SYNTHESIS OF TROPOMYOSIN IN CULTURES OF DIFFERENTIATING MUSCLE CELLS

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ABSTRACT

The accumulation of tropomyosin in cultures of differentiating muscle cells was quantitatively measured. Tropomyosin was isolated from cultured cells during and after myoblast fusion; both α- and β-subunits were present in myotube cultures. During fusion small amounts of tropomyosin were detectable, but, as fusion approached a maximum, tropomyosin accumulation began to increase. The increased synthesis of tropomyosin after the initiation of muscle cell fusion is consistent with the increased synthesis of other proteins characteristic of muscle, including myosin.

KEY WORDS fusion · muscle · myogenesis · tropomyosin · isotope dilution

Muscle cell differentiation is accompanied by changing patterns of gene expression, including those genes responsible for the synthesis of proteins involved in the energy metabolism characteristic of muscle (19–21) and proteins involved in the conduction of nerve impulses to muscle (6, 12). The cellular events that have received the most attention, however, have been the synthesis of myofibrillar proteins and subsequent assembly of these proteins into myofibrils. Myofibril assembly has been studied extensively with electron microscopy, and several detailed descriptions of myofilament aggregation and organization have been presented (see review by Fischman, [5]). Biochemical events associated with myofibrillar protein synthesis and assembly have not been described in comparable detail. Of the seven to nine myofibrillar proteins, myosin (3, 4, 14, 23) and actin (13, 17, 18) are the only ones the synthesis of which has been monitored in synchronous populations of differentiating muscle cells. These investigations have demonstrated that synthesis of both myosin and actin increases dramatically during or shortly after myoblast fusion in muscle cell cultures, although neither myosin synthesis (4) nor actin synthesis (Allen, unpublished observations) is dependent upon cell fusion.

Tropomyosin has not been studied in populations of differentiating muscle cells from culture, but its presence in developing embryonic muscle has been documented (8, 15, 16). Conflicting reports concerning the accumulation of tropomyosin during muscle development have been presented. Heywood and Rich (7) analyzed sucrose gradient polyribosome profiles from chick embryo leg muscle and noted differential changes, with increasing embryonic age, in the relative proportions of polyribosome fractions involved in the synthesis of myosin, actin, and tropomyosin. They concluded that tropomyosin synthesis increases later in development than the increase in actin and myosin synthesis. Hitchcock (8) monitored the development of Ca^{2+} sensitivity of actomyosin from embryonic chick muscle and reported reduced Ca^{2+} sensitivity and Ca^{2+} binding in muscle from embryos 14 days or younger. Because troponin and tropomyosin are required for Ca^{2+}

sensitivity, Hitchcock suggested that troponin or the troponin-tropomyosin complex is deficient in early embryonic natural actomyosin. Masaki and Yoshizaki (10), Potter and Herrmann (15), and Roy et al. (16), however, have suggested that the synthesis of tropomyosin is not delayed, but that tropomyosin is present early in development and can be detected as early as myosin or other myofibrillar proteins. We have used isotope dilution experiments to monitor accumulation of tropomyosin in synchronized populations of differentiating muscle cells in culture, thus enabling us to relate the tropomyosin synthesis pattern to skeletal muscle cell differentiation.

MATERIALS AND METHODS

Muscle Cell Cultures

Primary muscle cell cultures were prepared from leg muscles of 12-day chick embryos and were grown on 100-mm collagen-coated culture dishes as previously described (23). Because these cultures also contain fibroblasts, it was necessary to minimize the contribution of any fibroblast tropomyosin-like molecules to the net accumulation of tropomyosin in the culture. This was accomplished by adding fluorodeoxyuridine, to a final concentration of 2.5×10^{-7} M, to all muscle cultures at 48 h. By this time in culture, most myogenic cells had ceased DNA synthesis and either had fused or were in the process of fusing; therefore, they were not noticeably affected. Growth of the proliferating population of fibroblasts, however, was halted because of the inhibition of DNA synthesis, thereby limiting the fibroblast population to a constant level. On the basis of the final percentage fusion in these cultures, ~30% of the cells present at 48 h were either fibroblasts or proliferating myogenic cells that had not differentiated to a point of being competent to fuse.

The total number of nuclei and the percentage fusion were determined on randomly selected cultures that had been previously fixed in methanol for 5 min and stained with Giemsa stain for 15 min. The total number of nuclei in 10 random microscope fields in each dish was counted; most fields contained 50–150 nuclei, depending on the age of the culture. Nuclei were scored as being within either mononucleated cells or multinucleated fused cells, and the percentage fusion was calculated as the number of nuclei within multinucleated cells divided by the total number of nuclei counted.

Isotope Dilution Technique

Before isolation of tropomyosin from cultured muscle cells at different ages, a constant amount of radioactively labeled muscle culture myofibrils, having a known tropomyosin concentration and specific activity, was added to each culture sample. Knowing the mass (C_1) and specific activity (SA_1) of the labeled tropomyosin and the specific activity of tropomyosin in the final sample mixture (SA_2), the concentration of tropomyosin in the original muscle culture (C_2) could be calculated according to the formula:

$$C_2 = \frac{C_1(SA_1 - SA_2)}{SA_2}.$$

C_1 was determined by gel quantitation of a known volume of labeled myofibril homogenate, and SA_1 and SA_2 were determined by dividing the radioactivity in a tropomyosin band by the protein mass, as determined by gel quantitation.

Preparation of Radioactive Myofibrils

Cultures used as sources of radioactive myofibrils were labeled from 72 to 144 h in culture with L[4,5-^{3}H]leucine (5 mCi/mol) (New England Nuclear, Boston, Mass.) at a level of 5 μCi/ml of complete culture medium. Myofibrils used for isotope dilution were prepared by scraping labeled muscle cell cultures from the dish in 0.1 M KCl, 20 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, pH 6.8 (standard salt solution), and fragmenting the myotubes by vortexing for 2 min at top speed. Fragmented myotubes were sedimented at 3,000 g_{max} for 30 min. The supernate was discarded, and the pellet was resuspended in standard salt solution. The suspension was again vortexed at top speed for 1 min and resedimented as before. The pellet was then resuspended in 3 ml of 0.1 M NaCl, 5% Triton X-100, 9 mM tris-HCl, pH 7.6, and vortexed at top speed for 1 min. After sedimentation at 3,000 g_{max} for 15 min, the pellet was resuspended in the same solution with three strokes of a Dounce homogenizer (Knotes Co., Vineland, N. J., type B pestle) and sedimented at 3,000 g_{max} for 15 min. The myofibril pellet was resuspended in 0.1 M NaCl, 5 mM sodium phosphate, pH 7.0 with 10 strokes of a Dounce homogenizer.

Constant amounts of these labeled myofibrils were added to unlabeled cultures of muscle cells after they were scraped from the dish. An additional aliquot of labeled myofibrils was prepared for electrophoresis by adding one-half volume of 5.2% sodium dodecyl sulfate (SDS), 4.6 M 2-mercaptoethanol, 0.33% bromphenol blue, 60 mM sodium phosphate, 20% glycerol, pH 7.0, and placing the sample in a boiling water bath for 10 min. Triplicate gels of the aliquot at each of three different loading volumes were evaluated to determine the mass (C_1) and specific activity (SA_1) of tropomyosin in the aliquot of myofibrils added to the unlabeled cultures.

Isolation of Tropomyosin

Culture medium was decanted, and cultures were rinsed with 0.25 M sucrose. Then, a glycerination solution, composed of 50% glycerol, 0.1 M KCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.8, was added to each culture dish. Cells were scraped from the dish with a plastic spatula and were stored in

glycerination solution at 2°C for 48–96 h. The glycerinated cell suspension was centrifuged at 3,000 g_{max} for 30 min, and the supernate was discarded. Pellets were resuspended in 3 ml of 50 mM KCl, 10 mM Tris-HCl, 0.5 mM 2-mercaptoethanol, pH 7.6, and then centrifuged for 15 min at 3,000 g_{max}. To completely remove the glycerol and soluble proteins, the washing cycle was repeated two more times. In some experiments, all of the previous centrifugations were performed at 30,000 g_{max} for 30 min in order to sediment any tropomyosin that would have been bound to actin filaments or small filament aggregates that would not have been sedimented at 3,000 g_{max}. Identical results for tropomyosin synthesis were obtained with these two centrifugation methods. The residue was then subjected to two 3-ml washes with cold 95% ethanol followed by two 3-ml washes with cold anhydrous ether. The final pellet was dried for 4–5 h, resuspended in 1 ml of 1.0 M NaCl, 25 mM tris-HCl, 0.1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, pH 8.0, and extracted overnight at room temperature. The suspension was sedimented at 3,000 g_{max} for 15 min, and the supernate was removed and salted out between 40 and 60% ammonium sulfate saturation. The P_{40-60} precipitate was resuspended in 1 ml of 1 M NaCl, 20 mM sodium acetate, pH 4.6, and sedimented at 16,650 g_{max} for 30 min. The tropomyosin-rich pellet was prepared for electrophoresis by resuspending in 50 μl of 1% SDS, 10 mM sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.0, 25 μl of 4.6 M 2-mercaptoethanol, 0.03% bromphenol blue, 5.2% SDS, 60 mM sodium phosphate, pH 7.0, 20% glycerol, and then heating at 100°C for 10 min.

Two-Dimensional Electrophoresis

Muscle cells, grown in the presence of 5 μCi/ml [^{3}H]leucine, were glycerinated and washed as previously described. All supernates were pooled and the pH was adjusted to 4.6; after centrifugation at 30,000 g_{max} for 30 min, the pellet was retained and the remaining supernate was discarded. Carrier tropomyosin, which had been prepared from porcine skeletal muscle (2), was added to some of the samples before isoelectric precipitation.

The glycerinated pellet and the pH 4.6 precipitate from the original supernatant fractions were each resuspended and subjected to isoelectric focusing on 9-cm polyacrylamide gels according to the procedure of O'Farrell (11). Isoelectric focusing disc gels were removed from the tubes and prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by equilibrating with 0.065 M Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, pH 7.0 for 30 min at room temperature and 30 min at 80°C. SDS-PAGE was performed on 10% polyacrylamide gels (9) in the Ortec slab gel electrophoresis system (Ortec In., Oak Ridge, Tenn.). Dimensions of the slab gel were 8.5 × 10 × 0.3 cm. 3 ml of 1% agarose in equilibration solution (melted at 80°C) was added to the top of the polymerized slab gel; the equilibrated isoelectric focusing gel was immediately placed on the layer of agarose and covered with 1 ml of agarose solution. Gels were electrophoresed at 30 mA per slab for 5 h. The slab gels were stained overnight in 0.1% Coomassie Brilliant Blue R, 50% methanol, and 7% acetic acid, and were destained with several changes of 5% methanol, 7% acetic acid for 3–5 days.

Electrophoresis and Gel Quantitation

SDS-polyacrylamide disc gel electrophoresis was conducted as described by Weber and Osborn (22), and protein quantitation was performed according to Allen et al.[1] This procedure involved electrophoresis on 7½% SDS-polyacrylamide disc gels 8 cm in length and 0.5 cm in diameter. Tropomyosin was electrophoresed at 8 mA per gel until the tracking dye had traveled 6 cm into the gel. Gels were stained for 16–20 h in 0.1% Coomassie Brilliant Blue R 250, 50% methanol, and 7% acetic acid, and were destained electrophoretically in a Canalco Quick Gel Destainer (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) in 7% acetic acid. Gels were stored in destaining solution composed of 5% methanol and 7% acetic acid until they were scanned at a wavelength of 550 nm 7 days after electrophoresis.

Densitometry was performed with a Zeiss PMQ II spectrophotometer equipped with a thin-layer chromatogram scanning attachment that was modified to hold gel cuvettes. Peak areas were integrated by a Spectra-Physics System I Computing Integrater (Spectra-Physics Inc., Santa Clara, Calif.).

The amounts of tropomyosin obtained from samples in these experiments ranged from 0.2 to 5 μg which were included within the linear portion of the tropomyosin standard curve.

Gel Slicing and Liquid Scintillation Counting

The radioactivity in protein bands of disc gels was determined by freezing the gel and cutting 0.8-mm slices through the band with a Mickle gel slicer (Brinkmann Instruments, Inc., Westbury, N. Y.); protein spots in slab gels were cut from unfrozen gel slabs. Gel slices were placed in polyethylene mini-vials and dissolved in 0.2 ml of 30% H_2O_2 at 50°C for 3 h before adding 4 ml of Aquasol (New England Nuclear, Boston, Mass.). Samples were counted in a model 3320 Packard Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), and raw counts per minute were converted to disintegrations per minute by using an automatic external standardization method.

RESULTS

Tropomyosin was isolated from the extract of ethanol-ether-dried powder of cultured muscle

[1] Allen, R. E. Manuscript submitted.

cells by ammonium sulfate fractionation. The P_{0-40} ammonium sulfate fraction was heterogeneous but contained a major band that co-migrated with actin (Fig. 1, gel a). The P_{40-60} pellet, after a wash with 1 M NaCl, 20 mM sodium acetate, pH 4.6, contained primarily tropomyosin (Fig. 1, gel b). Considering the small quantities of the initial samples, the isolation procedure resulted in a preparation quite rich in tropomyosin with few contaminating proteins, therefore diminishing the possibility of measuring any contaminating protein that may have co-migrated with tropomyosin. When small quantities of tropomyosin from muscle cultures were electrophoresed on 10% SDS-polyacrylamide gels, the tropomyosin band was resolved into α- and β-tropomyosin subunits (Fig. 1, gel d) with an approximate α:β ratio of 2:1. Both α- and β-subunits were present in cultured muscle cells at all ages examined in these experiments.

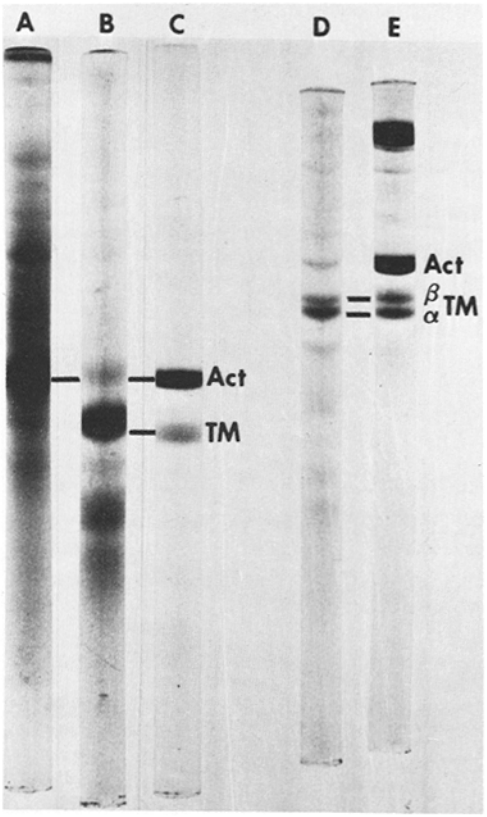

FIGURE 1 SDS-polyacrylamide gel electrophoresis of muscle cell culture tropomyosin. (*a*) 7½% gel of P_{0-40} ammonium sulfate fraction of muscle cell culture crude tropomyosin extract. This fraction contains several proteins; the major one co-migrates with actin. (*b*) 7½% gel of the insoluble tropomyosin pellet after P_{40-60} ammonium sulfate fractionation and subsequent extraction with 1 M NaCl, 20 mM sodium acetate pH 4.6. (*c*) Purified porcine skeletal muscle actin (*Act*) and tropomyosin (*TM*) electrophoresed on 7½% gels. (*d*) 10% gel of muscle cell culture tropomyosin showing evidence of α- and β-tropomyosin subunits. (*e*) 10% gel of purified porcine skeletal muscle actin and α- and β-tropomyosin subunits.

To determine whether significant amounts of tropomyosin were being lost during the glycerination process, two-dimensional gels were used to analyze the composition of the supernatant and pellet fractions remaining after the glycerination and washing steps. If large amounts of tropomyosin exist in these cells as free molecules or associated with free thin filaments, evidence for the presence of this tropomyosin pool should be found in two-dimensional gels of the pooled supernatant fractions. As demonstrated in Fig. 2, the supernatant gel (Fig. 2*a*) is practically devoid of staining in the tropomyosin region (arrows), as compared to the gel of the glycerinated cell pellet (Fig. 2*b*). In the gel of the cell pellet, actin isozymes (bracket) and both tropomyosin subunits (arrows) are readily visible. Radioactivity measured in the tropomyosin spots in the gel of the glycerinated cell pellet and in the tropomyosin region of the supernatant gel demonstrated that most of the tropomyosin in these cells remains with the cell residue. When carrier porcine skeletal muscle tropomyosin was added, 77.2 ± 7.4% of the radioactivity remained with the cell pellet, and, in the absence of carrier tropomyosin, 72.0 ± 6.0% of the cellular tropomyosin was isolated in the cell pellet after the glycerination and washing steps. The addition of carrier tropomyosin, therefore, did not significantly enhance the precipitation of tropomyosin from the pooled supernates. Because the radioactive tropomyosin was added before glycerination, much of the unlabeled tropomyosin that was lost during glycerination and washing would still have been included in the total tropomyosin measured in these cultures. A primary assumption associated with these isotope dilution experiments is that the added radioactive tropomyosin behaves the same as the unlabeled tropomyosin present in the original sample, with respect to subsequent purification steps. If a portion of the tropomyosin lost during the glycerination and washing steps actually existed as a free pool of soluble tropomyosin, it may not have

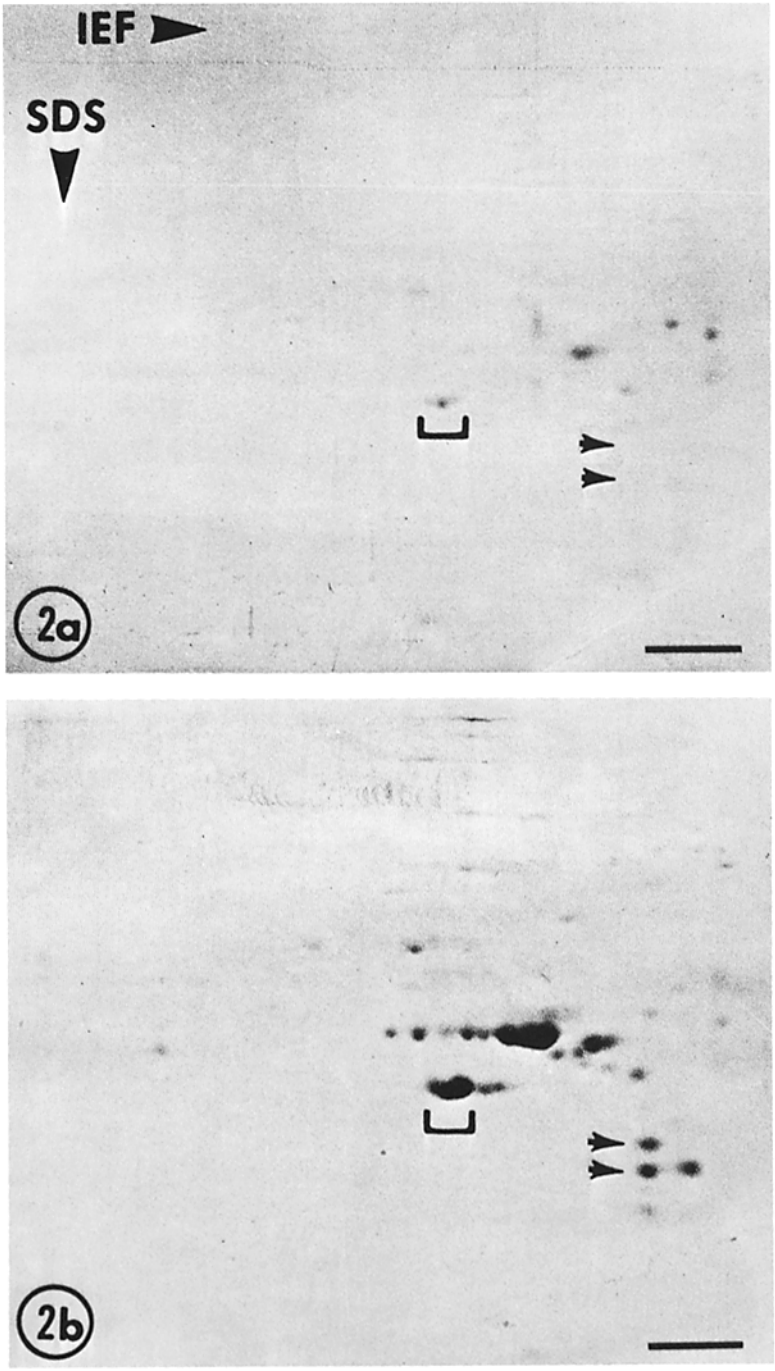

FIGURE 2 Two-dimensional gels of pooled supernates (*a*) and the residual pellet (*b*) remaining after glycerination and washing of muscle cultures. Isoelectric focusing (*IEF*) of cell proteins provided separation in the horizontal dimension; the pH gradient is decreasing from left to right. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (*SDS*) provided protein separation in the vertical dimension. Each gel contains 0.05 ml of the respective 0.5-ml supernatant and pellet fractions of one muscle culture from a 15-cm dish. Tropomyosin subunits are indicated by arrows in gel (*b*); the tropomyosin region of gel (*a*) is also identified with arrows. Actin (brackets) is present in both gels. In these experiments, myosin did not enter the *IEF* gels and was subsequently lost during equilibration in preparation for the second dimension. Bar, 1 cm.

behaved in the same manner as the actin-associated radioactive tropomyosin. Therefore, this pool might not have been measured in these experiments. However, if such a soluble pool existed, it would only represent a minor fraction of the total tropomyosin accumulating in the cells.

Under our culture conditions, the rapid burst of fusion occurred between 24 and 48 h and, in the presence of fluorodeoxyuridine, reached a maximum of ~70%. The inhibition of fibroblast proliferation by fluorodeoxyuridine resulted in a relatively stable cell population as indicated by the fusion curve (Fig. 3). Without fluorodeoxyuridine, percentage fusion would have reached a maximum at about 70 h and then declined throughout the remainder of the experiment as a result of continued proliferation of fibroblasts. Accumulation of tropomyosin, as measured by our isotope dilution techniques, began to increase about the time maximum fusion was reached (Fig. 3). Tropomyosin accumulation was linear between 72 and 120 h which indicates that the rate of accumulation was constant throughout the course of these experiments.

DISCUSSION

By applying isotope dilution techniques to the quantitation of tropomyosin in muscle cell cultures, only a portion of the total tropomyosin in the culture had to be isolated to obtain a specific activity determination. Consequently, the requirement of quantitative recovery of tropomyosin throughout the isolation procedure, including the glycerination and washing steps, was circumvented. Therefore, we were at liberty to apply conventional large-scale tropomyosin purification techniques to the isolation of a tropomyosin-rich fraction from the small quantities of muscle obtained from culture.

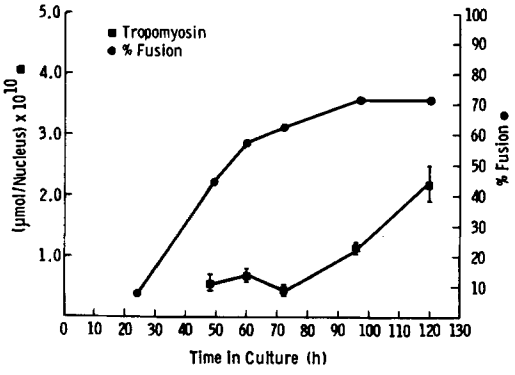

FIGURE 3 Accumulation of tropomyosin in differentiating muscle cell cultures. (■) Indicates number of molecules of tropomyosin subunit per nucleus. Each point represents the average of three determinations, the vertical bars encompass ±1 SEM. (●) The fusion curve serves as a reference point in muscle cell differentiation.

The accumulation of tropomyosin in differentiating embryonic skeletal muscle has been described (8, 15, 16); however, the relationship between tropomyosin synthesis and specific markers of muscle cell differentiation, such as cell fusion, has not been previously determined. Our observations provide evidence for the presence of tropomyosin in fusing myogenic cells and for the increased accumulation of tropomyosin shortly after fusion. In addition, both α- and β-tropomyosin subunits were synthesized in cultures started from leg muscle; this is consistent with the report by Roy et al. (16) and Amphlett et al. (1) of the presence of α- and β-tropomyosin subunits in developing embryonic chick leg muscle and embryonic rabbit muscle, respectively. Furthermore, the presence of radioactivity in the tropomyosin region of two-dimensional gels of the supernate remaining after glycerination indicates that a small fraction of the total tropomyosin may exist in a soluble pool, or at least in a fraction which is not associated with filament aggregates that are sedimented at centrifugal forces up to 30,000g.

Activation of myosin synthesis in culture has been reported to occur in association with myoblast fusion, either during the rapid burst of cell fusion (4, 23), at the peak of fusion (14), or shortly after cell fusion (3). Actin synthesis is also accelerated in fused cells (13, 17, 18). Because tropomyosin accumulation increases as the peak of cell fusion is approached, the relationship between tropomyosin synthesis and myoblast fusion appears to be identical to that described for myosin and actin synthesis. Recent experiments performed in our laboratory have actually demonstrated the simultaneous increase in tropomyosin and myosin heavy chain accumulation in differentiating muscle cells (Allen, unpublished observations). Therefore, the increase in tropomyosin synthesis is evidently not delayed compared with synthesis of actin and myosin as suggested by Heywood and Rich (7). Furthermore, evidence that tropomyosin is synthesized shortly after fusion and is therefore present throughout assembly strengthens the proposal that myofibrillar proteins assemble simultaneously into myofibrils, as op-

posed to the idea that different myofibrillar proteins are added sequentially to assembling myofibrils. Tropomyosin accumulation evidently reflects the same abrupt change in gene expression during the final phase of muscle cell differentiation that has been previously established for other proteins characteristic of skeletal muscle (3, 4, 12, 13, 19–21).

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