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Chick cytoplasmic actin and muscle actin have different structural genes

(mRNA/cell-free translation/peptide maps/myogenesis/gel electrophoresis)

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ABSTRACT **Actins isolated from embryonic chick brain and muscle differ in mobility when subjected to electrophoresis in gels containing urea and sodium dodecyl sulfate. Experiments were carried out to determine whether these actins are products of different structural genes and differ in primary amino acid sequence, or whether they are products of the same structural gene but are different because of post-translational modification. Messenger RNA from brain and muscle tissue was used to direct cell-free protein synthesis in wheat germ extracts. The synthesized actins were identified by conversion from globular to fibrous actin and by two-dimensional chromatographic analysis of tryptic peptides. The differences in electrophoretic mobility of brain compared to muscle actin were maintained in the cell-free protein synthetic products. Therefore, these mobility differences were not due to post-translational modification. It was concluded that brain and muscle actin are coded by different messenger RNAs and therefore arise from different structural genes. In addition, messenger RNA from 13- and 16-day embryonic thigh muscle directed the synthesis of both brain- and muscle-type actins, suggesting that muscle cell differentiation involves the regulation of at least two different actin genes.**

Actin is a major component of muscle tissue. In recent years actin has been identified in several non-muscle tissues, and it is now apparent that cytoplasmic actin is a ubiquitous protein of eukaryotes (1). Regardless of source, all actins appear to be structurally and physiologically very similar. This raises the question of whether all actins have evolved from the same structural gene and, in particular, whether actins from different tissues of the same organism are coded for by the same structural gene. This is particularly important with regard to the regulation of actin synthesis during cellular growth and differentiation, where it has been proposed that actin may have several different functions (2–4) and may thus have several different mechanisms controlling its synthesis.

We have developed a urea/sodium dodecyl sulfate (NaDodSO$_4$) polyacrylamide gradient slab gel system that distinguishes brain-type or cytoplasmic actin from muscle-type actin on the basis of electrophoretic mobility (5). This has stimulated us to ask whether these two forms of actin are products of different structural genes or are different because of post-translational modification of a single actin polypeptide precursor. Some preliminary information is already available on this question. Gruenstein and Rich (6) have analyzed all the tryptic peptides from actins obtained from chick brain and chick muscle tissue and report differences in eight peptides. Elzinga *et al.* (7) have reported a partial amino acid sequence from human platelet actin which differs from cardiac muscle actin by one amino acid residue. These reports suggest that actins may be coded by different structural genes.

We have answered this question by carrying out cell-free protein synthesis of chick brain and muscle actins in wheat germ extracts. Messenger RNA (mRNA) obtained from the brain directs the synthesis in these extracts of only the brain-type cytoplasmic actin, while the mRNA from muscle produces only the muscle-type actin. These results rule out post-translational modification for the observed differences in mobility. They demonstrate that these two forms of actin have different mRNAs and most likely are coded for by different structural genes. In addition, we show that early developing muscle appears to contain mRNA coding for both forms of actin.

Abbreviation: NaDodSO$_4$, sodium dodecyl sulfate.

MATERIALS AND METHODS

Preparation of RNA. Total cytoplasmic RNA was prepared from embryonic chick (Spafas, Inc., Norwich, Conn.) brain and thigh muscle dissected free of skin and bone. The tissue was disrupted by five strokes of a loose-fitting Dounce homogenizer in 10–20 volumes of 10 mM Tris·HCl, pH 7.4, 250 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40 (Shell Chemical Co., Ltd.). The homogenate was centrifuged at low speed to remove nuclei, and the supernatant was made 10 mM in EDTA and 1% in NaDodSO$_4$ and extracted with phenol-chloroform according to Singer and Penman (8). The RNA was precipitated twice with ethanol at −20° and stored at 4 mg/ml in water at −20°.

Cell-Free Translation in Wheat Germ Extracts. Wheat germ (Bar-Rav Mill, Tel-Aviv, Israel, or General Mills, USA) was prepared and protein synthesis was carried out as described by Roberts and Patterson (9) with the following exceptions: 76 mM potassium acetate was substituted for KCl and spermine was replaced by 250 μM spermidine in the reaction mix. Cell-free protein synthesis was monitored by the incorporation of [^{35}S]methionine (Amersham, specific activity >250 Ci/mmol) into hot trichloroacetic acid-insoluble material.

Electrophoresis. Cell-free products were analyzed by discontinuous NaDodSO$_4$ polyacrylamide gel electrophoresis according to Laemmli (10) using a 8–12% polyacrylamide gradient slab gel prepared according to Maizel (11). Urea/NaDodSO$_4$ gel electrophoresis in 8–12% polyacrylamide gradient slab gels was modified from NaDodSO$_4$ gels by the addition of urea in the stacking and separating gels to 2.7 M and 5.5 M, respectively. The details of this system have been reported elsewhere (5). The gels were stained with Coomassie blue, destained in acetic acid-methanol, dried under reduced pressure, and when necessary autoradiographed to detect radioactively labeled polypeptides.

Two-Dimensional Tryptic Peptide Analysis. [^{35}S]Methionine-labeled actin from embryonic thigh muscle was prepared in excised tissue slices as described previously (5). Brain and muscle actins synthesized in the cell-free system were purified by repolymerization to F-actin (12) (see legend to Fig. 3). The actins were subjected to electrophoresis in preparative urea/NaDodSO$_4$ slab gels, and the labeled polypeptides migrating

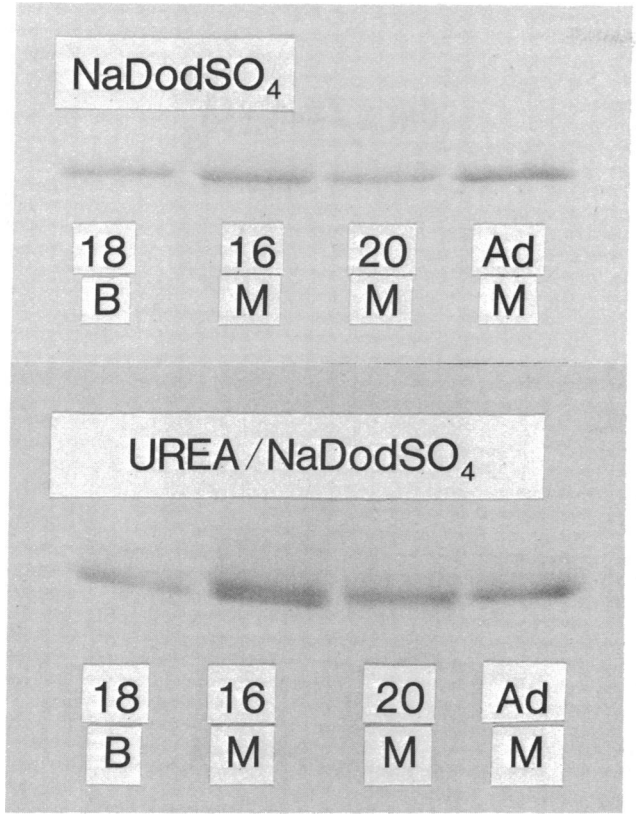

FIG. 1. $NaDodSO_4$ and urea/$NaDodSO_4$ polyacrylamide gel electrophoresis of purified actin from 18-day embryonic brain (18B); 16-day embryonic thigh muscle (16M); 20-day embryonic thigh muscle (20M); and adult muscle (AdM). The actin was purified by repolymerization of acetone powder extracts (5). The samples were subjected to electrophoresis in adjacent slots of the same slab gel, stained with Coomassie blue, destained, and dried under reduced pressure. The direction of migration is from top to bottom.

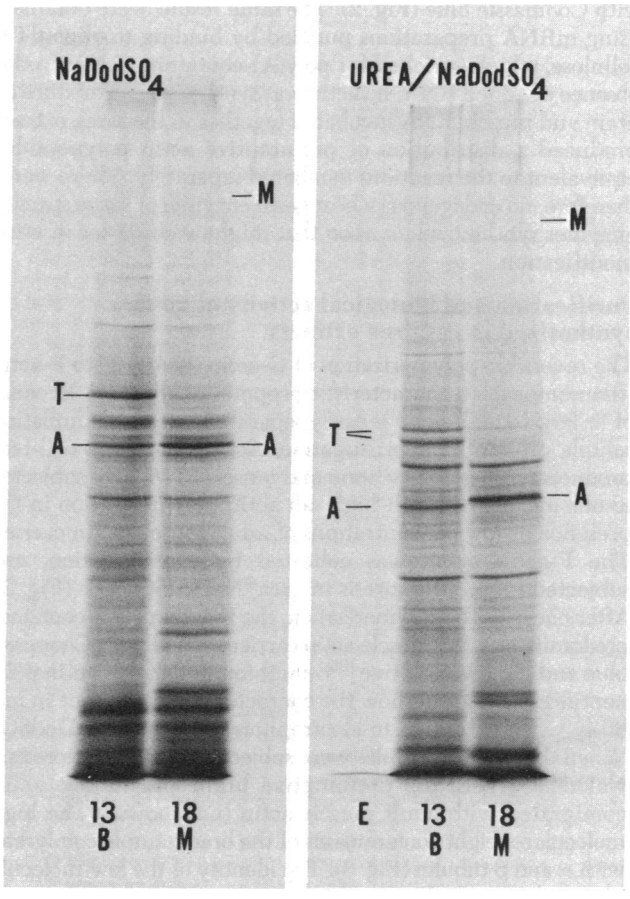

FIG. 2. Autoradiogram of [^{35}S]methionine-labeled cell-free products subjected to electrophoresis in $NaDodSO_4$ and urea/$NaDodSO_4$ gels. Reaction mixtures (25 μl) containing 13-day embryonic brain RNA (13B) and 18-day embryonic thigh muscle RNA (18M) were subjected to electrophoresis with unlabeled brain and adult muscle actin in adjacent slots. E represents the products of 25-μl reaction mixture with no added RNA. The gels were stained with Coomassie blue, destained, dried under reduced pressure, and exposed to x-ray film for one day. A, position of brain and muscle actin markers; T, tubulin marker; M, myosin marker.

with Coomassie blue-stained brain and muscle actin markers were located by autoradiography. The polypeptides were cut from the dried gels and eluted by electrophoresis. They were then precipitated with trichloroacetic acid, digested with trypsin (Worthington TPCK), and fractionated by electrophoresis and chromatography on 0.1-mm cellulose-coated plates (6) (see legend to Fig. 4).

RESULTS

Actins purified from embryonic or adult chick brain and adult thigh muscle comigrate as single polypeptides with a molecular weight of 43,500 when subjected to electrophoresis in polyacrylamide gels containing $NaDodSO_4$ (Fig. 1). However, when electrophoresis is performed in 8–12% polyacrylamide gradient slab gels containing both urea and $NaDodSO_4$, actin from late embryonic or adult thigh muscle migrates with a molecular weight of 43,500, whereas actin from embryonic or adult brain migrates more rapidly, with an apparent molecular weight of 42,000 (5) (Fig. 1). Actin from early embryonic thigh muscle, on the other hand, shows two actin components whose relative amounts change during development (5).

Cell-free protein synthesis directed by brain and muscle mRNA

The RNA-directed synthesis of the brain and muscle forms of actin *in vitro* was examined to determine if these electrophoretic differences in actin were encoded by different mRNAs. Accordingly, RNA purified from embryonic brain and muscle was used to direct protein synthesis in the heterologous cell-free system from wheat germ.

The incorporation of [^{35}S]methionine in wheat germ extracts to which total cytoplasmic RNA from embryonic brain and muscle had been added was 5- to 10-fold greater than in extracts without added RNA. Incorporation was linear for approximately 3 hr. Potassium acetate and spermidine, when added to the reaction mixture, increased stimulation 20- to 30-fold over extracts without added RNA and, in addition, increased the average size of polypeptides synthesized. Incorporation was maximal at an RNA concentration of 300–400 μg/ml. When subjected to electrophoresis in $NaDodSO_4$ polyacrylamide gels, the cell-free products consisted of polypeptides having molecular weights as large as 200,000. A major polypeptide synthesized in extracts directed by brain and muscle RNA had the same mobility as adult muscle actin stained with Coomassie blue (Fig. 2). When the cell-free products were subjected to electrophoresis in 8–12% polyacrylamide gradient slab gels containing urea and $NaDodSO_4$, the presumptive actin polypeptide of the brain RNA sample, which previously comigrated with adult muscle actin in $NaDodSO_4$ gels, now migrated more rapidly, with the same relative mobility as brain actin stained

with Coomassie blue (Fig. 2). The same results were obtained using mRNA preparations purified by binding to oligo(dT)-cellulose, which enriched for poly(A)-containing RNA. In the absence of added RNA, no actin was synthesized. A mixture of brain and muscle RNA incubated together in the same extract produced a distribution of presumptive actin polypeptides equivalent to the reactions incubated separately. There were, therefore, no endogenous wheat germ enzymes or tissue specific enzymes synthesized *de novo* that might account for *in vitro* modification.

Purification and biological activity of actin synthesized in cell-free extracts

The reversible polymerization of G-actin (globular) to F-actin (filamentous) is a characteristic property of all actins. Because of its large size, F-actin is easily separated from contaminating soluble protein by centrifugation. Accordingly, the cell-free products synthesized by brain and muscle RNA were subjected to one cycle of low salt-high salt actin polymerization in the presence of low concentrations of adult muscle actin carrier. The F-actin pellet was collected by centrifugation, and subjected to electrophoresis in urea/$NaDodSO_4$ gels (Fig. 3). After one cycle of polymerization, the F-actin pellets contained predominantly the muscle actin carrier stained with Coomassie blue and the presumptive [^{35}S]methionine-labeled actin polypeptides, which still show the characteristic difference in mobility when subjected to electrophoresis in urea/$NaDodSO_4$. When these same samples were subjected to electrophoresis in $NaDodSO_4$ gels, the presumptive brain and muscle actins comigrated with adult muscle actin (not shown). The high-molecular-weight contaminants of the brain sample comigrated with α and β tubulin (Fig. 3). The identity of the low-molecular contaminant is unknown.

Tryptic peptide two-dimensional chromatography of cell-free products

To confirm the identity of the presumptive actin polypeptides, we carried out a two-dimensional tryptic peptide fingerprint analysis. Cell-free synthesized presumptive brain and muscle actin and [^{35}S]methionine-labeled actin prepared from tissue slices of 18-day-old embryonic thigh muscle were analyzed. The actin from muscle tissue in this experiment was extracted by low salt homogenization, which preferentially solubilizes the brain-type or cytoplasmic actin (see ref. 5). The results in Fig. 4 show that there is coincidence of most of the major [^{35}S] methionine-labeled peptides, indicating a high degree of similarity in amino acid sequence between the cell-free synthesized brain and muscle actins and actin from whole muscle. It has already been shown that chick brain and muscle actins have very similar, although not identical, tryptic fingerprints (6).

RNA from developing embryonic muscle directs the cell-free synthesis of two different actins

We have observed that prior to 12 days of development embryonic thigh muscle contained primarily the brain-type or cytoplasmic actin. During myoblast fusion and differentiation, the muscle-type actin gradually appears, until by 18 days of development muscle actin predominates (5). Therefore, we examined RNA extracted from 13- and 16-day-old thigh muscle for the cell-free synthesis of actin. Figure 5 shows the cell-free products directed by these RNAs after one cycle of purification by repolymerization for F-actin. The polypeptides were subjected to electrophoresis in urea/$NaDodSO_4$ gels. The products synthesized in the cell-free system directed by 13- and 16-day muscle RNA each contained two actin polypeptides, one that comigrated with brain-type actin and one with muscle actin. By 18 days, only the muscle-type actin was seen. This suggests that mRNA for both forms of actin is present during early muscle development.

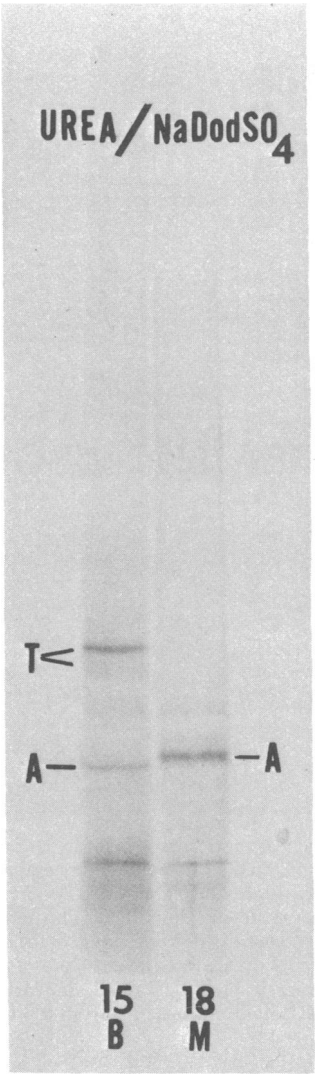

FIG. 3. Autoradiogram of [^{35}S]methionine-labeled cell-free products directed by brain and muscle mRNA. The products were purified for F-actin by repolymerization and then subjected to electrophoresis in urea/$NaDodSO_4$. The total cell-free products from 400–600 μl of reaction mixtures directed by RNA from 15-day embryonic brain (15B) and 18-day embryonic thigh muscle (18M) were dialyzed for 3 days against three changes of a 100-fold excess of depolymerization buffer (0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, pH 7.5). The samples were centrifuged at 100,000 × g for 2 hr at 4°, and 20 μg of purified carrier adult muscle G-actin was added to the supernatants. The solution was adjusted to 0.6 M KCl and 1 mM $MgCl_2$, and the samples were allowed to stand overnight at room temperature. The supernatants were centrifuged at 105,000 × g for 2 hr at 4°, and the F-actin pellets were resuspended in electrophoresis sample buffer. The gel was stained with Coomassie blue, dried under reduced pressure, and exposed to x-ray film for 4 days. A, position of actin markers; T, position of tubulin marker.

DISCUSSION

Total cytoplasmic RNA from embryonic chick brain and thigh muscle directed the synthesis of authentic actin in the heterologous cell-free system from wheat germ. The identity of the actin was established by coelectrophoresis with purified brain and muscle actin in polyacrylamide gels, the ability to repo-

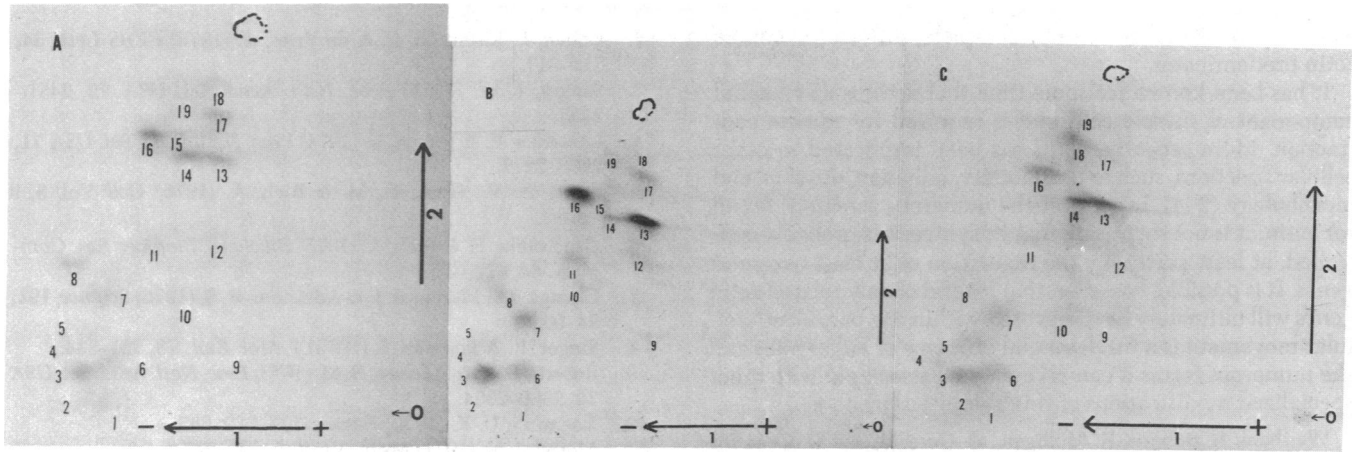

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled tryptic peptides from (A) actin synthesized in 18-day embryonic tissue slices (8000 cpm); (B) cell-free synthesized actin directed by 15-day embryonic brain RNA (3200 cpm); (C) cell-free synthesized actin directed by 18-day embryonic thigh muscle RNA (2800 cpm). The tryptic peptides were separated in the first dimension by electrophoresis in pyridine, acetic acid, water (30:1:270), pH 6.5, 950 V for 30 min for (A), and for 40 min for (B) and (C). The second dimension was chromatography in isoamyl alcohol, *sec*-butanol, propanol, pyridine, water (1:1:1:3:3). The cellulose plates were dried, chromatographed in 7% 2.5-diphenylaxazole in ether, and exposed to x-ray film at −70° for 1 week for (A) and 4 weeks for (B) and (C). The broken circle denotes the position of ε-dinitrophenyl lysine marker. O is the origin. The identities of peptides 17, 18, and 19 in (C) were assigned with some uncertainty.

lymerize to form F-actin, and by tryptic peptide fingerprint analysis (Figs. 2–4). The cell-free synthesis of muscle actin in wheat germ cell-free systems has been reported earlier (13–15) with results similar to that reported here.

Actins from chick brain and muscle tissues are not identical polypeptides when analyzed by electrophoresis in polyacrylamide gradient slab gels containing urea and $NaDodSO_4$ (5) (Fig. 1). Since both brain and muscle mRNAs were translated in the same wheat germ system, it is likely that their actin products were both subjected to the same post-translational modification, if any occurred at all. However, the mobility differences remained, so this cannot be the cause of the differences. Another possibility is that translation of one mRNA is simply terminated before the other, leading to a smaller protein. However, the tryptic peptide analysis of brain and muscle actin in which all of the peptides were labeled with lysine and arginine (6) showed several peptides that were not common to both proteins, thus ruling out early termination of translation as an explanation of the mobility differences. The results presented here strongly support the concept that brain and muscle actin are coded by different mRNAs and have different structural genes.

The coincidence of most of the tryptic peptides of brain and muscle actin suggests, however, that these actins have very similar amino acid sequences (Fig. 4). However, differences in at least eight lysine- and arginine-labeled peptides between chick brain and muscle actin have been reported by Gruenstein and Rich (6). Furthermore, Elzinga *et al.* (7) have reported one amino acid substitution in one peptide in a partial comparison of human cardiac and platelet actin. These differences could explain the difference in mobility we observe between brain and muscle actin when they are subjected to electrophoresis in urea/$NaDodSO_4$ gels.

The observation that cytoplasmic and muscle actin are coded for by different structural genes has important implications in the regulation of actin synthesis during cellular growth and differentiation. The demonstration of two actin genes may be particularly important during myogenesis, where one might expect that cytoplasmic actin is needed for cell division and mediates changes in cell shape and structure as the myoblasts fuse to form multinucleated myotubes. In addition, muscle actin is needed for the thin filament lattice of myofibrils. Both actin mRNAs appear to be present at the same time, as seen in the results of the cell-free synthesis of both cytoplasmic and muscle actin by RNA from 13- and 16-day embryonic thigh muscle (Fig. 5). Thus two different actin mRNAs are apparently both present during early myogenesis, but by day 18 of development,

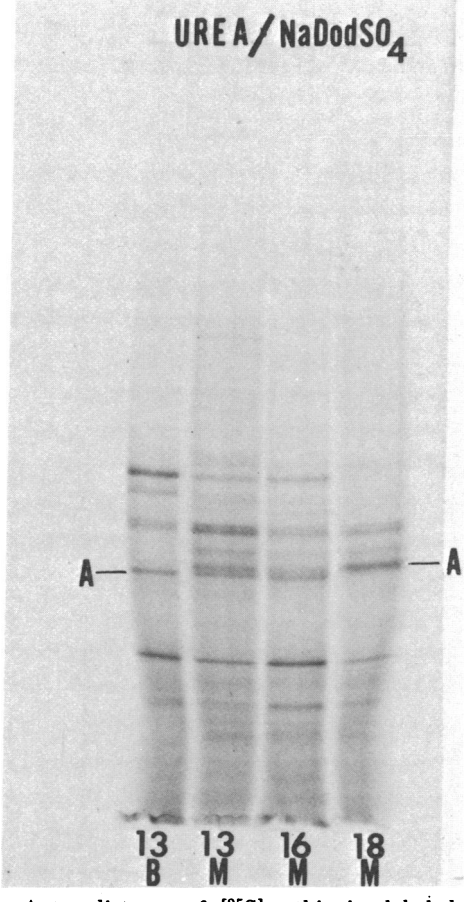

FIG. 5. Autoradiogram of [^{35}S]methionine-labeled cell-free products purified by polymerization to F-actin and subjected to electrophoresis in urea/$NaDodSO_4$. The cell-free products directed by 13-day embryonic brain (B) RNA and by 13-, 16-, and 18-day embryonic thigh muscle (M) RNA were purified for F-actin as described in the legend to Fig. 2. The dried gel was exposed to x-ray film for 1 week. A, the position of actin markers.

when myoblast fusion is nearly complete, mRNA for muscle actin predominates.

It has been known for some time that actin is an essential component of muscle cells and is required for muscle contraction. More recently, actin has been implicated in other cellular functions, such as cell motility, adhesion, division, and morphology (2–4). In view of the numerous functions found for actin, it is not surprising that its synthesis is probably controlled, at least partly, by the regulation of at least two actin genes. It is possible, however, that several closely related actin genes will ultimately be discovered within one organism. Cellular movement is a fundamental property of eukaryotes and the numerous forms it can take may be associated with other specialized modifications of this ubiquitous protein.

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