on a two-dimensional autoradiogram was quantitated by scanning densitometry (see text), and the sum of the optical densities from the five light chains on each autoradiogram was used to determine the percentage shown. Data for quail and chicken pectoral muscles represent averages of two determinations ± the deviation; ALD and clone data represent single determinations. All light chains contain an average of six methionines per molecule, so the relative intensities reflect the amount of protein present. Cultures were labeled for 3 hr with [^{35}S]methionine in all experiments, except where indicated, by using 80 μCi/ml in mass cultures and 8 μCi per clone. Quantitation for LC_2f is based on densities of both the LC_2f spot and the phosphorylated form of this subunit.

[^{35}S]methionine between 24 and 36 hr after plating. Labeled myosin light chains, as well as other skeletal muscle specific contractile proteins, were not detected after separation of myoblast proteins on two-dimensional gels (Fig. 4A). This gel contained more acid-precipitable radioactivity but was exposed for a shorter length of time than the gel of proteins from differentiated myotube cultures shown in Fig. 1. Longer exposures did not reveal spots corresponding to these proteins. The absence of detectable light chain synthesis in myoblast cultures, therefore, indicates that their synthesis is activated at some time during the primary differentiation processes of myoblast fusion and formation of multinucleated myotubes.

Quantitation of the Synthesis of Myosin Light Chains. The relative rates of synthesis of fast and slow myosin light chains were quantitated in mass cultures of embryonic chicken and quail muscles. Incorporation of [^{35}S]methionine into the individual light chains was measured by scanning densitometry of autoradiograms as well as by scintillation counting of light chain bands excised from the gels. Incorporation measured after a 3-hr labeling period (Table 1) indicates that LC_1f and LC_2f accumulate at consistently higher rates than do the other light chain isozymes in chicken pectoral and ALD muscles and in quail pectoral muscle. Also, LC_3f accumulates at a consistently lower rate than do the other isozymes. Rates determined from 15-min [^{35}S]methionine pulses agreed closely with those determined from 3-hr pulses. This indicates that the relative differences in incorporation reflect differences in initial rates of synthesis rather than a rapid, differential turnover of these isozymes. The rates of synthesis of LC_1f and LC_2f in muscle cultures have previously been shown to be very high, on the order of 30,000 molecules per min per 2*N* DNA (15, 21). Thus, even though LC_1s, LC_2s, and LC_3f are synthesized at one-quarter to one-fifth the rates of LC_1f and LC_2f, these isozymes are being synthesized at substantial rates by these muscle cells.

Effects of Embryonic Age on Synthesis of Light Chains in Culture. Light chain synthesis in differentiated cultures derived from day 9 and day 19 embryos was studied to determine whether synthesis of the light chain isozymes of fast and slow muscle becomes restricted with embryonic age. Differentiated cultures derived from muscles of these early and late developmental stages also synthesized the five light chains of fast and slow muscle. In addition, all five myosin light chain isozymes were synthesized by cultures immediately following fusion (72 hr after plating) and at late times when cultures are well-differentiated and fibers are undergoing spontaneous contractions (120 hr) (see *Discussion*).

Effects of Culture Medium on Light Chain Synthesis. We considered the possibility that the enriched medium used in all the experiments described above stimulates muscle cultures to synthesize all of the fast and slow myosin light chains rather than only those of a specific fiber type. To examine this possibility, myoblasts were allowed to differentiate in a completely defined medium (F12) without horse serum and embryo extract. In this medium, myoblasts fused to form multinucleated myotubes and synthesized the five myosin light chain isozymes (Fig. 4C), which shows that complex culture medium alone does not promote coexpression of the synthesis of the fast and slow isozymes.

Synthesis by Clonal Cultures. The myosin light chains present in whole muscle extracts and in mass cultures represent the accumulated products synthesized by a large number of nuclei within the myotubes. These fiber nuclei may have arisen from a heterogeneous population of myoblasts with differing potentials for synthesis of light chain isozymes. In order to examine the potential of a single myoblast for light chain synthesis, we assayed the light chains synthesized by myogenic clones derived from single myoblasts. Specifically, we asked whether a clone containing myofibers directs the synthesis of all light chains, only those light chains characteristic of either fast or slow muscle, or only a single isozyme of light chain. Using two-dimensional gel electrophoresis, we examined the proteins synthesized by more than 20 clones. All of these clones synthesized the five myosin light chains of fast and slow muscle. Fig. 4B shows the light chains synthesized by a single quail myogenic clone and is representative of the gels from clones of chicken ALD or pectoral muscle. There was some quantitative variation in the light chains synthesized by individual clones (Table 1) but, in general, the results of this clonal analysis were similar to those for mass cultures in that LC_1f and LC_2f accumulated at the highest rates, and LC_3f at the lowest rate. These data show, therefore, that individual myogenic cells in culture have the potential to synthesize the five fast and slow muscle light chain isozymes and indicate that the synthesis of all five isozymes by myotubes in mass cultures cannot be attributed to myoblast stem cell heterogeneity in embryonic ALD and pectoral muscles.

DISCUSSION

Our study shows that cultures of embryonic avian muscles, which differentiate in the absence of innervation, synthesize the five myosin light chains present in both fast and slow adult muscles. Clonal studies demonstrate that single myoblasts have the potential to express the synthesis of all of these adult myosin light chain isozymes. Expression is also qualitatively unaffected by the culturing conditions, because cells from different muscle tissues and embryos of different ages and cells grown in different media synthesize the fast and slow isozymes of myosin light chains. The synthesis of skeletal myosin light chain isozymes was not detected in cultures of dividing myoblasts,

implying that synthesis is activated during the process of myoblast fusion. Devlin and Emerson (21) have directly demonstrated that synthesis of LC_1f and LC_2f is activated coordinately with the other contractile proteins at the time of myoblast fusion. Reexamination of their data shows that the slow light chains LC_1s and LC_2s correspond to proteins 30 and 28, respectively, on their two-dimensional gels. Furthermore, their quantitative kinetic data show that the synthesis of these slow light chain isoenzymes is activated coordinately with LC_1f, LC_2f, and the other contractile proteins at myoblast fusion. Because LC_3f was not resolved on their gel system, we do not know precisely the developmental kinetics of its synthesis, although our data now show that LC_3f is synthesized by differentiated muscle cultures. Therefore, the primary events of muscle differentiation include activation of the synthesis of the entire set of fast and slow myosin light chains of adult muscles.

Our results are in contrast with those of earlier studies (9, 10, 12, 13, 28) which detected only LC_1f and LC_2f in partially purified myosin from embryonic muscle. However, our approach has differed in that we examined the total cellular proteins synthesized during short pulses without extraction and purification of actomyosin before electrophoresis on high-resolution two-dimensional gels. We think that the technical aspects of this approach have allowed us to detect the presence of fast and slow fiber-type light chains in single clones and cultures.

Our studies with cultured muscle are consistent with *in vivo* studies by Gauthier *et al.* (7) who reported that antigenic determinants of both fast and slow myosins can be detected by immunofluorescent antibody staining of embryonic rat diaphragm (fast) muscle fibers. Dhoot and Perry (29) used immunoperoxidase staining to demonstrate that adult slow type troponin and tropomyosin subunits also are present in both fast and slow muscle fibers of embryonic rat and mouse. These results, together with ours, suggest that synthesis of adult fast and slow isozymes for all contractile proteins is activated prior to innervation. The potential developmental significance of coexpression of contractile protein isozymes is suggested by a recent report (30) that single embryonic erythrocytes circulating at the time of the hemoglobin switch express both early embryonic and adult hemoglobins. These findings are consistent with a hypothesis that terminally differentiating cells become committed early to coexpression of gene families and later selectively express only specific gene subsets of these families.

Since the early expression of contractile proteins is not restricted, it is important to determine next whether all these fiber-specific proteins continue to be synthesized in adult muscles. Innervation patterns may, during later development, direct the continued synthesis of one subset of isozymes and selectively repress synthesis of the other subset. This question can be examined if newly synthesized proteins of high specific activity are prepared from adult muscle and examined with the minimal purification techniques required by two-dimensional electrophoresis. If all myosin light chains are found to be synthesized continually in fast and slow muscles, even in the adult, fiber-type specificity must be regulated through posttranslational mechanisms such as differential degradation of specific isoenzyme subsets or through selective assembly of isozymes into stable, complete myosin molecules.

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