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Determination of Actin Messenger RNA in Cultures of Differentiating Embryonic Chick Skeletal Muscle

[myogenic cultures/poly(A) mRNA/wheat germ extracts/protein synthesis/peptide analysis]

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ABSTRACT **Cytoplasmic polyadenylylated messenger RNA from differentiated muscle cultures, when incubated in a wheat germ cell-free system, directed the synthesis of a polypeptide indistinguishable from authentic chicken skeletal muscle actin, as judged by mobility on sodium dodecyl sulfate–polyacrylamide gels, tryptic peptide analyses, and biological activity. The synthesis of actin in the cell-free system was used to assay levels of translatable actin mRNA in cultures of fibroblasts, pre- and post-fusion myoblasts, and myoblasts grown under conditions that prevent fusion. In all cases the amount of actin polypeptide synthesized in the cell-free system was proportional to the rate of actin synthesis in the cultures from which the RNA was extracted. It is suggested that actin synthesis is regulated by the actin mRNA content of the cell and that an increase in the cytoplasmic level of translatable actin messenger RNA is mediated by cell fusion rather than by the terminal round of DNA synthesis.**

It is now apparent that a fibrillar protein very similar to skeletal muscle actin is a structural component of several diverse cell types and may play an essential role in cellular differentiation with regard to cell morphology, motility, and function (1–4). Cultures of embryonic skeletal muscle provide a model system in which to study the regulation of actin synthesis during differentiation.

Wheat germ extracts direct the synthesis of skeletal muscle actin in response to total cytoplasmic polyadenylylated messenger RNA from cultures of embryonic chick skeletal muscle. The level of this synthesis was used as an assay for the relative amount of translatable actin messenger RNA in cultures grown under conditions that regulate the process of myogenesis (2, 5, 8).

MATERIALS AND METHODS

Whatman cellulose powder CF-12 was obtained from Reeve Angel; Thymidine 5′-monophosphate, from Sigma Biochemicals; *N,N′*-dicyclohexylcarbodiimide, from Fluka; [*methyl*-^{3}H]methionine (specific activity 9 Ci/mmol) and [^{35}S]methionine (specific activity > 175 Ci/mmol), from Amersham. Biochemicals for the cell-free system were all from Sigma Biochemicals; trypsin (TPCK trypsin) was obtained from Worthington; Aminex A-7 cation exchange resin from Bio-Rad; oligo(dT)-cellulose was prepared according to Gilham (7).

Cultures. Cultures of embryonic chick breast muscle and of fibroblasts were prepared as described previously (5). Muscle fibroblasts were isolated from well fused muscle cultures and serially passaged to the necessary density in growth medium used for primary muscle cultures. To arrest myoblast fusion (8) cultures were treated with ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid (EGTA), as detailed previously (5). Where stated, BrdU was added to the cultures at plating, at a final concentration of 10 μg/ml (2).

Preparation of Polyadenylylated Messenger RNA. Total cytoplasmic and polyadenylylated RNAs were isolated from 10 to 60 cultures (2.5 to 5.0 $\times$ 10^{6} cells per 100 mm culture dish at plating) by a modification of the procedures of Singer and Penman (9). TNM buffer (0.01 M Tris·HCl, pH 7.5, 0.25 M NaCl, 5 mM $MgCl_2$) was used for rinsing and scraping (plus 1% NP-40) the cultures; polyadenylylated RNA was prepared with 0.3 g of oligo(dT)-cellulose per centrifuge tube and binding and eluting were carried out at 32° without tRNA. The polyadenylylated messenger RNA prepared in this way contained less than 10% ribosomal RNA, as judged by electrophoresis on formamide gels (10).

Cell-Free Translation in Wheat Germ Extracts. Preincubated extracts were prepared and protein synthesis assays were carried out as described by Roberts and Paterson (11). Reactions were incubated at 22° for 90–120 min.

Isolation of Labeled Actin from the Cultures. Well-fused muscle cultures (72 hr after plating) were labeled for 4–6 hr with either 100 μCi per dish of [*methyl*-^{3}H]methionine or 10 μCi per dish of [^{35}S]methionine in 3 ml of Eagle's medium made with a 200-fold reduction in the methionine concentration. Thereupon, cultures were rinsed with phosphate-buffered saline (PBS) and glycerinated as described by Schroeder (3). Glycerinated cells were rinsed in PBS, pelleted at 2000 $\times$ *g* for 5 min, the pellet dissolved in electrophoresis sample buffer (100 μl), and the extract run on preparative sodium dodecyl sulfate–polyacrylamide slab gels for subsequent actin extraction as described below. Actin prepared by the glycerol procedure was identical to actin prepared from acetone powders of culture material, as judged by molecular weight on sodium dodecyl sulfate–polyacrylamide gels and tryptic analysis.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; PBS, phosphate buffered saline; BrdU, 5-bromodeoxyuridine.

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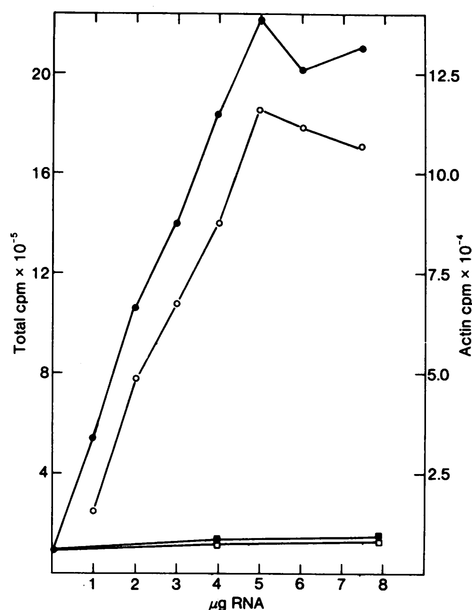

FIG. 1. [35S]Methionine incorporation into total protein or actin in response to RNA from differentiated muscle cultures (60 hr after plating). Assays (100 µl) incubated with (●) polyadenylylated messenger RNA; (■) total cytoplasmic RNA before oligo(dT)-cellulose treatment; (□) RNA not retained by oligo(dT)-cellulose; and (O, right-hand ordinate) incorporation into actin polypeptide with polyadenylylated messenger RNA.

For measurement of the rates of actin synthesis in the cultures the cells were given a 1-hr pulse with [35S]methionine (3.3 µCi/ml of low methionine Eagle's medium), extracted with glycerol, and run on analytical sodium dodecyl sulfate–polyacrylamide gradient slab gels (see below). The actin band was cut from the dried gel for scintillation counting.

Preparation of Actin and Myosin. Myosin was purified from the breast muscle of adult Leghorn chickens according to Finck (12). Actin was prepared from the acetone powders of the same muscle according to Spudich and Watt (13).

ANALYTICAL PROCEDURES

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The cell-free products were analyzed on 10–20% gradient sodium dodecyl sulfate–polyacrylamide gels as described previously (14). For quantitative determination of the radioactivity in a particular gel band it was cut from the dried gel after autoradiography and swollen in a mixture of NCS and water (9:1) at 50° for 6 hr; its radioactivity was measured in toluene scintillant.

Peptide Analysis. [35S]Methionine labeled actin synthesized in cultures and in wheat germ extracts was isolated from preparative 10% sodium dodecyl sulfate–polyacrylamide gels as described (13), mixed with 250–500 µg of purified actin, and digested with trypsin as described by Mathews *et al.* (15). The tryptic digest was either subjected to high voltage two-dimensional paper ionophoresis as previously described (11), or run on a 1 × 20-cm column of Aminex A-7 cation exchange resin at 55° in the pyridine acetate buffer system described by Aviv and Leder (16). The peptides were eluted from the column with a linear gradient made from 200 ml of 0.05 M pyridine–acetate, pH 2.5, and 200 ml of 1.20 M pyridine–

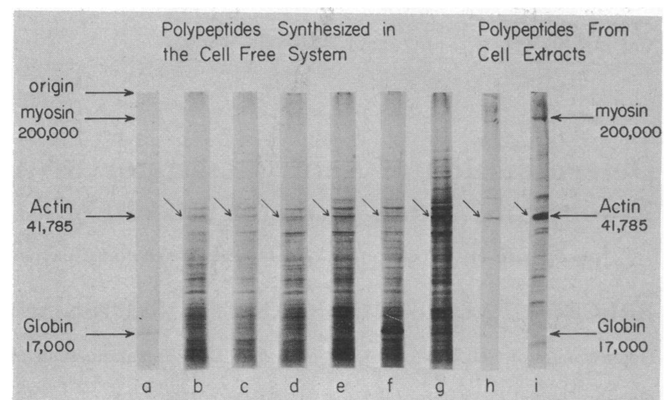

FIG. 2. Autoradiogram of [35S]methionine-labeled cell-free products fractionated on sodium dodecyl sulfate–polyacrylamide gels. Incubations (100 µl) were carried out (a) without added RNA; with (b) RNA from chick fibroblasts; (c) RNA from muscle fibroblasts; (d) RNA from 72-hr muscle cultures grown in 10 µg/ml of BrdU; (e) RNA from 72-hr fusion arrested myoblasts, blocked with EGTA (1.85 mM) at 20 hr; (f) RNA from 26-hr prefusion myoblasts, and (g) RNA from 72-hr differentiated muscle cultures. RNA from fusion arrested cultures, 20 hr after restoring the calcium concentration in the medium, gave a pattern identical to (g). Actin synthesized in cultures during a 1-hr pulse with [35S]methionine is shown in (h) for 26-hr prefusion myoblasts and (i) for 72-hr well-differentiated muscle cultures. The diagonal arrow marks the actin polypeptide. The autoradiography film was exposed to the dried gel for 48 hr. The molecular weight of actin is that derived from the published sequence (17), however, estimates on sodium dodecyl sulfate–polyacrylamide gels vary from 42,000 to 45,000.

acetate, pH 5.0. Samples of 1.40 ml were collected and disintegrations were counted directly in 10 ml of Triton X-100–toluene scintillation fluid. Where indicated, peptides were stained with cadmium ninhydrin reagent.

RESULTS

Protein Synthesis Directed by Polyadenylylated Messenger RNA from Differentiated Muscle Cultures. Polyadenylylated messenger RNA isolated from well-differentiated muscle cultures (60 hr after plating) greatly enhanced the incorporation of [35S]methionine into hot-acid-insoluble material when added to a wheat germ cell-free system (Fig. 1). Incorporation was maximal at an RNA concentration of 50 µg/ml, and this was taken as the standard RNA concentration for subsequent cell-free incubations. The level of incorporation at this concentration of RNA was approximately 30 times greater than in reactions incubated for the same period in the absence of added RNA. Total cytoplasmic RNA and RNA not retained by oligo(dT)-cellulose were ineffective in promoting [35S]methionine incorporation at all the concentrations tested (Fig. 1).

The proteins synthesized *in vitro* were analyzed on sodium dodecyl sulfate–polyacrylamide gradient slab gels. The cell-free products consisted of a large number of polypeptides ranging in molecular weight from 10,000 to 100,000 (Fig. 2g). One of the major polypeptide bands, indicated by the small diagonal arrow, co-migrated with purified actin marker. This presumptive actin polypeptide represented approximately 8.5% of the total radioactivity on the gel when reactions were carried out with polyadenylylated RNA from 72-hr-old cultures. The proportion of actin polypeptide synthesized re-

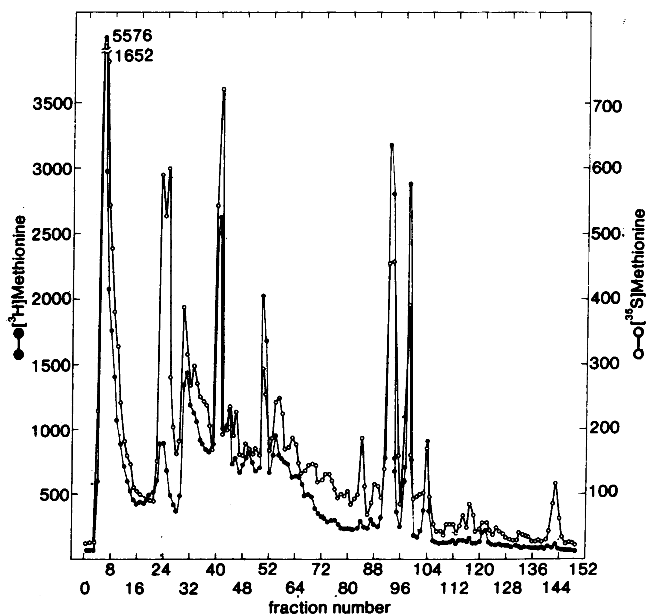

FIG. 3. Cation exchange chromatography of peptides derived from the presumptive [35S]methionine labeled actin polypeptide synthesized in the wheat germ cell-free system (○), and peptides derived from [*methyl*-3H]methionine actin synthesized in differentiated muscle cultures (●). Fractions of 1.40 ml were collected for scintillation counting.

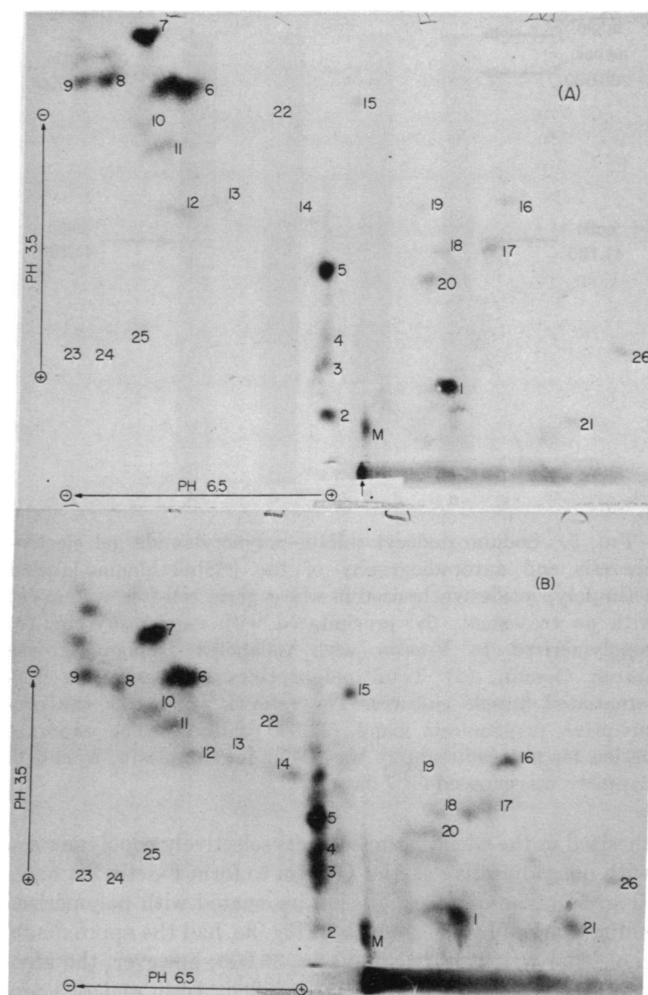

FIG. 4. Autoradiographs of electropherograms of [35S]methionine-labeled tryptic peptides derived from (A) actin synthesized in differentiated muscle cultures (300,000 cpm), and (B) the presumptive actin polypeptide synthesized in the cell-free system (500,000 cpm). The peptides were fractionated by ionophoresis on Whatman 3MM paper at pH 6.5 at 3 kV for 60 min in the first dimension and at pH 3.5 at 3 kV for 90 min for (A) and 60 min for (B) in the second dimension. The arrow marks the origin. The spot M corresponds to methionine sulfone. Autoradiography of the dried papers was for 8 days. As shown previously endogenous synthesis yields no detectable peptides (11).

mained constant over the range of RNA concentrations used for translation, extrapolating to the origin in the absence of added RNA (Fig. 1).

Identification of Actin. Actin extracted from cells labeled either with [*methyl*-3H]methionine or [35S]methionine, and the [35S]methionine labeled presumptive actin polypeptide synthesized in the cell-free system, were each purified by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and digested with trypsin.

The digest derived from [*methyl*-3H]methionine labeled actin prepared from cultures and [35S]methionine labeled peptides prepared from the presumptive actin polypeptide translated in the cell-free system were analyzed by cation exchange chromatography (Fig. 3). The elution profiles were coincident with respect to 10 major peaks. Theoretically, from the published amino-acid sequence of skeletal muscle actin (17), 11 methionine-containing tryptic peptides can be derived from the protein.

As a final analysis, [35S]methionine labeled tryptic peptides prepared from actin synthesized in the cultures and the presumptive actin polypeptide were subjected to two-dimensional high voltage paper ionophoresis. As shown in Fig. 4, the peptide patterns were coincident for most major peptides in two dimensions. Peptides numbered 1, 2, 3, 4, 5, 8, and 9 were coincident with ninhydrin-stained peptides derived from the purified actin used as carrier for the trypsinization procedure.

Biological Activity of Actin Synthesized in the Wheat Germ Extracts. Actomyosin formation and the globular (G-actin) to fibrillar (F-actin) transformation are two inherent properties of actin and were used to further characterize the actin polypeptide. Cell-free reaction products containing the actin polypeptide were clarified at 10,000 × *g* for 10 min and incubated with RNase A (50 μg/100 μl per reaction) at 37° for 30 min. One aliquot (100 μl) was adjusted to 0.4 M KCl, mixed with 50 μg of purified carrier chicken myosin, and precipitated by the addition of 10 volumes of ice-cold water to form actomyosin. The precipitate was collected at 8000 × *g* for 5 min, resuspended and pelleted three times in 1.5 ml of cold PBS containing 1% Triton X-100. Another aliquot (400 μl) was treated as crude F-actin extract and was depolymerized, mixed with carrier G-actin (500 μg), polymerized, and purified as described (13). The final pellets of actomyosin and F-actin were dissolved in electrophoresis sample buffer.

Prior to precipitation with myosin, the actin polypeptide represented approximately 8% of the total radioactivity in cell-free products (Fig. 5a). After actomyosin formation and precipitation 25% of the radioactivity in the precipitate was associated with actin polypeptide (Fig. 5b). The actin syn-

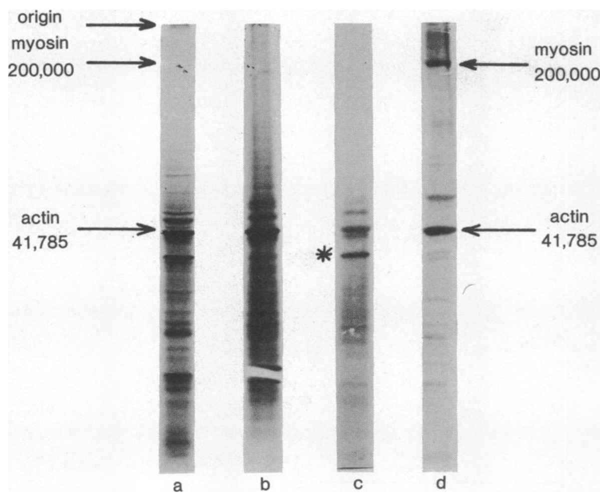

FIG. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of the [^{35}S]methionine-labeled actin polypeptide synthesized in wheat germ cell-free system (a) with no treatment, (b) precipitated with carrier myosin, (c) copolymerized to F-actin with unlabeled tropomyosin-free carrier G-actin. (d) Total polypeptides synthesized in differentiated muscle cultures. The asterisk (*) marks the presumptive tropomyosin band (36,000 daltons). The exposure period for autoradiography was 48 hr for samples a, b, and d. Sample c was exposed for 7 days.

thesized in the wheat germ extracts selectively copolymerized with nonradioactive carrier G-actin to form F-actin filaments (Fig. 5c). The other component associated with polymerized actin, marked by the asterisk in Fig. 5c, had the approximate molecular weight of tropomyosin, 36,000; however, the identity of this polypeptide was not verified. Actin and this presumptive tropomyosin polypeptide accounted for more than 80% of the radioactivity in the final F-actin pellet.

Actin Template Activity of Polyadenylylated Messenger RNA from Myogenic and Nonmyogenic Cultures. Actin comprised almost 9% of the total product synthesized in wheat germ extracts (Table 1 and Fig. 2) when incubations were carried out with polyadenylylated messenger RNA from well-differentiated muscle cultures (72 hr after plating). In contrast, the corresponding level of actin synthesis was in the order of 1% of the total cell-free product for messenger RNA isolated from chick fibroblasts, muscle fibroblasts, and prefusion myoblasts (26 hr after plating). This proportion was also reflected in the relative rate of actin synthesis in the corresponding muscle cell cultures (Table 1).

If myogenesis was blocked either by exposure to BrdU (10 μg/ml) or by low calcium fusion arrest (Table 1 and Fig. 2), polyadenylylated messenger RNA extracted from these cultures directed levels of actin synthesis in wheat germ extracts comparable to the low levels seen with mRNA from fibroblasts or prefusion myoblasts. However, when fusion was initiated in low calcium blocked cultures with the addition of calcium to the medium and the RNA was extracted 20 hr later, approximately 8% of the total product synthesized in response to the messenger RNA from these cultures was actin polypeptide. This increase in the cytoplasmic level of translatable polyadenylylated actin messenger RNA was unaccompanied by an additional round of DNA synthesis, as fusion arrested cells are post mitotic (5, 8).

DISCUSSION

Polyadenylylated messenger RNA isolated from differentiated cultures of embryonic chick breast muscle directs the synthesis of a polypeptide in a wheat germ cell-free system, which is indistinguishable from authentic skeletal muscle actin.

TABLE 1. *The level of cell-free actin polypeptide synthesis directed by RNA from cultures compared to the rate of actin synthesis in cultures*

Cultures	Actin synthesis in the cell-free system (5 μg of RNA per 100 μl of reaction)			Actin synthesis in cultures with a 1-hr pulse label	
	^{35}S cpm*	% of total†	Relative increase	^{35}S cpm*	Relative increase
1. Chick fibroblasts. RNA extracted at a cell density of 10^7 cell per 100-mm dish	2,340	1.46	—	—	—
2. Muscle fibroblasts. RNA extracted at a cell density of 10^7 cells per 100-mm dish	1,472	0.92	—	—	—
3. Myoblasts plated in 10 μg/ml of BrdU. RNA extracted at 72 hr. Fusion < 1%‡	1,645	1.03	1.00	2,131	1.00
4. Low calcium fusion-arrested myoblasts. RNA extracted at 72 hr. Fusion = 8%.	2,949	1.84	1.79	3,213	1.54
5. Low calcium fusion-arrested myoblasts, restored to standard medium at 72 hr. RNA extracted at 92 hr. Fusion = 65%.	12,831	8.01	7.81	21,132	10.00
6. Prefusion myoblasts. RNA extracted at 26 hr. Fusion < 5%.	1,734	1.08	1.05	2,654	1.24
7. Differentiated muscle cultures. RNA extracted at 72 hr. Fusion = 70%.	13,150	8.21	8.10	22,164	10.50

* [^{34}S]Methionine counts were determined by direct solubilization of the actin band cut from dried gels after autoradiography. The sample layered for gel analysis corresponded to the tissue in one tenth of a culture.

† This is taken as the ratio of radioactivity in actin polypeptide divided by hot trichloroacetic acid precipitable radioactivity layered on the gel.

‡ Fusion is given at the time of RNA extraction (5).

The synthesis of skeletal muscle actin in a heterologous cell-free system derived from plant tissue suggests that tissue specific factors are not essential for the translation of specific messenger RNAs, in agreement with previous observations for this and other heterologous cell-free systems (reviewed in ref. 11).

The relative amount of actin polypeptide synthesized in the wheat germ extracts in response to polyadenylylated messenger RNA from myogenic and nonmyogenic cultures is closely correlated with the rate of actin synthesis in these cultures (Table 1 and Fig. 2), and tentatively provides a direct assay for the level of translatable polyadenylylated actin messenger RNA in the cytoplasm of differentiating muscle cells.

If actin messenger RNA were present in the cytoplasm of undifferentiated or fusion-arrested myoblasts in a "masked" or untranslatable form, the inability to detect such a class of RNA could be explained on the basis of a translational control mechanism inherent in the primary structure of the RNA and/or the absence of polyadenylate residues on the message. However, it is unlikely that the regulation of actin synthesis involves the adenylylation of a cytoplasmic pool of messenger RNA lacking poly(A) since, in somatic cells, polyadenylylation of mRNA seems to occur prior to or coincident with nuclear export (18). Furthermore, using inhibitors of ribosomal RNA synthesis (19, 20), we found no evidence for a class of poly(A) minus messenger RNA in muscle cells (unpublished observations).

Fusion-arrested myoblasts contain four to six times less polyadenylylated actin messenger RNA, as judged by translation, than well-fused control cultures of the same age, or cultures allowed to fuse by increasing the calcium concentration in the medium (Table 1). These fusion blocked cells are arrested as post mitotic single cells in the G_1 or G_0 phase of the cell cycle (5) and in the absence of DNA synthesis. These cells do not synthesize myosin (5) or actin (Table 1) at the linearly increasing rates observed in fused cultures unless fusion proceeds. Furthermore, several cytoplasmic enzymes associated with differentiated muscle (6) are also not found at appreciable levels in fusion-arrested cultures, and increase only if fusion takes place (19, 21). These findings, taken together, suggest that cell fusion mediates the transcription and/or the nuclear processing (export) of the messenger RNA species that code for actin, and possibly other proteins associated with the contractile apparatus of muscle. This mediation can occur after those events associated with a terminal round of DNA synthesis.

The low level of actin synthesis in response to polyadenylylated messenger RNA from fibroblasts and undifferentiated myoblasts is consistent with those reports that indicate that actin or an actin-like protein is present in a variety of cell types, as these cells would also be expected to contain low levels of actin messenger RNA. Although the actin polypeptide has not been rigorously characterized in these cases, its precise mobility with actin polypeptide suggests that it is indeed actin. The basic approach described above should be applicable to cells that synthesize increased amounts of actin during differentiation.

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