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Protein synthesis and actin heterogeneity in calf muscle cells in culture

(myogenesis/cell-free protein synthesis/two-dimensional electrophoresis/nonmuscle actin)

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ABSTRACT **Pulse-labeled cytoplasmic proteins from cultured fetal calf muscle cells at various stages of development were analyzed by one- and two-dimensional gel electrophoresis. The high resolution of the two-dimensional technique allows the determination of those protein species that begin to be synthesized after cell fusion. In addition, actin has been found to exist in three forms possessing similar biochemical properties and identical molecular weights but having slightly different isoelectric points. Two of the forms are found in prefusion dividing myoblasts and also in cultured kidney cells. The third form is the only one found in fetal muscle tissue and is predominant in cultures of fused muscle cells. Thus, it would seem that actin can exist in several isozymic forms of which one is specific to fused muscle tissue.**

The effect of environmental factors on the physiological characteristics of muscle fibers is a question of long-standing interest (1). The influence of so-called "trophic factors" on the expression of the muscle phenotype has been clearly demonstrated in experiments in which conversion of fast to slow muscle traits occurs upon interchange of the respective nervous innervation (2). Likewise, the embryological development of muscle tissue may be subject to environmental and trophic influences. Two of the problems encountered in studying such phenomena are the biochemical definition of the muscle phenotype and how its genetic expression is regulated. For nerve cross-innervation, the characteristic pattern of myosin light chains has been used as a biochemical marker of a given muscle phenotype. In addition, myosin heavy chain or tropomyosin might exist in different forms in various muscle fibers or fetal muscle, given the available evidence on structural variation of these proteins (3–6).

The ability to grow muscle cells in tissue culture offers possibilities for studying the muscle phenotype and its expression either while following the formation of muscle fibers from mononucleate myoblasts or by examining myotubes in the presence or absence of various trophic influences such as nerve cells. Muscle cells in culture seem to recapitulate many of the events that accompany terminal differentiation of muscle tissue *in vivo;* e.g., an increase in myosin heavy chain synthesis (7, 8), the appearance of the acetylcholine receptor (9–11), increased production of acetylcholinesterase (12), and the induction and isozymic conversions of muscle specific enzymes (13). Although cultured muscle cells thus represent a well controlled model system, very little is known about the overall patterns of protein synthesis.

This report concerns an analysis of protein synthesis in cultured calf embryo muscle cells. A high-resolution two-dimensional electrophoretic technique has been used to document those differences between proteins synthesized in dividing and fused muscle cell cultures. Even though a significant number of differences are found, the observation can be made that most protein species are synthesized at both stages. Furthermore, actin exists in three forms, with one predominating after cell fusion. This form appears to be muscle specific, whereas the others are found in nonmuscle tissue and dividing myoblasts. This study thus defines some of the protein components involved in the phenotypic expression of replicating and fused muscle cells.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

MATERIALS AND METHODS

Cell Culture and Protein Labeling Procedures. Fetal calf muscle cells were grown as described (14) in culture medium composed of Dulbecco's modified Eagle's medium and medium 199 (3:1). The medium was supplemented with 10% fetal calf serum (Gibco, Grand Island, NY) or 15% horse serum (Gibco) in addition to 1% chicken embryo extract (Eurobio, Paris, France). Culture dishes were labeled at 37° for 1 or 2 hr either by adding L-[^{35}S]methionine (200 Ci/mmol, Amersham) directly to the existing medium or by washing the dishes with warmed phosphate-buffered saline and adding serum-free modified Eagle's medium containing a total of 5 μM unlabeled methionine in addition to [^{35}S]methionine. The final concentration of radioactive methionine was 30–70 μCi/ml. After labeling, the dishes were washed with cold phosphate-buffered saline and the cells were scraped into 0.25 M NaCl, 0.02 M Tris·HCl at pH 7.6, 0.005 M $MgCl_2$, and 0.5% (vol/vol) Nonidet P-40. After 10 min at 0° with occasional mixing by pipetting, the lysed cells were centrifuged at 1000 × *g* for 10 min to remove nuclei. To prepare samples for sodium dodecyl sulfate ($NaDodSO_4$) electrophoresis, we precipitated the supernatant with four volumes of acetone. For two-dimensional electrophoresis, the supernatant was treated with ribonuclease A (10 μg/ml, 10 min at 0°), solid urea (Schwarz/Mann) was added to 9 M, and an equal volume of solution A [9.5 M urea, 2% (vol/vol) Nonidet P-40, 2% ampholines (LKB) (1.6% at pH 5–7 and 0.4% at pH 3.5–10), and 5% 2-mercaptoethanol] was added as described by O'Farrell (15).

Electrophoresis. Slab gel electrophoresis was done in gels of 15% acrylamide and 0.087% bisacrylamide containing 0.1% $NaDodSO_4$ (BDH, specially pure) (16). After the gels were stained with Coomassie blue and destained, they were dried and autoradiograms were prepared by exposure to Kodirex x-ray film. Samples that had been precipitated with acetone were resuspended in 2% $NaDodSO_4$, 0.065 M Tris·HCl at pH 6.5, and 5% 2-mercaptoethanol and heated for 3 min at 100° before analysis. Isoelectric focusing and two-dimensional electrophoresis were performed as described by O'Farrell (15), with a mixture of ampholines of 1.6% at pH 5–7 and 0.4% at pH 3.5–10. The first-dimension isoelectric focusing gels were either stained with Coomassie blue or equilibrated for 2 hr in 2.3% $NaDodSO_4$, 0.065 M Tris·HCl (pH 6.5), and 5% 2-mercaptoethanol, and then applied to a second-dimension $NaDodSO_4$

slab gel of 12.5% acrylamide and 0.1% bisacrylamide. These gels were stained, destained, dried, and autoradiographed as above.

Cell-Free Protein Synthesis. The cell-free system of Schreier and Staehelin (17) was used with initiation factor fractions IF-A and IF-B. Total cytoplasmic RNA, extracted as described (14), was used to direct incorporation *in vitro* with [^{35}S]methionine (200 Ci/mmol) at 180 μCi/ml as the isotope. The final ionic conditions were 90 mM KCl, 2.0 mM Mg acetate, and 0.8 mM spermidine (16). After incubation at 35° for 2 hr, the reaction mixtures were treated with ribonuclease A (20 μg/ml, 10 min at 35°), precipitated with acetone, and analyzed as above on $NaDodSO_4$ slab gels.

Actin Purification. Acetone powders prepared from fetal calf muscle tissue, cultured muscle cells at various stages, and confluent cultured calf kidney cells were extracted with 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM Tris base, and 0.5 mM 2-mercaptoethanol (final pH 7.5) at 0° for two 10-min periods (18). The extracts were centrifuged at 27,000 × *g* for 2 hr, and the ionic strength of the supernatant was adjusted to 0.1 M KCl and 0.001 M $MgCl_2$. After incubation for 2 hr at room temperature and 4° overnight, the F-actin was centrifuged at 130,000 × *g* for 2 hr. The resulting pellets were dissolved directly in solution A (15) for isoelectric focusing.

RESULTS

Analysis of Major Protein Species. Mononucleate fetal calf muscle cells synthesize DNA and divide during the first 2–3 days in tissue culture, at which time they cease cell division and begin to fuse to form multinucleate myotubes. By the fourth or fifth day they attain maximal values of 50–65% of total nuclei in fused myotubes (14, 19). To ascertain what changes in major polypeptide species might occur during this period, we have electrophoretically analyzed cytoplasmic proteins. The spectrum of labeled proteins from differentiating muscle cells on $NaDodSO_4$-containing slab gels is shown in Fig. 1a–e. In general, most of the bands seen are synthesized at all stages of culture, although some significant quantitative and qualitative changes can be observed. As expected, an increase in the synthesis of the myosin heavy chain (200,000 molecular weight) begins during cell fusion and continues to increase even after a plateau of cell fusion is reached (Fig. 1c–e). In contrast, the synthesis of actin (molecular weight 43,000 in Fig. 1a–e) does not seem to increase greatly, comprising 12% of total radioactivity incorporated before fusion and about 17% after, as estimated by direct determination of radioactivity of the actin band cut from a similar gel. One polypeptide species of molecular weight 50,000 is synthesized only after cell fusion and appears to be stable, since its accumulation can be noted on stained gels. Furthermore, this band is clearly enriched in preparations of glycerinated cells (Fig. 1f) as well as in glycerinated fetal calf muscle tissue (not shown, similar to Fig. 1f), and thus may have some structural role. A few other changes can be observed in proteins smaller than 40,000, although the identity of these polypeptides is not known. Since a significant number of cells do not fuse under these culture conditions, it is possible that proteins synthesized by these mononucleate cells obscure changes normally associated with myogenesis. However, cultures treated with FdUrd or AraC at the onset of cell fusion to kill cells continuing to synthesize DNA (8, 20), exhibit labeling patterns essentially identical to those of untreated cultures (not shown).

Additional experiments have been done in which total cytoplasmic RNA from muscle cells before and after fusion was isolated and translated in the cell-free protein-synthesizing system described by Schreier and Staehelin (17) (R. G. Whalen, manuscript in preparation). Analysis of the cell-free products by $NaDodSO_4$ gel electrophoresis (Fig. 1g–i) indicates that virtually the same major polypeptides are synthesized regardless of the stage at which the RNA was isolated. Thus large changes in the translatable cytoplasmic messenger RNAs do not seem to accompany cell fusion, a result consistent with the labeling patterns *in vivo* shown in Fig. 1a–e.

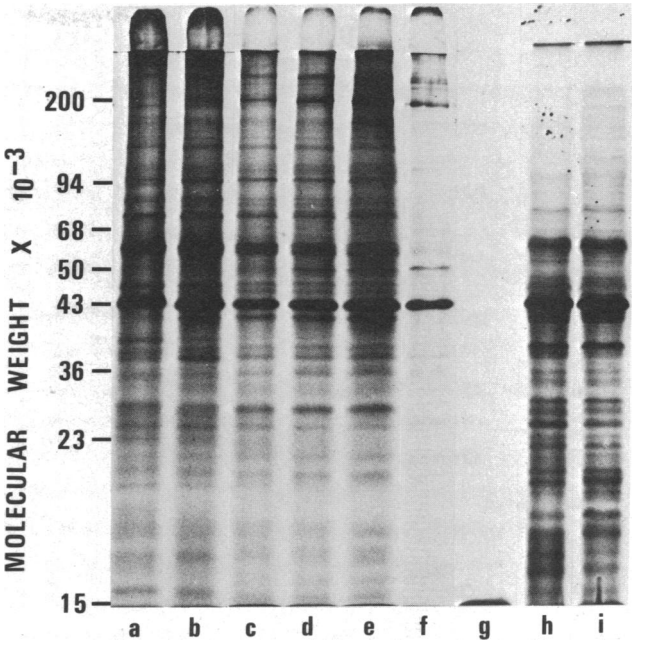

FIG. 1. Autoradiogram of ^{35}S-labeled proteins analyzed by $NaDodSO_4$ slab gel electrophoresis. Cytoplasmic extracts were prepared from muscle cell cultures grown in 15% horse serum and labeled for 1 hr at 26 hr (a), 46 hr (b), 71 hr (c), 93 hr (d), and 114 hr (e) as described in *Materials and Methods*. Sample f represents ^{35}S-labeled fused cells that had been glycerinated, washed (18), and analyzed similarly. In samples a–e approximately equal cpm were applied to the gel, and the autoradiogram was exposed for 22 hr. Samples g–i are equal aliquots of the cell-free translation system programmed with no RNA (g), 28 μg of cytoplasmic RNA from 44-hr cultures (h), and 35 μg of RNA from 115-hr cultures. The amount of radioactivity applied to the gel was 32,000 cpm (g), 375,000 cpm (h), and 362,000 cpm (i), and the autoradiogram was exposed for 45 hr. The standard proteins used for molecular weight calibration were myosin, phosphorylase *b*, bovine serum albumin, gamma globulin heavy and light chains, actin, lactate dehydrogenase, and hemoglobin.

Two-Dimensional Gel Analysis. The one-dimensional gel analysis used above is clearly not sufficient to reveal the synthesis of minor protein components. Furthermore, isozymic conversions or minor secondary modifications of proteins occurring during myogenesis may not be detected in the analysis based uniquely on molecular weight separation. The two-dimensional electrophoretic technique described by O'Farrell (15), involving isoelectric focusing under disaggregating conditions followed by $NaDodSO_4$ gel electrophoresis, gives a relatively uniform two-dimensional distribution of labeled proteins from calf muscle cells (Fig. 2). Many species that appeared as single bands in the one-dimensional analysis seemed to separate into several spots of similar molecular weight but with different isoelectric points. The two-dimensional analysis reveals very clearly those changes that occur between the first (Fig. 2A) and sixth day (Fig. 2B) of culture.

The myosin heavy chain (Fig. 2B, coordinates 1.5 to 3.6 × 1.1) does not produce distinct spots, possibly due to aggregation

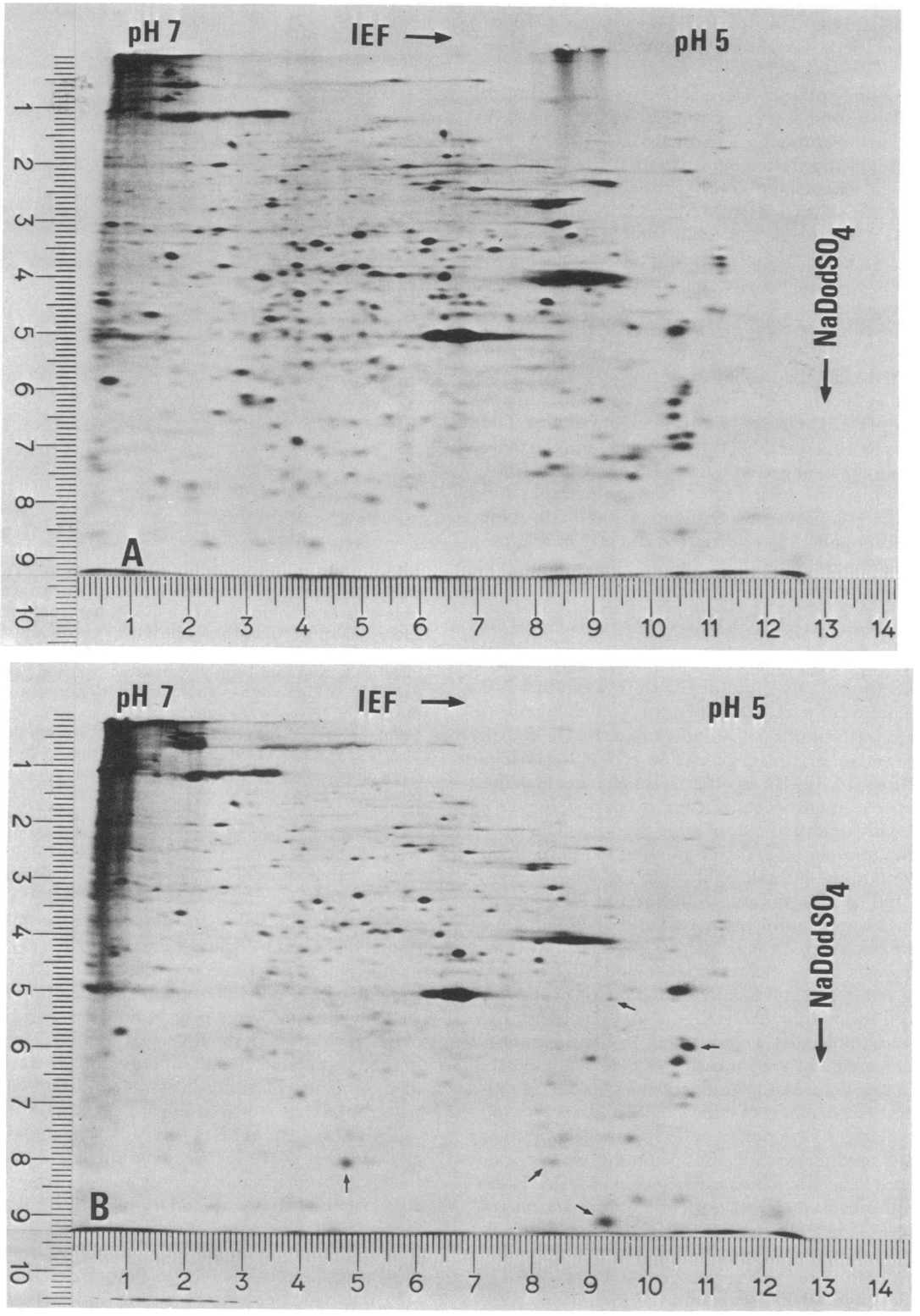

FIG. 2. Autoradiogram of ^{35}S-labeled cytoplasmic proteins separated by two-dimensional electrophoresis. Cultures grown in 10% fetal calf serum were labeled for 2 hr on day 1 (A) and day 6 (B). Fusion began in this culture on day 3. In (A), 410,000 cpm were applied to the gel and the autoradiogram was exposed for 361 hr. In (B), 350,000 cpm were applied and the autoradiogram was exposed for 506 hr. The coordinates referred to in the *text* are given as horizontal × vertical, as established by the metric rulers photographed with the gels. IEF, isoelectric focusing.

under the conditions of low ionic strength of the isoelectric focusing, and its expected quantitative increase after fusion (see Fig. 1a–e) is not clearly evident. The 50,000 molecular weight protein (Fig. 2B, coordinates 6.7 × 4.3) is labeled more intensely on day 6 than on day 1. The two major tropomyosin components (Fig. 2B, coordinates 10.5 × 6.2 and 10.7 × 6.0) likewise are more strongly labeled after fusion than before. Some of the spots appearing only after fusion are marked with an arrow in

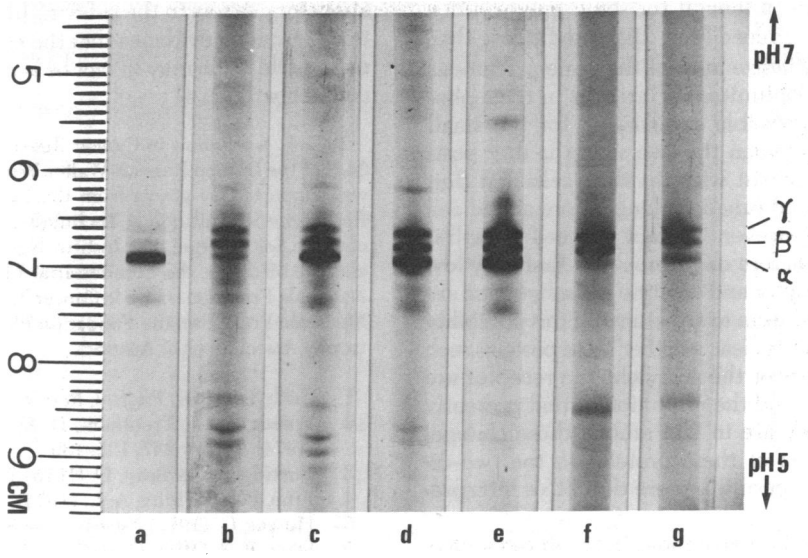

FIG. 3. Stained isoelectric focusing gels of purified actin from various sources. Actin was prepared as described in *Materials and Methods* from fetal calf muscle tissue (a), day 2 muscle cultures (b), day 4 muscle cultures (d), day 8 muscle cultures (e), and confluent kidney cell cultures (f). Muscle tissue actin was added to day 2 cultured cell actin (c) and to confluent kidney cell actin (g) to indicate the precise location of α-actin. Only the middle part of the original 12 cm gel is shown.

Fig. 2B. Surprisingly, even among the less abundant protein species revealed by this technique, a great deal of similarity is seen before and after fusion, although quantitative variations can be noted in the labeling of certain polypeptides. A pulse-labeled sample prepared from the same muscle culture at the onset of fusion (day 3) and analyzed by the two-dimensional technique showed a pattern intermediate between the two shown in Fig. 2. Thus, there do not appear to be protein species synthesized only at the time of cell fusion that cease to be synthesized after fusion is completed.

At the position of actin in Fig. 2B (coordinates 6.4 to 7.0 × 5.1), a series of multiple spots can be seen. In addition, the most acidic of these spots is intensified after fusion. These species, abundantly synthesized before and after fusion in agreement with the observations of Fig. 1, might represent multiple forms of actin. This point was investigated further.

Heterogeneity of Actin. Several unique properties of actin facilitate its purification and identification. Acetone powders were prepared from fetal calf muscle tissue and cultured calf muscle and kidney cells. Actin, which resists denaturation by acetone, was extracted from the powder as G-actin and then polymerized in the presence of KCl to yield F-actin (18). The resulting F-actin was collected by centrifugation and analyzed by isoelectric focusing under disaggregating conditions. The actin purified from fetal muscle tissue gives essentially one component (Fig. 3a), whereas actin from prefusion cultured muscle cells yields predominantly two other components (Fig. 3b). That it is the muscle tissue form of actin that is diminished in prefusion cells is shown by mixing of the two samples (Fig. 3c). These three major forms are tentatively called α, β, and γ-actin; α-actin is the muscle tissue form and the species with the most acidic isoelectric point. As the muscle cultures develop, α-actin becomes the predominant form, consistent with its presence in well-fused muscle tissue (Fig. 3d and e). Actin prepared from confluent cultured kidney cells is composed almost entirely of β- and γ-actin (Fig. 3f), as shown by the mixing with muscle tissue actin (Fig. 3g). Thus, the β and γ forms may occur in nonmuscle tissue as well as in prefusion muscle cells. The possibility that the observed heterogeneity is an artifact of the electrophoresis conditions is rendered less likely by the fact that the presence of one, two, or three forms can be obtained, depending on the source of the material. Furthermore, the appearance of multiple spots of closely similar isoelectric point is clearly not a general feature of the two-dimensional technique (Fig. 2). When labeled actin was purified from fused muscle cultures and subjected to the complete two-dimensional separation, the three forms migrated with exactly the same apparent molecular weight, similar to their behavior in Fig. 2 (result not shown). These three forms contained 83% of all the radioactivity on the gel; 14% was associated with the two tropomyosin components copurifying with the actin. (A further 3% was associated with an unknown component of molecular weight 35,000.) Thus the multiple species must be derived from actin heterogeneity, since all three forms possess very similar biochemical properties and isoelectric points and have identical molecular weights.

DISCUSSION

The results described here represent a basis for further study of protein synthesis in developing muscle cells in culture. The high resolution of the two-dimensional electrophoresis technique will permit the simultaneous determination of rates of synthesis for various polypeptides. Such resolution is necessary since it is likely that many of the characteristic markers of muscle cells may be present as very minor components or synthesized in relatively small amounts. For example, the acetylcholine receptor comprises only 0.005–0.05% of radioactivity incorporated during labeling experiments (ref. 10, and J. Merlie, unpublished results), and the muscle form of creatine phosphokinase may make up only about 0.1% of total protein after cell fusion, based on activity and total protein measurements (19). For several such specific markers, it seems that cell fusion is not required to trigger their appearance although, morphologically, fusion serves as a useful indication of normal differentiation. Whether some of the protein species whose synthesis accompanies myotube formation are in fact coupled to cell fusion can be tested under conditions that block this process (19).

One qualitative observation that can be drawn from the present results is that most of the changes in protein synthesis after fusion represent the appearance of new protein species

rather than disappearance, although for some polypeptides labeling is quantitatively reduced (Fig. 2B). The finding that replicating myoblasts synthesize most of the same proteins as myotubes demonstrates the limited differences in their phenotypic expression. One possible explanation for the small number of differences between the two stages is that post-mitotic myoblasts could coexist with dividing cells and thus might express differentiated functions long before actual cell fusion takes place (21). However, the fact that cell growth is considerable in the days before cell fusion and that only low levels of acetylcholine receptor and creatine phosphokinase are found prior to fusion would seem to argue against this possibility (19). For the moment, it is not clear whether those proteins such as muscle specific enzymes or the acetylcholine receptor are in an abundancy class beyond the resolution of that presently achieved, or whether they are in fact among those changes actually seen. Localization of these proteins in the two-dimensional separation will permit a more definitive interpretation of these results.

The heterogeneity of actin preparations from various sources had not until recently been reported, although a protein with many of the functional and structural properties of muscle actin has been identified in several nonmuscle cell types (22). Recently, Gruenstein and Rich (23) found significant differences in the peptide maps of chicken brain and muscle actin. In a preliminary report, Storti *et al.* (24) also suggest that two forms of actin are found in developing chick muscle cultures with one form predominating in fused cultures, similar to the situation described here for calf muscle cells. The presence of multiple species of calf actin in kidney cells and prefusion myoblasts strongly suggests that actin from fused muscle is only one of several isozymic forms. Peptide analysis will be required to ascertain how the β and γ components of calf actin differ from the muscle tissue α form. The differences may be due either to variations of primary structure or to secondary modifications, although in either case they may prove to be quite subtle because of the very similar isoelectric points of the three species.

It can be inferred that α-actin is located exclusively in myotubes, since both nonmuscle cells and prefusion myoblasts contain the β/γ forms. Likewise, well developed myotubes may contain only α-actin, since actin from fetal muscle tissue shows only traces of the β/γ forms (Fig. 3a). The low but significant level of α-actin in prefusion cultures may be a basal level or may arise from a certain percentage of post-mitotic muscle cells present in these cultures. Various possibilities exist concerning the β/γ forms of actin. They may be found in microfilament type structures (25) rather than in sarcomeric structures or, alternatively, they may have cellular locations different from that of the α form. If the differences among the three species are secondary modifications, then the β/γ forms may be metabolic precursors to the α-form. In any case, these results add to the growing evidence that the contractile proteins found in nonmuscle tissue may in fact be isozymic forms of their muscle counterparts (3, 4).

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