SYNTHESIS OF MYOSIN HEAVY AND LIGHT CHAINS IN MUSCLE CULTURES

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ABSTRACT

The weight ratio of myosin/actin, the myosin heavy chain content as the percentage of total protein (wt/wt), and the kinds of myosin light chains were determined in (*a*) standard muscle cultures, (*b*) pure myotube cultures, and (*c*) fibroblast cultures. Cells for these cultures were obtained from the breast of 11-day chick embryos. Standard cultures contain, in addition to myotubes, large numbers of replicating mononucleated cells. By killing these replicating cells with cytosine arabinoside, pure myotube cultures were obtained. The myosin/actin ratio (wt/wt) for pure myotube, standard muscle, and fibroblast cultures average 3.1, 1.9, and 1.1 respectively. By day 7, myosin in myotube cultures represents a minimum of 7% of the total protein, but about 3% in standard cultures and less than 1.5% in fibroblast cultures. Myosin from standard cultures contains light chains LC1, LC2, and LC3, with a relative stoichiometry of the molarity of 1.0:1.9:0.5 and mol wt of 25,000, 18,000 and 16,000 daltons, identical to those in adult fast muscle. Myosin from pure myotubes exhibits light chains LC1 and LC2, with a molar ratio of 1.5:1.6. Myosin from fibroblast cultures possesses two light chains with a stoichiometry of 1.8:1.8 and mol wt of 20,000 and 16,000 daltons. Clearly, the faster migrating light chain, LC3, found in standard cultures is synthesized not by the myotubes but ty the mononucleated cells. In myotubes, both the assembly of the sarcomeres and the interaction between thick and thin filaments required for spontaneous contraction occur in the absence of light chain LC3. One set of structural genes for the myosin light and heavy chains appears to be active in mononucleated cells, whereas another set appears to be active in multinucleated myotubes.

During the past several years, there have been many reports complementing the electron microscopic observation of Ishikawa et al. (24), that actin molecules are synthesized by a variety of cell types (2, 6, 14, 15, 23, 28, 45, 49, 61). There is less agreement regarding the presence of myosin molecules in different types of cells. Though myosin heavy chains have been reported in a variety of normal and abnormal cells (34, 35, 37, 38, 57), Yaffe and co-workers (58, 59, 60) and Strohman and co-workers (36, 39, 40) have claimed that mononucleated, replicating presumptive myoblasts do not synthesize myosin. In contrast, Rubinstein et al. (41) have reported that replicating, presumptive myoblasts as well as BudR-suppressed myoblasts do, in fact, synthesize myosin and actin. These investigators also demonstrated that the myosin heavy chain/actin ratio (wt/wt)

and the stability of the myosin and actin differed between presumptive myoblasts and nonmyogenic cells on the one hand and terminally differentiating myotubes on the other (19, 41).

The myosin light chains are an important part of the contractile system (13, 42, 56). Sreter et al. (46) reported that myotubes derived in vitro from replicating myogenic cells of chick breast muscle synthesized the definitive fast myosin light chains: LC1, LC2, and LC3. These light chains were assembled in the total absence of neural influences and presumably are an intrinsic expression of the myogenic program.

The experiments to be reported in this paper confirm the finding that the postmitotic myotubes derived from replicating muscle precursor cells synthesize the myosin light chains LC1 and LC2. However, additional experiments suggest that the LC3 light chain found in these standard muscle cultures is not, in fact, synthesized by the myotubes, but is a contribution of replicating presumptive myoblasts and replicating fibroblasts. Furthermore, the slower migrating light chain synthesized by fibroblasts differs in mobility from the LC2 in myotubes. We found that both the assembly of the basic sarcomere pattern and the interaction between thick and thin filaments in myotubes required for spontaneous contraction occur in the absence of LC3. A preliminary account on some of these observations has been reported elsewhere (7).

MATERIALS AND METHODS

Muscle cultures were prepared from 11-day old chick breast as has been described (5). Mononucleated muscle cells were grown on collagen-coated dishes at a density of $3-5 \times 10^6$ cells per 100-mm dish and fed daily for 6-7 days. In these "standard" cultures, cells proliferate and some yield postmitotic myoblasts which then fuse to form multinucleated myotubes. In addition to the postmitotic myotubes, standard cultures contain large numbers of mononucleated cells. To obtain cultures consisting almost exclusively of myotubes, the cultures were treated with cytosine arabinoside (Ara-C; 1 μg/ml), an inhibitor of DNA synthesis, from day 2 through 5. The resultant cultures contained virtually no mononucleated cells (11, 19). Fibroblast cultures were established with the mononucleated cells obtained by trypsinizing 9-day secondary or tertiary muscle cultures as described (1); these cells synthesize Type I collagen chains and hyaluronic acid. Fig. 1 *a-c* are light micrographs of the "standard", "pure myotube," and "fibroblast" cultures respectively.

To study the time-course of the accumulation of myosin heavy chains in pure myotube and standard cultures, cells were rinsed and removed from each dish daily and homogenized in a Potter tissue grinder in 0.1-0.3 ml of a solution containing 1% sodium dodecyl sulfate (SDS), 2 mM dithiothreitol (DTT), 10 mM phosphate buffer (pH 7), 10% glycerol. The samples were heated in a boiling water bath for 5 min. After centrifuging at 2,000 *g* for 20 min to remove undissolved material, the resultant supernates were used for total protein determination and SDS-polyacrylamide gel electrophoresis.

Proteins were measured by the method of Lowry et al. (29), using bovine serum albumin as a standard. DNA was measured using Burton's modification of the Dische diphenylamine reaction and purified calf thymus DNA as a standard (10). Actomyosin was isolated by the method of Adelstein et al. (3, 4). Crude actomyosin obtained after three precipitations was used to determine the myosin/actin ratio. For myosin light chain analysis, the myosins were purified by KI-Bio Gel A 15 M column as described by Pollard et al. (37). For the determination of myosin as a percentage of total protein (wt/wt) as well as the relative mass ratio of myosin/actin and of myosin light chains, samples were electrophoresed on 7% SDS-polyacrylamide gels, whereas 12.5% gels were used for myosin light chain analysis (53). Gels were stained with Coomassie brilliant blue R 250 (0.25% made in 50% methanol-10% acetic acid), destained, and scanned on a Gilford spectrophotometer at 600 nm. The areas under the myosin heavy and light chain and actin peaks were cut out and weighed. It was previously established that the weight per unit area of chart paper was constant.

To obtain radioactive actomyosin, cultures were labeled with 10 μCi/plate [^{3}H]leucine (50 Ci/mmol) or 3 μCi/plate [^{14}C]leucine (280 mCi/mmol) for 24 h on day 6, and the actomyosins were extracted on day 7. The distribution of radioactivity was determined as follows: the gel was sliced into 1-mm thick pieces. Slices were placed in scintillation vials with 5 ml of a 90:10:2 mixture of scintillation fluid (1 × Liquifluor, New England Nuclear, Boston, Mass.): NCS (Nuclear Chicago tissue solubilizer):water (or 4M NH_4OH). Vials were tightly capped and agitated at room temperature, or in a 37°C incubator overnight, until the gel slices swelled and turned clear. Samples were counted on a Beckman liquid scintillation counter (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.).

ATPase was assayed at 25°C in 10 mM imidazole-HCl buffer (pH 7.0), 2 mM ATP, 0.6 M KCl, and either 2 mM EDTA, 10 mM $CaCl_2$, or 5 mM $MgCl_2$ (4). Inorganic phosphate (Pi) was measured by the modified method of Fiske and SubbaRow (12). The molecular weight of the entire myosin molecule and the percent total light chains or heavy chains in myosin were not determined in the present studies. The relative stoichiometry of the molarity of the light chains was estimated according to the equation of Lowey and Risby (27), assuming 17% total light chains in myosin and 470,000 daltons for the molecular weight of the myosin molecule. The molecular weights of myosin light chains were

calibrated from a standard curve prepared from a mixture of proteins of known molecular weights.

Nomenclature

Standard, primary muscle cultures contain a heterogenous population of cells. These include replicating cells in various compartments of the fibrogenic and myogenic lineages, as well as varying numbers of postmitotic myoblasts and postmitotic multinucleated myotubes (17). Currently there is no reliable method of discriminating among the various types of replicating mononucleated cells (1). Presumptive myoblasts are the replicating myogenic cells that generate the postmitotic mononucleated myoblasts. The myoblasts are the only cells in the myogenic lineage which have the capacity to fuse to form multinucleated myotubes. During the first 2 or 3 days of culture, approximately 80% of the replicating monunucleated cells are by definition presumptive myoblasts. As the cultures mature, the numbers of fibrogenic cells and myotubes increase whereas the numbers and proportion of presumptive myoblasts decrease (9).

RESULTS

Myosin and Actin in Standard Muscle Cultures and in Pure Myotube Cultures

Autoradiographic studies of these cultures have demonstrated that approximately 90% of the mononucleated cells will divide at least once, and some twice during the first 48 h in culture (5, 33). Fusion into multinucleated myotubes does not begin until early on day 3. Accordingly, any measurements made on day 1 and day 2 are measurements largely of the activity of presumptive myoblasts. By day 4, between 50–70% of the nuclei are in multinucleated myotubes, and by day 5 striated myofibrils can be observed in the living myotubes under the phase microscope. Fusion diminishes during day 5 and by day 6 is virtually completed. The mononucleated, replicating cells, however, still constitute a heterogenous, and changing population of fibrogenic and myogenic cells about which little is known. Because of their continued replication, the number of nuclei in the mononucleated cells often exceed the number of nuclei in the myotubes by day 6 or 7. These cultures will be referred to as "standard cultures". Obviously, the total contractile proteins from such cultures (Fig. 1) will be a mixture of those extracted from myotubes plus those extracted from the mononucleated cells.

To distinguish between the contractile proteins synthesized by mononucleated cells and the contractile proteins synthesized only in myotubes, the mononucleated cells were selectively killed by adding ara-C to standard cultures from day 2 through 5. There was no evidence that ara-C affected the normal differentiation of the myotubes in any manner. Spontaneous contractions were initiated around day 5 in both standard and ara-C treated pure myotube cultures.

Figs. 2 *a* and *b* are electron micrographs of myotubes in cultures initiated with equal aliquots of the same cell suspension; one set was treated with ara-C. In all structural details the myotubes in the two types of cultures are indistinguishable. From these two observations, we conclude that the ara-C had no significantly deleterious effect on either the assembly of thick and thin filaments into myofibrils or on the interaction between the thick and thin filaments required for contraction.

Fig. 3 shows the total protein per culture in both standard and pure myotube cultures. The difference between these curves reveals the impact of the inhibition of DNA synthesis from day 2. The block to DNA synthesis has two distinct effects: (*a*) fewer mononucleated cells survive, and (*b*), since fewer myoblasts are generated in treated cultures, the resulting myotubes contain fewer nuclei. Fig. 4 depicts the difference in total DNA between the two types of cultures.

To determine the weight percentage of total protein that was myosin in these cultures, the same protein concentration of whole-cell lysate (75 μg protein) was electrophoresed on SDS-polyacrylamide gels. Fig. 5 *a*, *b* depict densitometric scanning profiles of SDS-gel runs of total cell protein from 6-day standard muscle and pure myotube cultures, respectively. Myosin heavy chain and actin were localized by co-electrophoreing the whole-cell lysate with labeled actomyosin. Tracings of gel scans were cut out and weighed. The approximate percentage of total cell protein represented by myosin heavy chain (M) was estimated by comparing the weight under the M peak with that of the stained gel scan. The myosin heavy chain clearly separated from other large molecule weight proteins. The weight percentage of total protein that was myosin in both myotube and standard muscle cultures is shown in Fig. 6. The figures shown in Fig. 6 represent the minimum values. In standard muscle cultures, the percent weight myosin heavy chain hovers between 2 and 5% of the total protein. In the pure myotube cultures myosin, at a minimum, constitutes approximately 7% of the total protein. The maximum myosin value ever obtained was

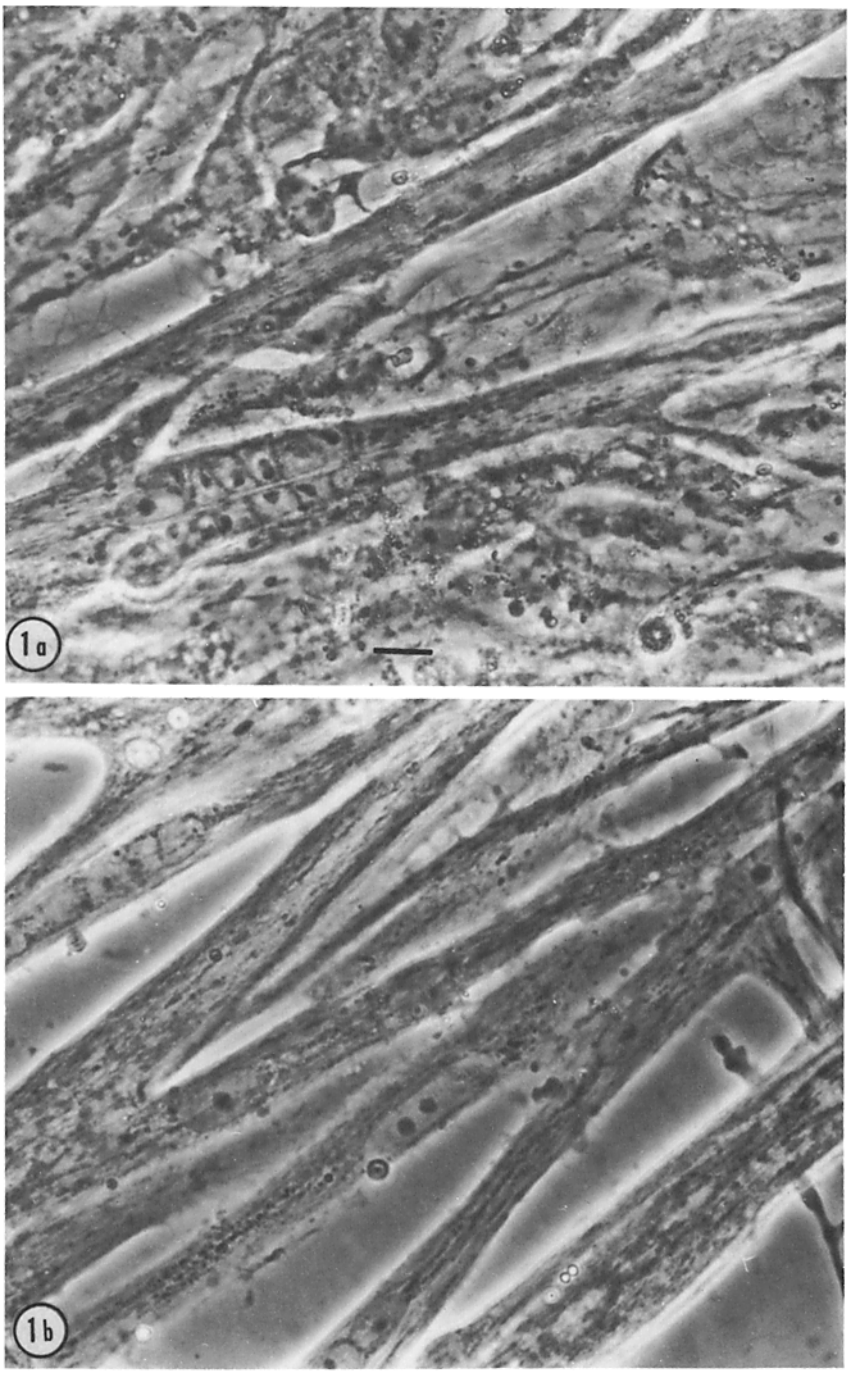

FIGURE 1 Micrographs of living, (*a*) 6-day standard muscle culture, (*b*) 6-day pure myotube culture. Pure myotube cultures were prepared by adding Ara-C to standard cultures from day 2 through 5; the fibroblast cultures were 3rd or 4th generation cultures prepared from primary cultures as described in Materials and Methods. Observe how the large numbers of mononucleated cells in Fig. 1 *a* tend to obscure the elongated multinucleated myotubes. In the older standard muscle cultures the mononucleated cells are a mixture of mononucleated myogenic and fibrogenic cells. The virtual absence of mononucleated cells in muscle cultures treated with Ara-C is shown in Fig. 1 *b*. This inhibitor of DNA synthesis kills the great majority of replicating myogenic and fibrogenic cells, but has no discernable effect on the myotubes that form from the postmitotic myoblasts. Cross-striations were evident in all myotubes after 4 days in culture and they contract spontaneously. Bar, 10 μm. $\times$ 780.

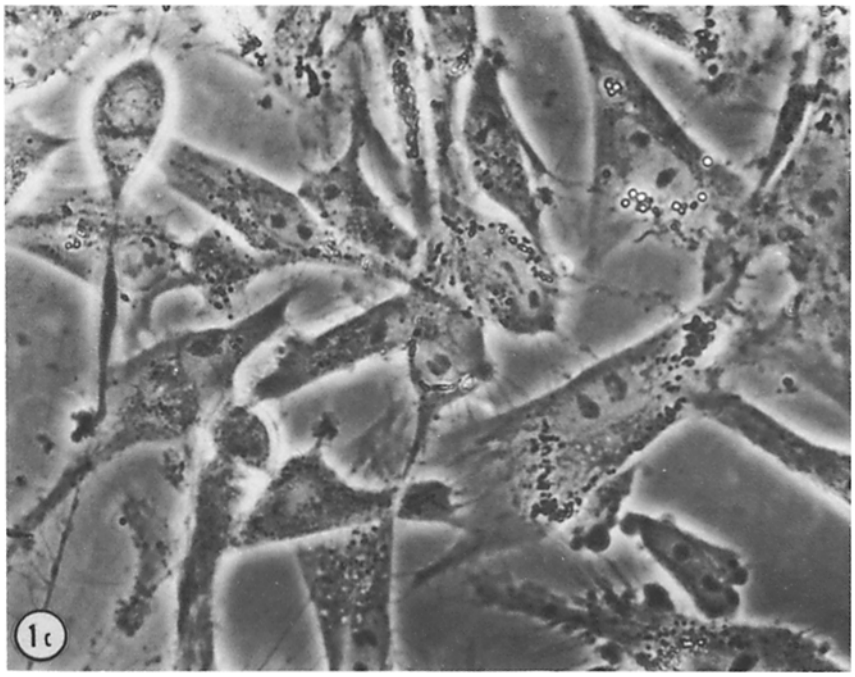

FIGURE 1 *c* Micrograph of living 4-day fibroblast culture. The mononucleated cells shown in here will form a dense, multilayered mat after 6 days in culture. These mononucleated cells secrete Type I collagen chains and hyaluronic acid(1). Fibroblastic cells have been cultured for a total of six passages and have never fused to form multinucleated myotubes. ×780.

about 15% of the total protein. As others have shown, there is a rise in the accumulation of myosin after fusion (36). If the data in Figs. 3, 4, and 6 are combined and the accumulation of myosin is expressed in terms of myosin/μg DNA then the amount of myosin in the pure myotube cultures is about 5- to 10-fold greater than that in the standard cultures. Although the increase of percent weight of myosin heavy chain in standard muscle cultures is not as apparent as in myotube cultures, the total content of myosin heavy chain per dish in standard muscle culture is higher than that in myotube cultures. The greater total amount of myosin from standard cultures over pure myotube cultures is due to, (*a*) the large number of fibroblasts in the former and (*b*) the fact that myotubes are more robust in standard than in pure myotube cultures.

It was of interest to determine whether the replicating mononucleated cells in the 2-day cultures—of which at least 70% are replicating presumptive myoblasts (5, 33)—synthesize a greater quantity of myosin heavy chain than do authentic fibroblasts. Accordingly, the quantity of myosin/total protein in 2-day old standard cultures was compared with 2-day old fibroblast cultures. The values for both cultures were between 1.5 and 2%. This is the same value obtained for cultured chondroblasts, BrdU-suppressed myoblasts, and gizzard cells, and is similar to that reported for a variety of other replicating normal and abnormal cells (57).

The ratio of myosin heavy chain to actin (wt/wt) in chondroblasts, fibroblasts, and the presumptive myoblasts in 1- or 2-day muscle cultures is approximately 0.8. In standard muscle cultures, between days 5–7, on the other hand, the ratio (wt/wt) is consistently around 1.5. These figures are identical to those previously published (41). Fig. 7 shows the radioactivity profile of actomyosin extracted from 7-day old standard and pure myotube cultures. The graph only coincidentally shows equal counts per minute for myosin heavy chains in both types of cultures. This is merely a result of the concentrations of ^{14}C and ^{3}H labels and does not reflect identical amounts of myosin heavy chain synthesized in both cases. Table I gives the weight ratio of myosin heavy chain to actin from the standard muscle, pure myotube, and pure fibroblast cultures.

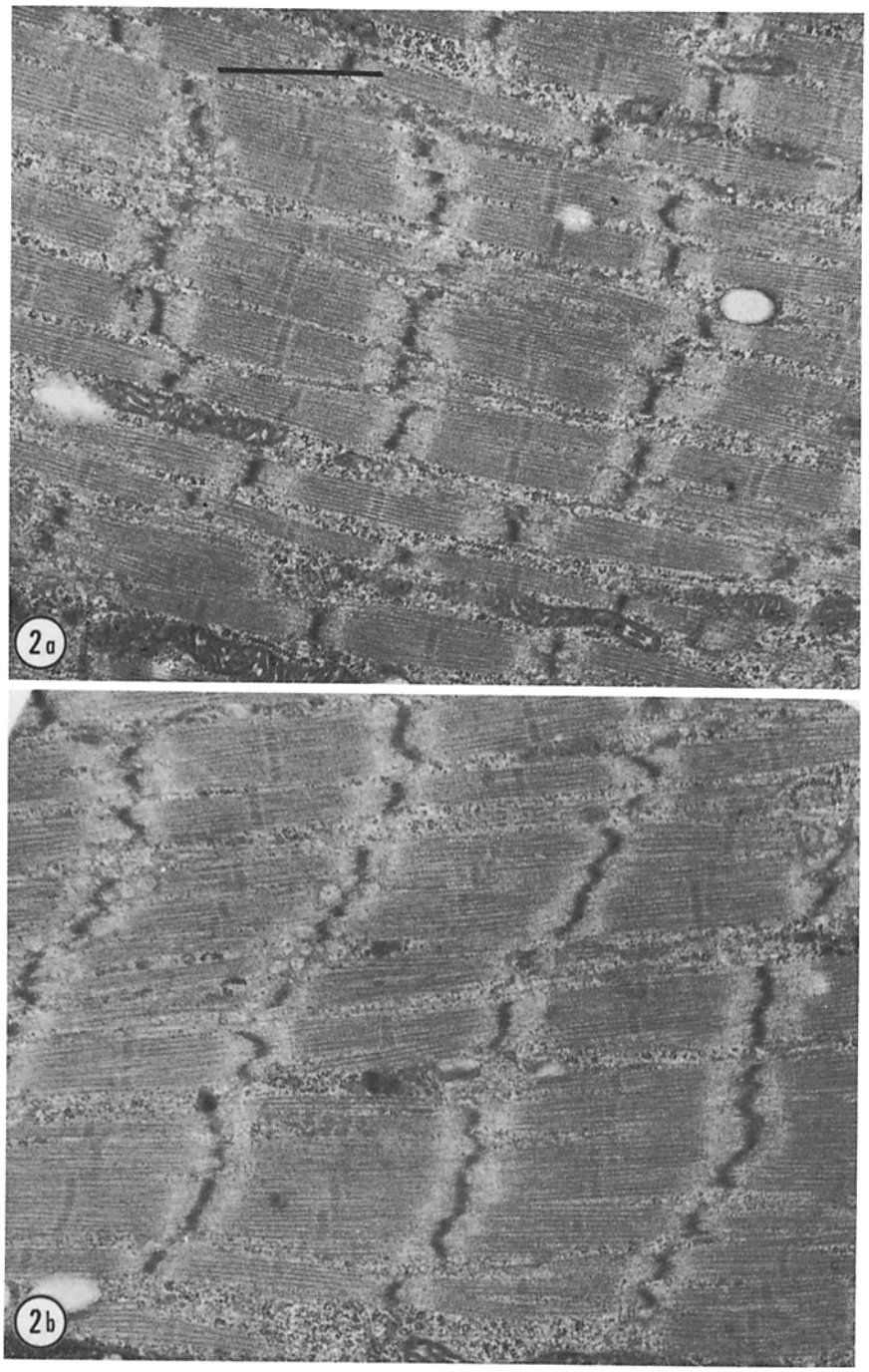

FIGURE 2 Electron micrographs of a myotube from (*a*) standard muscle culture and (*b*) Ara-C treated culture. At day 7, the cultures were fixed in 3% glutaraldehyde and prepared for electron microscopy. There are no detectable differences in structural details between the myotubes in the two types of cultures. Bar, 1 μm. × 21,000.

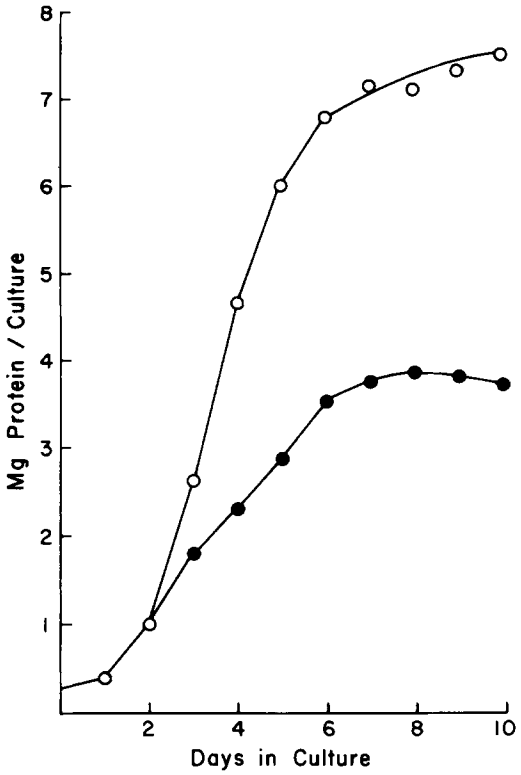

FIGURE 3 Total protein per culture in standard (O—O) and pure myotube (●—●) cultures.

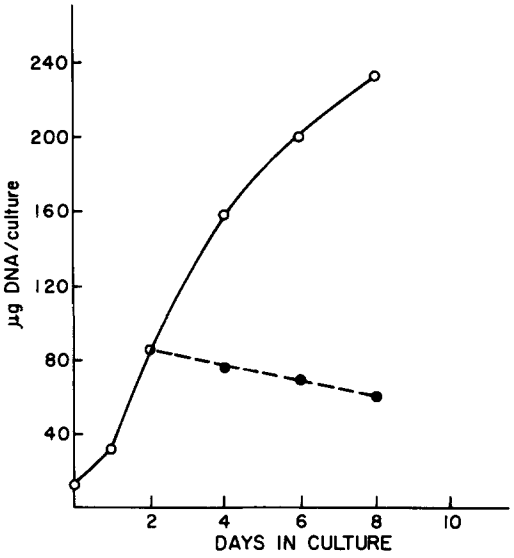

FIGURE 4 Total DNA per culture in standard (O—O) and pure myotube (●—●) cultures.

If one assumes that the myosin heavy chain comprises approximately 83% of the total myosin molecule (50), then the myosin/actin ratio (wt/wt) for pure myotube cultures averages 3.1, while the ratio (wt/wt) for the standard muscle cultures is 1.9. Clearly, the large number of mononucleated cells lowers the myosin/actin ratio in standard muscle cultures. It should be noted that addition of excess cold myosin to homogenates of fibroblast cultures did not change the ratio of labeled myosin/actin.

Myosin Light Chains in Standard and Pure Myotube Cultures

Distinctive patterns of light chains have been reported for fast, slow, cardiac, and smooth muscle myosins, as well as for platelet, fibroblast, and slime mold myosins (25, 27, 32, 35, 44, 51, 54). Sreter et al. (46) reported that myosins extracted from embryonic or adult chicken breast muscle as well as from myotubes that formed in vitro, contained light chains LC1, LC2, and LC3. The quantity of the light chain with the highest mobility, LC3, was variable in the muscle cultures and in the embryo. In these experiments, the contributions of the mononucleated cells to the light chain pattern was unknown. To examine this question, myosins from standard muscle cultures and pure myotube cultures were purified on Bio Gel A-15 M columns. Fig. 8 shows the elution pattern of myosin from pure myotube cultures from the Bio Gel column plus the ATPase activity of the eluted fractions. The ATPase activity of myosin from myotubes was slightly higher than that from standard muscle cultures. Fibroblast myosin, however, had a significantly lower ATPase activity (Table II). Similar elution profiles were obtained from preparations of myosin from standard muscle cultures and from pure fibroblast cultures. In each case the ATPase activity coincided with the first eluted peak.

Fig. 9 *a* is the typical pattern of myosin light chains from standard cultures prepared from embryonic breast myogenic cells. The three light chains are identical in mobility to those from adult breast muscle and have mol wt of approximately 25,000, 18,000 and 16,000 daltons. These are LC1, LC2 and LC3 respectively, and have a molar ratio of 1.0:1.9:0.5. Myosin from pure myotube cultures, however, reveals only two light chains (Fig. 9 *b*) which correspond to LC1 and LC2. The molar ratio of these two chains is 1.5:1.6. These findings demonstrate that the LC3 found in control muscle

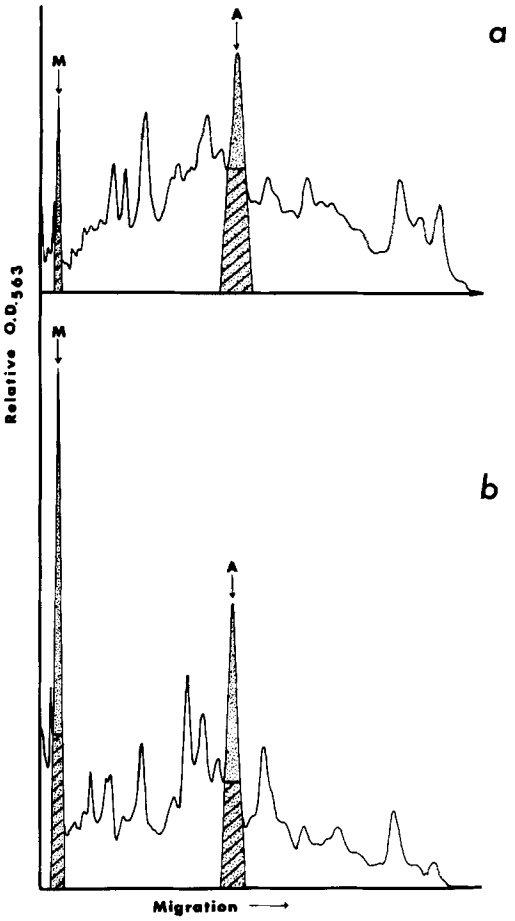

FIGURE 5 Densitometry tracings of total cell proteins from (*a*) 6-day standard muscle culture, and (*b*) 6-day myotube culture on 8% SDS-polyacrylamide gels. Cell pellets were dissolved in 1% SDS, 10 mM phosphate buffer (pH 7.0), 2 mM DTT, and 10% glycerol at 37°C for 1 h and then in a boiling water bath for 5 min. The shaded areas correspond to the position of myosin heavy chain (M), and actin (A). Sections of gel scans were cut up and weighed to determine the approximate percentage of cell protein that was myosin heavy chain. Minimum values are obtained from the stippled areas and maximum values are obtained from the stippled and striped areas respectively.

cultures is not in fact synthesized by the myotubes, but must be synthesized by the mononucleated cells that are present in these cultures. That, the mononucleated cells do contribute LC3 is shown in Fig. 9 *d*. The two light chains from the pure fibroblast myosin correspond to LC2 and LC3 from the standard muscle myosin and have a stoichiometry of 1.8:1.8. A trace of a band around 25,000 daltons was also noted. When myosins from fibroblast cultures and pure myotube cultures are combined, the light chain pattern of control muscle cultures is observed (Fig. 9 *c*). When these two myosins—from fibroblasts and from myotubes—are run on split gels (Fig. 9 *e*), the respective LC2's do not co-electrophorese, suggesting that these molecules are not identical. In summary: in standard muscle cultures it is probable that LC1 is synthesized only in the myotubes, whereas it is likely that LC3 is synthesized only in the mononucleated cells. LC2 is found in both myotubes and mononucleated cells, but these differ in molecular weight. The stoichiometry and molecular weights of the light chains from myotubes, mononucleated cells, and fibroblasts are given in Table III.

It should be noted that a protein band just ahead of LC2 can be seen in the gels of Fig. 10 *a*, *b*. The formation of this faster band can be prevented when the myosin samples were reduced with sufficient amount of DTT or mercaptoethanol in a closed system. This result indicated that the faster band was derived from LC2 due to the formation of a new form of LC2 with intramolecular S-S bridges. Similar results have been reported in rabbit skeletal myosin (43).

It is possible that the appearance of only two light chains in myotubes was a result of either (*a*) differential extraction or (*b*) the effect of cytosine arabinoside. To rule out these possibilities, the following experiments were performed.

(*a*) Pure myotube cultures were labeled with

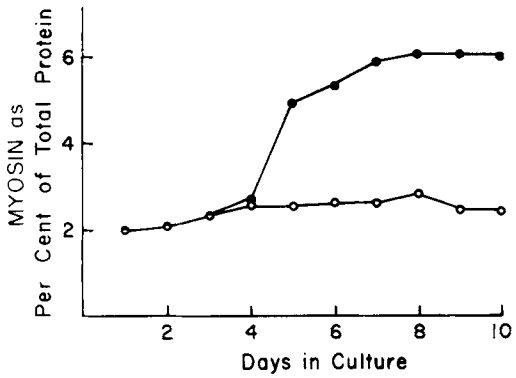

FIGURE 6 Myosin as percent of total protein in standard (O—O) and pure myotube (●—●) cultures. The percent weight myosin per pure myotube culture varies among experiments. However, the overall pattern of increase in both types of cultures is quite consistent. The percent weight of myosin ranges from 6 to 15%.

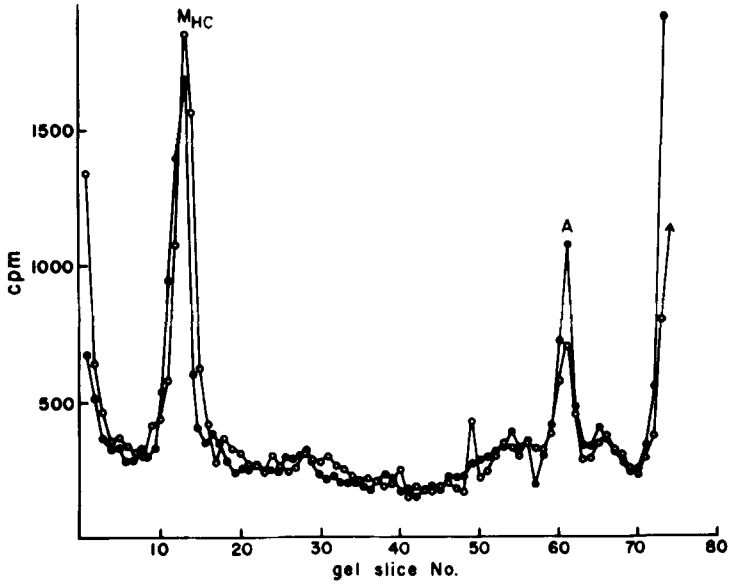

FIGURE 7 The radioactivity profile of actomyosin extracted from 7-day old standard (O—O) and pure myotube (●—●) cultures. For co-electrophoresis pure myotube cultures were labeled with [^{14}C]leucine and standard cultures were labeled with [^{3}H]leucine. On day 7, the cultures were rinsed and the living cells collected and mixed. The actomyosins from the mixed cells were isolated. The methods for SDS gels electrophoresis and radioactivity determination are described under Materials and Methods. M_{HC} = myosin heavy chain, A = actin.

TABLE I

*The Weight Ratio of Myosin Heavy Chain to Actin (M_{HC}/A)**

Methods	Type of culture		
	Standard (myotube + fibroblast)	Pure myotube	Fibroblast
Densitometry tracing of SDS-gels	1.53 ± 0.23	2.36 ± 0.60	0.82 ± 0.14
Radioactivity‡ pattern	1.64 ± 0.47	2.63 ± 0.81	0.93 ± 0.29

Addition of excess unlabeled myosin to labelled homogenates did not cause a significant change in the ratio of extracted labeled myosin/actin ratio.

* Average of five-eight determinations + SE.

‡ Numbers have been corrected for the amount of leucine in the heavy chain (9.5 leucine/100 amino acids) vs. that in actin (8.4 leucine/100 amino acids).

[^{3}H]leucine for 24 h while standard cultures were not labeled. The living cells were mixed and the myosins purified together. As shown in Fig. 10, the electrophoresis pattern illustrates three protein bands by spectrophotometry (LC1, LC2, and LC3) and only two bands (LC1 and LC2) labeled with [^{3}H]leucine. A similar experiment with standard muscle cultures labeled with ^{14}C-amino acids and myotube cultures with ^{3}H-amino acids showed three light chains for standard muscle cultures and two light chains for myotube cultures (data not shown). This demonstrates that the appearance of two bands in myotube preparations is not an artifact of extraction.

(*b*) Tertiary cultures of fibroblasts were grown to confluency. At that time, cytosine arabinoside was added for 3 days under the same conditions as for myotubes. Myosins were extracted and purified. The same light chain pattern was obtained as for control fibroblast cultures; hence the ara-C did not interfere specifically with the synthesis of the 16,000 dalton light chain.

(*c*) FudR, an inhibitor of DNA synthesis, was added to standard cultures on days 2 through 5. The resulting cultures contained almost pure populations of myotubes. Only two myosin light chains, identical to those obtained in myotubes from ara-C treated cultures, were found.

(*d*) EGTA (1.8 mM) was added to the muscle cultures from day 0 to day 4 to block cell fusion (36). The replicating mononucleated cells were removed by the ara-C treatment from day 2 to day 4. After day 4, the cultures were grown in the

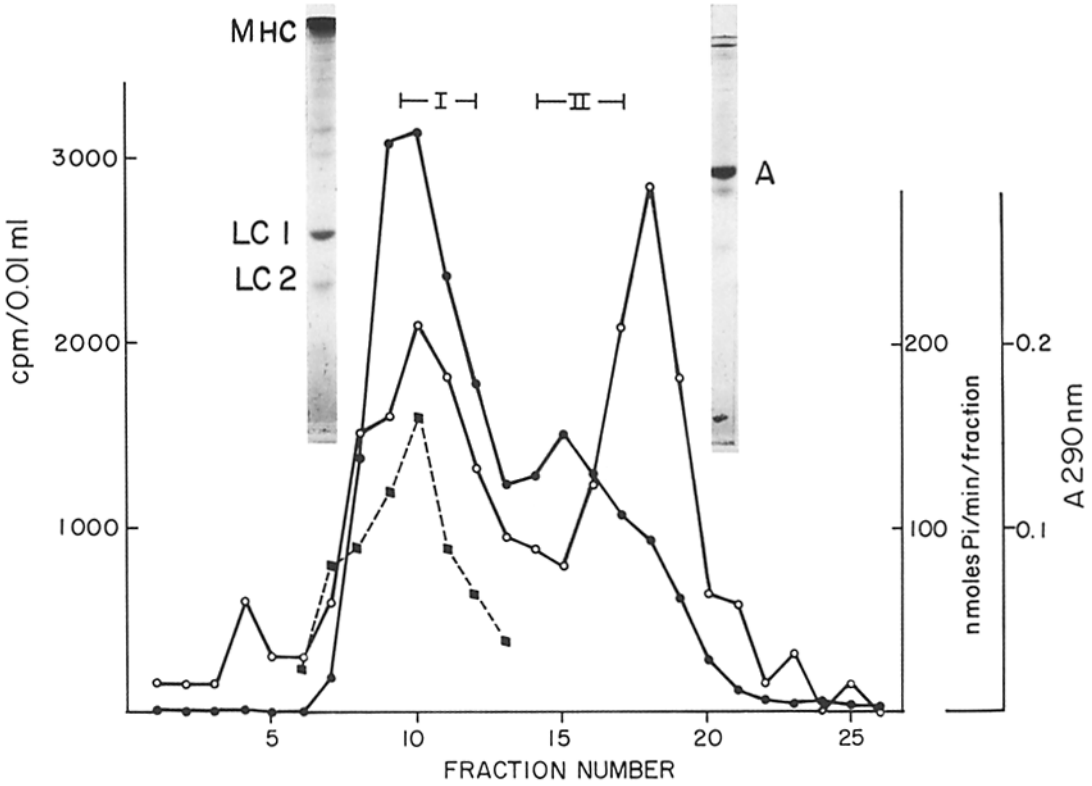

FIGURE 8 Chromatographic purification of myosin isolated from the 7-day old pure myotube cultures. About 5 mg protein was applied to a 22 × 0.7 cm Bio Gel A-15 column equilibrated with column buffer containing 0.6 M KCl, 10 mM imidazole (pH 7), 1 mM DTT, 0.5 mM ATP, and 0.1 mM $MgCl_2$. Crude actomyosin obtained after three precipitations was dissolved in a small volume of KI-ATP buffer (0.6 M KCl, 5 mM ATP, 5 mM DTT, 1 mM $MgCl_2$, and 20 mM imidazole pH 7.0) and clarified by centrifugation. Aliquots of the supernate were applied to the top of the column which had been pre-run with a 15% bed volume of KI-ATP. After washing on the sample with an appropriate volume of KI-ATP buffer, the protein was eluted with column buffer. Radioactivity ([^{3}H]leucine cpm/0.01 ml, ●—●), absorbence at 290 nm (A_{290nm}, O—O) and ATPase activity (nmol Pi/min/fraction ◆---◆) were determined. Fractions 9-12 (I) and 15-18 (II) were pooled, dialyzed against 5 mM phosphate buffer pH 7.0, and concentrated by freeze-drying. Protein was dissolved in 1% SDS-1% mercaptoethanol-10 mM phosphate buffer, 35% glycerol and analyzed by SDS-polyacrylamide gel electrophoresis.

standard medium for 3 more days in the absence of ara-C. In these experiments, in contrast to those previously described, fusion occurred and the myotubes developed after ara-C had been removed. The myotubes that formed in these cultures synthesized only LC1 and LC2.

DISCUSSION

These experiments confirm our earlier report (41) that replicating, presumptive myoblasts and fibroblasts do in fact synthesize readily detectable quantities of myosin heavy and light chains and actin. These findings render untenable the claims of Strohman and co-workers that replicating myogenic cells do not transcribe myosin mRNA (36, 39, 40), or the claims of Yaffe and co-workers that though myosin mRNA is transcribed as an informosome in replicating myogenic cells, it is translated only in multinucleated myotubes (39, 58, 59, 60).

In standard muscle cultures the increase in myosin in terms of percentage of total protein is relatively slight, but only because it is obscured by other kinds of proteins produced by the large number of presumptive myoblasts and fibroblasts. A more accurate appreciation of myosin heavy chain content in maturing myotubes is provided by

TABLE II

ATPase Activity of Purified Myosins from Myotube plus Fibroblast, Pure Myotube, and Fibroblast Cultures

Cell types	Specific activity	
	EDTA-K-ATPase	Ca-ATPase
	nmol/mg protein/min	
Standard (myotube + fibroblast)	686 551	479
Pure myotube	747 626	521
Fibroblast	290 281	223

ATPase was assayed at 25°C for 10 min. The reaction mixture contains 10 mM imidazol-HCl (pH 7.0), 2 mM ATP, 0.6 M KCl, and either 2 mM EDTA or 10 mM $CaCl_2$, and protein in a final vol 1.0 ml. Pi was measured by the modified method of Fiske and SabbaRow (12), and protein by the method of Lowry et al. (29).

the cultures treated with ara-C. It will now be of considerable interest to determine whether the rate of heavy chain synthesis/DNA/unit of time is, in fact, significantly different in postmitotic myoblasts vs. myotubes.

The determination of the ratio of myosin/actin is affected by the presence of mononucleated cells in standard muscle cultures. In our standard muscle cultures, the large number of mononucleated cells with a low myosin/actin ratio is capable of reducing the overall myosin/actin ratio to approximately 1.6. Although there may be fewer mononucleated cells in muscles in vivo, measurements of myosin/actin ratios made on a piece of muscle must be viewed with some caution. Under conditions where the muscle mass is reduced, as in various diseased states, the relatively large number of connective tissue cells could contribute significantly to the myosin/actin ratio.

Our findings with respect to light chains also stress the contribution of the mononucleated population when extracts of "muscle" are analyzed. The addition of ara-C or FudR to standard muscle cultures produces populations of pure myotubes without perceptibly influencing fusion, the synthesis of contractile proteins, or their assembly into thick and thin filaments. The normal appearance of sarcomeres and the fact that the pure myotubes begin to contract at the same time as do control cultures demonstrate that LC3 is dispensible for both the structure and function of the thick, myosin filament.

In the myotubes each myosin molecule consists of two heavy chains and two light chains, LC1 and LC2. Several investigators have reported that LC2 does not contribute to ATPase activity (13, 26, 27, 42). If the LC2 synthesized in myotubes is similar to that found in mature muscle, then it follows that the spontaneous contractions observed in in vitro myotubes is mediated by LC1 alone. The lack of LC3 in myotube cultures is unusual. Clearly, in the standard muscle cultures, LC3 must be a contribution only of fibroblasts. Morimoto and Harrington (30, 31) found that purified thick filaments from mature rabbit psoas, a fast muscle, contained LC1, LC2, and LC3; the smallest chain being present in the least amount. Weeds and Hall (55) also

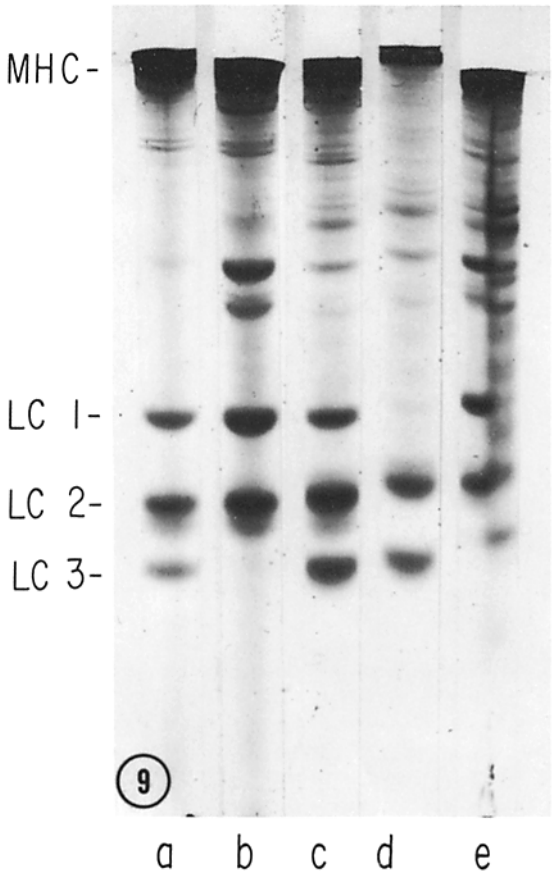

FIGURE 9 Electrophoresis of myosin in 12.5% polyacrylamide gels: (*a*), standard culture myosin; (*b*), pure myotube culture myosin; (*c*), co-electrophoresis mixture of pure myotube and fibroblast myosins; (*d*), fibroblast culture myosin; and (*e*), split gel; left, pure myotube myosin and right, fibroblast myosin. Note that the LC2 from myotube does not co-electrophorese with the heavier light chain from fibroblasts.

TABLE III

Stoichiometry and Molecular Weights of Myosin Light Chains

Light chains mol wt	Type of culture					
	Standard		Myotube		Fibroblast	
daltons	*% Mass**	*(Mol.)‡*	*% Mass*	*(Mol.)*	*% Mass*	*(Mol.)*
25,000	35 ± 3.5	(1.0)	57 ± 0.9	(1.5)	—	—
20,000	—	—	—	—	55 ± 2.0	(1.8)
18,000	52 ± 3.7	(1.9)	43 ± 0.9	(1.6)	—	—
16,000	13 ± 5.9	(0.5)	—	—	45 ± 2.0	(1.8)

(Moles) of light chain in myosin = (17%) (% mass) (mol wt of myosin)/(mol wt of light chain).

* The figure represents average of four–six determinations + SE.

‡ The relative stiochiometry of the molarity of light chains were estimated according to the equation of Lowey and Risby (23) assuming that total light chains in myosin is 17% and the molecular weight of entire myosin molecule is 470,000 daltons.

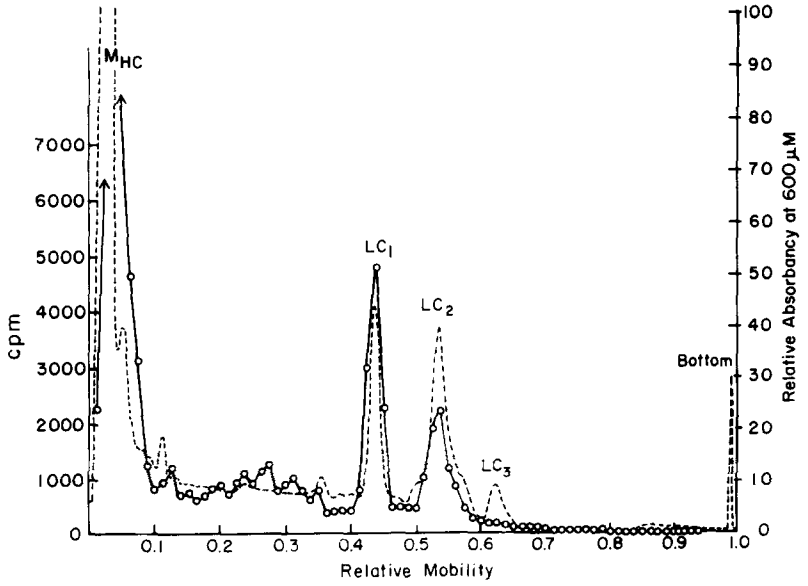

FIGURE 10 Co-migration of myosins from control and pure myotube cultures. The pure myotube cultures were labeled with 10 μCi/plate [^{3}H]leucine (30 Ci/mmol) for 24 h at day 6. The labeled cells were collected and mixed with the unlabeled control cells. Actomyosins were isolated from the mixed cells and myosins were purified by Bio Gel A-15 M column as described under Fig. 8. Myosins were subjected to polyacrylamide gel electrophoresis in the presence of SDS. The gel was scanned and then sliced. The radioactivity was determined as described. (O—O), radioactivity of pure myotube myosin light chains; (-----), densitometry tracing at 600 nm.

demonstrated that a single mature rabbit muscle fiber contains all three light chains. If LC3 is an integral part of the thick filament in all fast muscles, including mature breast muscle, then it follows that: (*a*) this light chain is synthesized only in relatively mature muscle, and (*b*) the synthesis of the three light chains is not tightly coupled. This is consistent with the finding that the myosin molecules of adult fast muscle consist of a heterogenous population as suggested by Sarkar (42) and Starr and Offer (47). Conceivably, early myotubes synthesize only LC1 and LC2, whereas in older muscle either all three or only two of the light chains are synthesized in any one muscle fiber. For example, some fibers might synthesize LC1 and LC2, whereas others only synthesize LC1, and

LC3, or LC2 and LC3. The absence of LC3 is not due to lack of innervation or to limiting in vitro conditions, for it is also lacking in breast muscle from 16- to 20-day chick embryos (Chi and Holtzer, unpublished results).

An alternative explanation of the presence of LC3 in isolated thick filaments is that, during the protracted glycerination the LC3 from the connective tissue cells present in whole muscle becomes associated with the thick filaments secondarily. This interpretation is rendered less probable by the observation that slow muscles, which also contain associated fibroblasts, do not exhibit a light chain with a mol wt around 16,000. However, when there are large changes in the proportions of muscle fibers to connective tissue cells as in various degenerative diseases or in experiments involving nerve-muscle switches, simple analysis of the quantity or quality of light chains could be misleading.

In contrast to finding LC1 and LC2 in myotubes, presumptive myoblasts and fibroblasts only synthesize two light chains of different molecular weights. The light chain pattern for these mononucleated cells is similar to that reported for other types of replicating cells (35).

The interesting issue now is to determine the relationship between the myosin heavy and light chains synthesized by presumptive myoblasts and nonmyogenic cells on the one hand, with the kinds of myosin synthesized by myotubes on the other hand. Of significance in this context is the observation that antibodies prepared against skeletal myosin and against skeletal tropomyosin do not cross-react with the myosins or tropomyosins from presumptive myoblasts, chondroblasts, nerve cells, or even smooth muscle cells (16–18, 22). Conversely, antibody prepared against platelet myosin, chicken gizzard myosin, as well as against mouse L929 and uterus myosins, do not precipitate skeletal myosin (37, 52, 57). Elsewhere (8, 20–22), we have demonstrated that antibody against light meromyosin in Ouchterlony tests will not precipitate myosin heavy chains from presumptive myoblasts, fibroblasts, chondroblasts, or BudR-treated myogenic cells. Similarly, fluorescein-labeled antibodies against light meromyosin are not bound by presumptive myoblasts or nonmyogenic cells; such antibody is bound only along the lateral edges of emerging and definitive A bands. Equally interesting is the fact that fluorescein-labeled antibody to light meromyosin is not bound to either the external or internal aspects of the cell surface of myogenic or nonmyogenic cells (8). These findings strongly suggest that the structural genes regulating the synthesis of the myosin heavy and light chains in presumptive myoblasts and nonmyogenic cells are different from those active in myoblasts and myotubes. The former might be the "constitutive" myosins (18, 22) synthesized by all types of cells, the latter only synthesized in terminally differentiated fast, slow, cardiac, or smooth muscle cells. According to this view, the transition from replicating presumptive myoblast to daughter, postmitotic myoblasts would initiate the translation of the definitive myosins mRNAs (8, 20, 21). This view of myogenesis stresses the qualitative differences between presumptive myoblasts and postmiotic myoblasts in contrast to the quantitative differences proposed by others (48). This view is also consistent with the notion of a quantal cell cycle separating replicating presumptive myoblasts from their daughter, postmitotic, mononucleated myoblasts (9, 18, 20, 21).

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