Proc. Nat. Acad. Sci. USA
Vol. 71, No. 3, pp. 662–666, March 1974

Myosin Heavy Chain Messenger RNA from Myogenic Cell Cultures

[oligo(dT)–cellulose/poly(A)/reticulocyte lysate/chick embryo]

ALAN PRZYBYLA AND R. C. STROHMAN*

Department of Zoology, University of California, Berkeley, Calif. 94720

Communicated by Daniel Mazia, October 21, 1973

ABSTRACT **The appearance of messenger RNA for myosin heavy chains in chick-embryo myogenic cell cultures was investigated. Total polyribosomes were isolated from cultures at various times of development and were purified in sucrose step gradients. These polysomes were either extracted with phenol or were treated with puromycin. The ribonucleoprotein particles and ribosomal subunits released by puromycin were fractionated on sucrose gradients. RNA from polysomes or from puromycin-dissociated subunits was fractionated on oligo(dT)–cellulose columns, and the bound and unbound RNA was assayed for activity of myosin heavy chain messenger RNA in a rabbit reticulocyte cell-free system. RNA stimulating myosin heavy-chain synthesis was found predominantly in the unbound fractions of the oligo-(dT)–cellulose columns. After puromycin treatment of polysomes, the myosin heavy chain messenger RNA, which sediments at 18–26 S, was associated with a ribonucleoprotein particle sedimenting between 30 and 40 S. Myosin heavy chain messenger RNA was obtained from cultures containing well-developed myotubes and from cultures undergoing myogenic cell fusion. This messenger RNA was not detectable in early, unfused cultures, or in later cultures in which myogenic cell fusion had been prevented by treatment with ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid. These experiments demonstrate that messenger RNA for myosin heavy chains becomes associated with ribosomes only after myogenic cell fusion has begun.**

During the differentiation of the multinucleated, skeletal muscle fiber the time of myogenic cell fusion and the formation of the embryonic myotube is correlated with the time of intensive synthesis of myosin and the other muscle-specific enzymes (1–4). Presently at issue is whether cell fusion is actually coupled to the regulatory mechanisms that control this intensified protein synthesis. Evidence from this laboratory on fusion-arrested chick myogenic cell cultures strongly suggests that such a coupling mechanism exists (2), and a recent report from another laboratory (5) suggests that in rat-muscle cell cultures there is a post-transcriptional regulatory event that is correlated with cell fusion.

In this paper we report on the isolation of an mRNA fraction derived from polysomes of cultured chick-embryo myogenic cells. This RNA directs the incorporation of radioactive leucine into myosin heavy chains (MHCs) in a cell-free system. It is obtained from cultures of mature myotubes and from cultures in which the main burst of myogenic cell fusion is occurring. This mRNA is not obtainable from cultures in which fusion has failed to occur. In other words, the appearance of detectable amounts of a specific ribosome-associated mRNA for MHCs parallels closely the time course of the actual synthesis of MHCs, which we have reported as following cell fusion with a lag-time of 4 hr (2). The experiments reported here suggest, therefore, that cell fusion in chick myogenic cultures promotes the synthesis of new mRNA and that the coupling suggested above between cell fusion and regulation of myosin synthesis operates ultimately at the level of gene transcription. Furthermore, we find that MHC mRNA does not bind to oligo(dT)–cellulose and, therefore, may not contain a long tract of poly(adenylic acid).

Abbreviations: poly(A), RNA composed predominantly of poly-(adenylic acid); MHC, the 200,000-molecular-weight subunit of the myosin molecule; TKM, Tris·HCl, potassium, magnesium buffer pH 7.5 at indicated concentrations; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

MATERIALS AND METHODS

Cultures. Primary cultures of 12-day embryonic chick breast muscle were obtained as described (6).

Fusion Arrest. Methods for blocking fusion of myogenic cultures with ethyleneglycol bis(β-aminoethyl ether)-*N N'*-tetraacetic acid (EGTA) have been described (2).

Labeling of Cultures. All cultures in 100-mm culture dishes were labeled in 2.0 ml of minimal essential medium (Gibco) containing 100 μCi/ml of [8-^{3}H]adenosine (15 Ci/mmol; Schwartz/Mann). Label was equilibrated in the culture incubator before use. The duration of labeling is noted where necessary.

Isolation of Polysomes. After cultures were labeled, dishes were rinsed with cold balanced salts solution, cells were removed from the dish in TKM-I (250 mM KCl–1.5 mM $MgCl_2$–10 mM Tris·HCl, pH 7.5), and homogenized with a loose-fitting Dounce homogenizer. Lysates were spun at 10,000 × *g* for 10 min. The supernatant was layered on step gradients containing 2.0 ml of 2.0 M sucrose and 2.0 ml of 1.35 M sucrose in TKM-II (250 mM KCl–5 mM $MgCl_2$–50 mM Tris·HCl, pH 7.5) and centrifuged for 24 hr at 104,000 × *g* at 4°.

Puromycin Release of Polysomes. Polysome pellets from step gradients were dispersed in 0.2 ml of TKM-IV (500 mM KCl–5 mM $MgCl_2$–50 mM Tris·HCl, pH 7.5) by swirling for 30–60 min at 4°. Puromycin dissociation mixtures contained 0.2 ml of polysomes in TKM-IV (from ten 100-mm dishes), 0.025 ml of 10 mM puromycin (Nutritional Biochemical Co.), and 0.025 ml of TKM-III (1.0 M KCl–10 mM $MgCl_2$–100 mM Tris·HCl, pH 7.5). Reaction mixtures were incubated 10 min at 4°, 15 min at 37°, layered on 5–20% sucrose gradients in TKM-IV, and centrifuged at 40,000

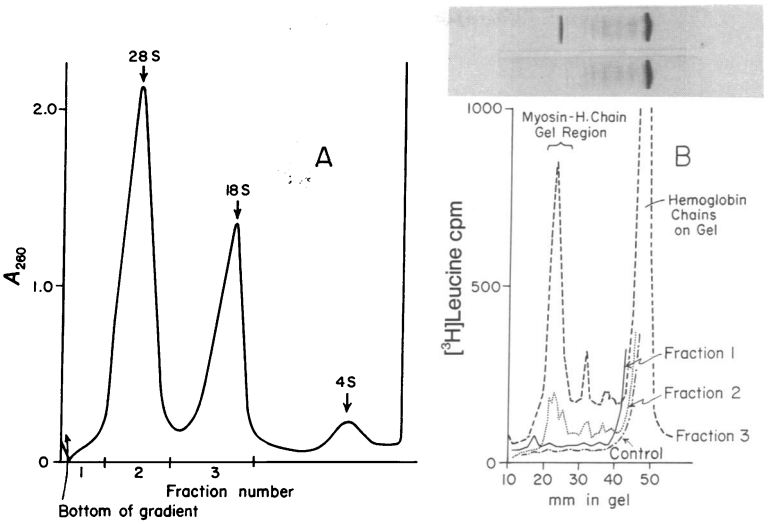

FIG. 1. Translation of 18–26S RNA for myosin heavy chains in a reticulocyte cell-free system. (*A*) Phenol-extracted RNA from polysomes of 72-hr cultures displayed on 15–30% sucrose gradients (see *Methods*). Fractions 1, 2, and 3 were collected after passage through a UV absorbance monitor. (*B*) Fractions 1, 2, and 3 were assayed in a cell-free reticulocyte lysate for MHC mRNA activity, after which myosin was extracted from the lysate and identified on 3.36% acrylamide gels. [³H]Leucine cpm in MHCs as programmed by RNAs from fractions 1, 2, and 3 (*A*) are shown. *Control* refers to radioactive protein obtained from lysates to which no muscle RNA was added.

rpm for 4 hr at 20° in a Beckman SW41 rotor (7). Gradients were monitored and fractionated with a UV photometer (Chromatronix model 200). Fractions were adjusted to 1% Sarkosyl and precipitated with 2.5 volumes of 95% EtOH.

Phenol Extraction. EtOH precipitates from sucrose gradients or polysomal pellets were resuspended in extraction buffer (50 mM sodium acetate–10 mM EDTA–0.5% sodium dodecyl sulfate pH 5.0), and an equal volume of redistilled phenol–chloroform isoamyl alcohol (25:24:1) with 0.1% 8-hydroxyquinoline was added. The aqueous phase was reextracted twice at 45° with the phenol mixture. A final extraction was with chloroform–isoamyl alcohol at 45°. The aqueous phase was adjusted to 200 mM potassium acetate and precipitated with 2.5 volumes of 95% EtOH (8).

Sucrose Gradients. Sucrose gradients (15–30%) were made in 10 mM Tris·HCl, pH 7.5–10 mM EDTA–100 mM NaCl–0.2% sodium dodecyl sulfate, and RNA dissolved in this buffer was layered on the gradients (10). Gradients were centrifuged for 6 hr at 40,000 rpm at 20° in a Beckman SW41 rotor.

Oligo(dT)–Cellulose Columns. RNA in 2.0 ml of binding buffer (500 mM KCl–10 mM Tris·HCl pH 7.8) was passed through a column of oligo(dT)–cellulose (1 g) (Collaborative Research, Inc.) and the column was washed three times with 2.0 ml of binding buffer. Bound RNAs were eluted with four 2.0-ml washes of elution buffer (10 mM Tris·HCl, pH 7.8) (9). Bound fractions were made 200 mM in potassium acetate and all fractions were precipitated with 2.5 volumes of 95% EtOH.

Reticulocyte Lysate. Rabbit reticulocytes were prepared and lysed by the method of Evans and Lingrel (13) and stored at −80°. Cell-free protein synthesis was done according to Lockard and Lingrel (14) with these modifications: the KCl concentration of the lysate was raised to 0.15 M and no 2-mercaptoethanol was used in the reaction. After incubation for 60 min with [³H]leucine, 50 Ci/mmol, at 25° the lysate was adjusted to 0.5 M in KCl and centrifuged at 104,000 × *g* for 2 hr at 4° to sediment polysomes. The supernatant was removed, 20 μg of column-purified carrier myosin was added, and 10 volumes of ice-cold distilled water was added to precipitate the myosin.

Electrophoresis of Myosin. Myosin was subjected to electrophoresis on 3.36% SDS–polyacrylamide gels as described (2).

RNase Digestion. RNA was resuspended in 0.3 M NaCl–0.03 M sodium citrate, pH 6.7. 50 μg/ml of pancreatic ribonuclease (Worthington Biochemicals) and 10 units/ml of T1 nuclease (Calbiochem) was added to the RNA and incubated at 37° for 30 min. The reaction mixture was then extracted with phenol, and the RNA was precipitated with 95% EtOH (11).

Formamide Gels. 10% formamide gels were cast and subjected to electrophoresis as described (12).

RESULTS

MHC mRNA from Total Polysomes and Puromycin-Dissociated Polysomes of 72-Hr Myogenic Cell Cultures. When polysomes from well-fused, 72-hr cultures are extracted with phenol, the RNA obtained shows the sedimentation pattern on a sucrose gradient given in Fig. 1*A*. When this displayed RNA is assayed, the mRNA activity for MHCs is found predominantly in fraction 3 of Fig. 1*A*, in that RNA sedimenting between 18 and 26S (Fig. 1*B*). If we isolated MHC mRNA from puromycin-dissociated polysomes (7, 15), then the MHC mRNA activity is found predominantly associated with the ribonucleoprotein sedimenting between 30 and 40S. The puromycin treatment dissociates the muscle polysomes to ribosomal subunits. In our procedure the 60S ribosomal subunit is pelleted (Fig. 2*A*) and the material under the 30–40S peak is taken for extraction of RNA and assay in the reticulocyte lysate for MHC subunits (Fig. 2*B*).

HMC mRNA Does Not Bind to Oligo(dT)–Cellulose Columns. In all cases in which we assayed for MHC mRNA activity we

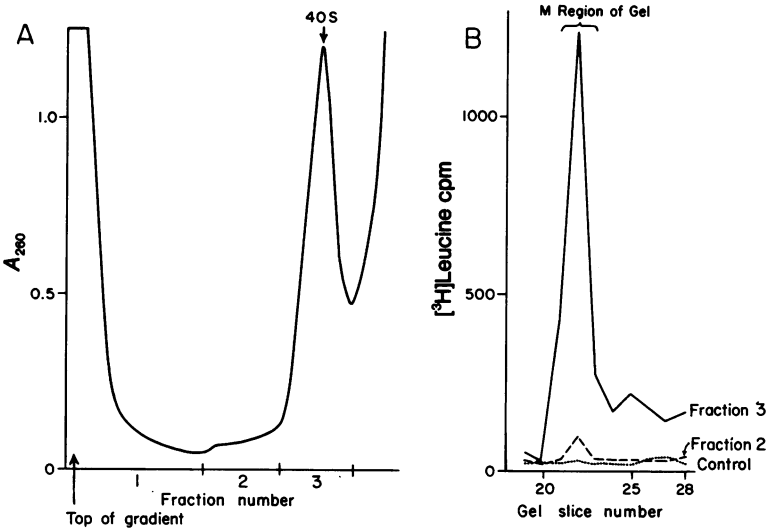

FIG. 2. Translation of RNA derived from puromycin-dissociated 30–40S ribonucleoprotein particles for myosin heavy chains. (*A*) Total polysomes from 72-hr cultures were treated with 1 mM puromycin and the material was sedimented on 5–20% sucrose gradients in TKM-IV. Fractions 2 and 3 were collected after passage through a UV absorbance monitor. (*B*) Fractions 2 and 3 were extracted with phenol, purified, and assayed in a cell-free reticulocyte lysate for ability to direct the incorporation of [³H]leucine into MHCs. The assay was processed as in *Methods*. The labeling pattern of nascent polypeptides in sodium dodecyl sulfate–acrylamide gels is shown. *M* = myosin.

found that this mRNA did not bind to oligo(dT)–cellulose columns. This result was the same whether we started with phenol-extracted RNA from total polysomes or Sarkosyl-extracted RNA from puromycin-dissociated 30–40S material. The conditions used for binding these RNAs to oligo(dT)–cellulose columns gave quantitative binding of synthetic poly(adenylic acid). The results are given in Fig. 3*A*. This figure represents the outcome for RNA derived from the 30S to 40S puromycin-dissociated subunits. The RNA from the columns was concentrated by EtOH precipitation and assayed in the cell-free lysate. 95% of the MHC mRNA activity was obtained from RNA that elutes from the columns in the binding (wash) buffers.

We determined the average size of poly(A) tracts of the RNA bound and not bound to the oligo(dT)–cellulose columns. The cultures were labeled for 1 hr with [³H]adenosine, and

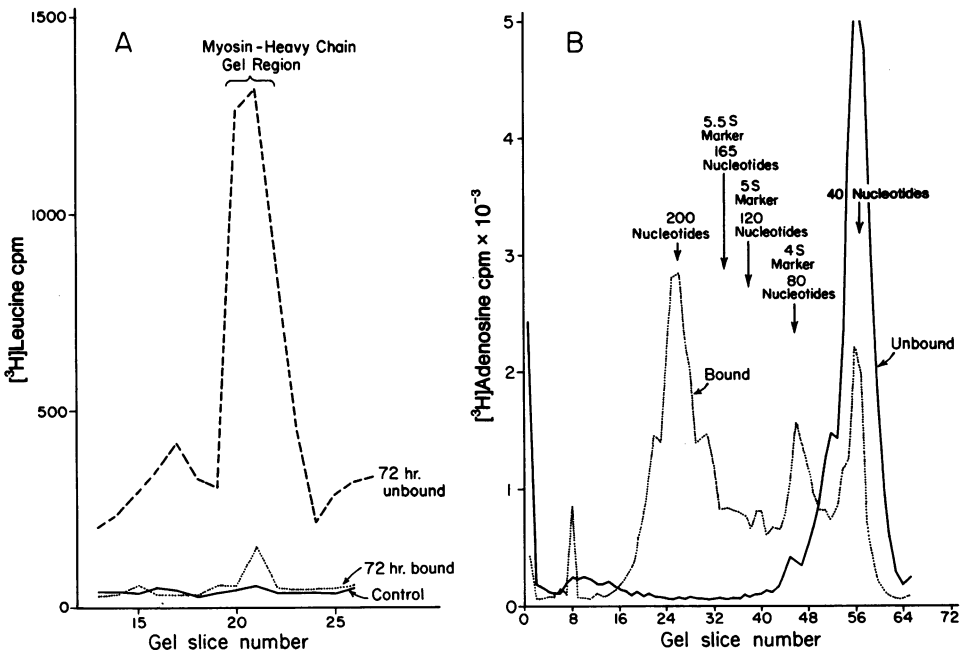

FIG. 3. mRNA for MHCs does not bind to oligo(dT)–cellulose columns. (*A*) RNA as derived in Fig. 2*A* is passed over oligo(dT)–cellulose columns before assay in a cell-free reticulocyte lysate. The ability to direct incorporation of [³H]leucine into MHCs is almost completely restricted to the fraction that does not bind to oligo(dT)–cellulose columns. (*B*) Poly(A) sequences associated with unbound material are smaller than 50 nucleotides long. RNA bound and not bound to oligo(dT)–cellulose was digested with RNase. The size of the undigested polyadenylate chains was determined by electrophoresis on 10% formamide gels. Values for cpm have been multiplied by 10^{-3}.

RNA was prepared from the puromycin-dissociated material. Of the total cpm in RNA placed on an oligo(dT)–cellulose column, approximately 20% bound to the column. Labeled bound and unbound RNA was subjected to procedures that digest RNA but that leave the poly(A) tracts intact (11). The digested material was then run on 10% formamide acrylamide gels (12); the result is shown in Fig. 3*B*. The RNA binding to the oligo(dT)–cellulose column displays a large size spectrum of poly(A) tracts and clearly RNA containing poly(A) tracts of greater than 100 nucleotides is isolated from the muscle-culture polysomes. The RNA that does not bind to these columns and that contains the mRNA activity for MHCs shows a major component on the acrylamide gels with an average length of 40 nucleotides. A poly(A) length of 50 nucleotides is the minimum for stable binding of RNA to these columns (19).

MHC mRNA Is Not Detected in Fusion-Blocked Myogenic Cells. We determined the earliest time during myogenesis in culture that we could detect mRNA activity for MHC. In these experiments the RNA obtained from puromycin-dissociated polysomes was passed over oligo(dT)–cellulose columns before assay for mRNA activity in the cell-free system. In all cases almost 100% of the relevant mRNA activity was associated with RNA not bound to the oligo(dT)–cellulose column. The result is given in Fig. 4. It is clear that MHC mRNA activity is detected as early as 46 hr of total culture time. At this time the main burst of cell fusion is 50% complete (2) and small myotubes are observed randomly on all culture plates. There is no detectable MHC mRNA associated with cells 36 hr in culture. At this time, there is present a small (less than 10%) population of myotubes that is synthesizing myosin (2), but the efficiency of our procedures for recovering and translating mRNA is evidently not sufficient to allow detection of the small amounts of mRNA required for that synthesis.

The next question was whether myogenic cells that had been prevented from fusing would show detectable levels of MHC mRNA. EGTA added to myogenic cell cultures will block cell fusion reversibly (2) and will prevent the normal rise in rates of myosin synthesis associated with cell fusion. Myogenic cell cultures blocked in this way do not yield detectable amounts of MHC mRNA (Fig. 4). The fusion-blocked cultures were assayed at 72 hr, which is a peak time in normal muscle cultures for production of the MHC mRNA.

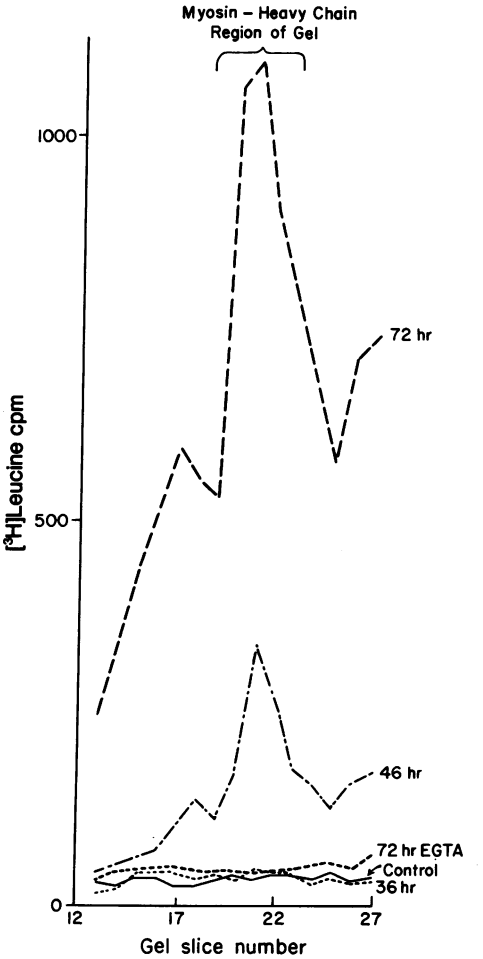

FIG. 4. Appearance of mRNA for MHCs as a function of myogenic cell fusion time in culture. RNA was prepared as in Fig. 3*A* from normal cell cultures at 72, 46, and 36 hr and from 72-hr cultures that had been prevented from fusing by addition of EGTA to the culture medium. The figure shows [³H]leucine cpm incorporated into MHCs as directed by these RNAs.

DISCUSSION

This is the first report on the isolation of an active mRNA for myosin heavy polypeptide chains (MHC mRNA) from developing skeletal muscle cells *in vitro*. This RNA has been isolated from whole chick-embryo muscle and has been characterized in several earlier reports (17, 18, 22). We confirm, with the MHC mRNA from cultured cells, much of what has been found for the mRNA from whole muscle developing *in vivo* or *in ovo*. Thus, we find that the mRNA is very large and sediments in sucrose gradients between 18 and 26S. In addition we find that the MHC mRNA may be obtained from puromycin-dissociated polysomes and that in the dissociated state it sediments together with the small ribosomal subunit between 30 and 40S. This mRNA may therefore be associated with specific protein, as reported for the heterogeneous mRNA obtained from polysomes of L cells and rat hepatocytes (16). On the other hand, the MHC mRNA obtained by us does not bind to oligo(dT)–cellulose columns, and if it contains tracts of poly(adenylic acid), these tracts are smaller than 50 nucleotides in length (Fig. 3*B*). This finding does not confirm an earlier report (22) which assigns a poly(adenylic acid) tract of 170 nucleotides to MHC mRNA isolated from whole muscle. Our attempts to enrich RNA fractions for MHC mRNA activity by binding to oligo(dT)–cellulose are not successful.

The experiments discussed above are all performed on 72-hr cultures. At this time myogenic cell fusion is complete, myotubes are well developed, and some are cross striated. Myosin synthesis is well under way and the rate of myosin synthesis is increasing linearly (2). We have suggested that myosin synthesis is causally related to cell fusion in these cultures since procedures that block cell fusion but that do not affect leucine intracellular pools or the rates of synthesis of total RNA and total protein nevertheless prevented the normal increase of myosin synthesis rates. Other studies have also pointed to the fact that myosin synthesis and the appearance of enzyme activities characteristic of terminally differentiated muscle are not demonstrable until after myogenic cell fusion (1, 3, 4). For the mass of skeletal muscle, therefore, it is clear that a direct coupling might exist between cell fusion on the

one hand and the regulation for terminal differentiation on the other. The level of this coupling, however, remains undefined. We have suggested earlier (2) that the synthesis of myosin is coupled to cell fusion at the level of transcription of new mRNA. Yaffe and Dym (5) have presented evidence, for rat myogenic cells in culture, that the fusion-coupled level of regulation is posttranscriptional. They suggest that presumptive myoblasts that have not fused contain significant amounts of mRNA for myosin that is not translated until after cell fusion. Several workers have observed in unfused myogenic cells the presence of large, helical polyribosomes, and the suggestion has been made that these may indeed be the sites of latent myosin synthesis (2, 20, 21).

We have examined isolated polyribosomes from fused and unfused myogenic cells and we find no evidence for the presence, in unfused cells, of MHC mRNA. The results of these experiments are given in Fig. 4. At 46 hr, cell fusion has occurred to about the 50% level and the maturation of the myotubes has scarcely begun. At this stage, our previous studies on the direct labeling of nascent proteins have shown that myosin synthesis is just beginning to change to the linearly increasing rate. As the figure shows, we are also able to detect at this stage the presence of MHC mRNA associated with polysomes. At 36 hr, which is about 4–5 hr before the major burst of cell fusion, there are only scattered small myotubes, and while these precociously fused cells are most probably synthesizing myosin, we are not able to detect the small amount of MHC mRNA that must be present. This result suggests two things. First, the vast majority of the unfused myogenic cells in the culture (90%) do not contain polysome-associated MHC mRNA, or second, these single cells at 36 hr do contain such mRNA but we are unable to detect it. It may be, for example, that the cells have recently exited from the last division before terminal differentiation and that they are just beginning to produce MHC mRNA independently of cell fusion but at levels too low to detect at this stage.

We therefore measured the polysome-bound MHC mRNA that might be present in single myogenic cells that have completed the last division before terminal differentiation but that have been prevented from fusing by addition of EGTA to the culture medium. These cells whose fusion is blocked by EGTA are arrested in G1 of the cell cycle, they show normal intracellular leucine pools, they synthesize total RNA and total protein at a normal rate, and they proceed to grow (2). Most of these cells become highly bipolar and quite thick. These cells do not however enter into the linearly increasing rate of myosin synthesis typical of the normal fused cells. If the unfused myogenic cells began to regulate for myosin synthesis after the last mitotic division, independently of cell fusion, then by 72 hr there should be an extensive store of untranslated MHC mRNA in the cytoplasm of these cells. The results of the experiment are given in Fig. 4. We are not able to detect any MHC mRNA associated with polysomes in fusion-blocked cells after 72 hr of total culture time. We may conclude therefore that the model for control of terminal differentiation in skeletal muscle that involves the presence of latent, polysome-associated mRNAs may be ruled out. The simplest model for a fusion-coupled regulation of terminal differentiation as marked by myosin synthesis, is one that results in the synthesis of new mRNA. There are of course other models. The use of chicken-embryo myogenic cultures should provide some basis for resolving doubts about these models and for developing insights into modes of regulating differentiation in eukaryotic cells.

The studies reported so far point to cell fusion as a critical time in which regulatory events for the synthesis of muscle structural proteins are best studied. It is interesting to note however that the development of the contraction and excitation systems of skeletal muscle may be distinctly separate in time. The elaboration of acetylcholine receptor sites on myogenic cells does not require cell fusion (23). These receptors appear on fusion-blocked myogenic cells that otherwise fail to progress in differentiation, as marked by morphological change and by myosin synthesis. In addition, the specific molecules required for cell fusion are also elaborated before myosin synthesis (2, 4). This apparent separation of controls for the synthesis of membrane components from controls regulating synthesis of cytoplasmic structural proteins may afford a unique opportunity to probe relationships between events at the cell surface and subsequent and possibly dependent intracellular events.

We thank Mrs. Julie Micou-Eastwood for technical assistance and acknowledge the very helpful advice and assistance of Dr. Fred H. Wilt. This work was supported by Grant GM 13882 from the National Institutes of Health. A. P. is a fellow of the USPHS GM47394-03.

1. Coleman, J. R. & Coleman, A. W. (1968) *J. Cell Physiol.* **72**, Suppl. 1, 19–34.
2. Paterson, B. & Strohman, R. C. (1972) *Develop. Biol.* **29**, 113–138.
3. Yaffe, D. (1971) *Exp. Cell Res.* **66**, 33–48.
4. Shainberg, A., Yagil, G. & Yaffe, D. (1971) *Develop. Biol.* **25**, 1–29.
5. Yaffe, D. & Dym, H. (1973) *Cold Spring Harbor Symp. Quart. Biol.* **37**, 543–547.
6. O'Neill, M. & Strohman, R. C. (1969) *J. Cell Physiol.* **73**, 61–68.
7. Blobel, G. & Sabatini, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 390–394.
8. Aronson, A. I., Wilt, F. H. & Wartiovaara, J. (1972) *Exp. Cell Res.* **72**, 309–324.
9. Aviv, H. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1408–1412.
10. Penman, S. (1966) *J. Mol. Biol.* **17**, 117–130.
11. Wilt, F. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2345–2349.
12. Staynov, D. V., Pinder, J. C. & Gratzer, W. B. (1972) *Nature New Biol.* **235**, 108–110.
13. Evans, M. J. & Lingrel, J. B. (1966) *Biochemistry* **8**, 829.
14. Lockard, R. E. & Lingrel, J. B. (1969) *Biochem. Biophys. Res. Commun.* **37**, 204–212.
15. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 832–835.
16. Blobel, G. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 924–928.
17. Morris, G., Buzash, E., Rourke, A., Tepperman, K., Thompson, W. & Heywood, S. M. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 535–541.
18. Heywood, S. M. & Nwagwu, M. (1969) *Biochemistry* **8**, 3839–3845.
19. Sullivan, N. & Roberts, W. K. (1973) *Biochemistry* **12**, 2395–2403.
20. Fischman, D. (1972) in *Structure & Function of Muscle* (Academic Press, New York), Vol. I, pp. 75–132.
21. Allen, R. & Pepe, F. A. (1965) *Amer. J. Anat.* **116**, 115–148.
22. Mondal, H. & Sarkar, S. (1973) *Fed. Proc.* **32(3)**, pt 1, 456 Abstr. 1307.
23. Paterson, B. & Prives, J. (1973) *J. Cell Biol.* **59**, 241–245.