Myosin Messenger RNA: Studies on Its Purification, Properties and Translation during Myogenesis in Culture

GLENN E. MORRIS,* ELIZABETH A. BUZASH, ARTHUR W. ROURKE, KATHERINE TEPPERMAN, WILLIAM C. THOMPSON, AND STUART M. HEYWOOD

Biological Sciences, Genetics and Cell Biology Section, University of Connecticut, Storrs, Connecticut 06268

Although a great deal is known concerning the chemistry and function of the contractile proteins, it has been only recently that progress has been made in attempts to gain a detailed understanding of the biosynthesis of these proteins. It is our ultimate goal to elucidate the control mechanisms involved in myofibrillar protein synthesis, to understand the role of these mechanisms in the development of muscle, and to establish the relationship between the synthesis of these proteins and their ultimate assembly into the contractile apparatus. Although we are currently working on the synthesis of many of the structural proteins of chick muscle, the relative ease by which polysomes synthesizing myosin heavy chain can be isolated on sucrose density gradients (Heywood and Rich, 1968) has led us to analyze myosin synthesis in some detail.

An RNA fraction, sedimenting at approximately 26S, can be extracted from the large myosin-synthesizing polysomes. This RNA fraction has been shown by a number of criteria to contain messenger RNA (mRNA) coding for the large molecular weight subunit (200,000) of myosin (Heywood and Nwagwu, 1969; Heywood, 1969; Rourke and Heywood, 1972). Peptide analysis by two-dimensional high voltage electrophoresis as well as by ion exchange chromatography has suggested that the myosin mRNA is translated with a high degree of fidelity in a cell-free amino acid incorporating system (Rourke and Heywood, 1972).

We report here some further studies on myosin synthesis in cell-free systems and some observations on the relationship between cell fusion and myosin biosynthesis in differentiating cell cultures.

We also describe a method for the isolation of polysomal myosin mRNA by removal of 28S ribosomal RNA (rRNA) and some preliminary studies on its properties and biosynthesis in chick muscle cell cultures. We hope that this approach will be useful in analyzing directly the role of mRNA synthesis during muscle differentiation.

* Present address: School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex, Great Britain.

Results and Discussion

It has been amply demonstrated that most, if not all, proteins incorporate methionine into the N-terminal position during the initiation of protein synthesis (Lucas-Lenard and Lipmann, 1971; Lengyel and Soll, 1969). In prokaryotes formylmethionine is utilized, whereas in eukaryotes methionine is incorporated into the N-terminal position. In a significant number of cases in both prokaryotes and eukaryotes, the N-terminal methionine is cleaved off during polypeptide chain growth or soon after completion of the synthesis of the polypeptide (Lucas-Lenard and Lipmann, 1971). In order to determine if myosin heavy chain synthesis also initiates with methionine, 14-day embryos were pulse-labeled in vivo with a mixture of [^{35}S]methionine and [^{3}H]serine. Small nascent chains, selectively isolated by phenol extraction (Jackson and Hunter, 1970), were subsequently subjected to Edman degradation (Gray, 1967). As shown in Table 1, a fraction of the nascent peptides in both polysome fractions have an N-terminal methionine. The amount of serine present may imply that some of the longer chains have already been cleaved of their N-terminal amino acid. The fact that the Edman degradation is only 75% effective (Jackson and Hunter, 1970) accounts for the small amount of methionine observed in the

Table 1. N-Terminal Amino Acids Obtained from Myosin-synthesizing Polysomes and Small Polysomes

Polysome Fraction	Edman Degradation Step	[^{35}S]Met (cpm)	[^{3}H]Ser (cpm)
Myosin-synthesizing polysomes	1	18	460
	2	7	1530
Small polysomes	1	72	400
	2	20	460

Embryonic chicks (14-day) were pulse-labeled with a mixture of [^{35}S]methionine and [^{3}H]serine for 5 min. Myosin-synthesizing polysomes and smaller polysomes were prepared by sucrose density gradient centrifugation (Heywood and Rich, 1969). Short nascent chains were recovered by phenol extraction (Jackson and Hunter, 1970) and subsequently submitted to Edman degradation (Gray, 1967).

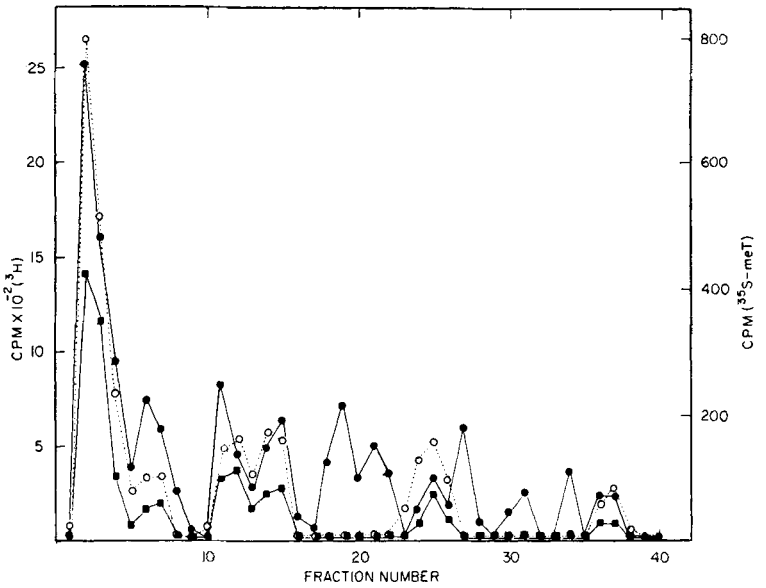

Figure 1. Analysis of the proteolytic digest of the 200,000 mol wt subunit of myosin by ion exchange chromatography on Aminex A-5 resin (Bio-Rad Laboratories). Cell-free synthesis of myosin, proteolytic digestion of the large myosin subunits, and elution of the peptides from the ion exchange column were as previously described (Rourke and Heywood, 1972). Peptides from myosin labeled in vivo with ^{3}H-amino acid mixture (●—●) were counted by liquid scintillation. A low background gas flow counter was used to count the [^{35}S]methionine-labeled peptides from myosin labeled in vivo (○—○) and in the cell-free system (■—■).

second step of degradation. The large increase in serine in the nascent chains from myosin-synthesizing polysomes at the second cleavage suggests an N-terminal sequence of Met-Ser for growing myosin polypeptide chains. This agrees well with findings of Offer (personal communication) suggesting an N-terminal sequence for myosin heavy chain as *N*-acetyl-Ser-Ser-Asp-.

If a proteolytic digest of [^{35}S] methionine-labeled myosin, synthesized in a cell-free amino acid incorporating system, is chromatographed on an ion exchange column and compared to in vivo labeled [^{3}H]myosin (using a mixture of ^{3}H-amino acids) and in vivo labeled [^{35}S]methionine, a good correspondence of in vivo and in vitro labeled myosin peptides is obtained (Fig. 1). These results suggest that, within the limits of the column separation of the peptides, myosin is synthesized with a high degree of fidelity in the cell-free system and contains no additional methionine peptides. Therefore, unless there is an extra methionine peptide in the unresolved first peak, the N-terminal methioinine is cleaved off in the in vitro system as well as in the intact cell.

The myosin message is by far the largest eukaryotic mRNA that has been used to program a cell-free system and once its translation is initiated, we might expect a considerable lag, as nascent chains grow, before completed chains are released from the ribosomes. As shown in Fig. 2, there is a lag period of about 8 minutes before completed myosin chains are released, while endogenous globin synthesis is measurable at the earliest times tested.

Assuming that initiation occurs at the start of the incubation (addition of mRNA to the incubation mixture), myosin chain growth occurs at the rate

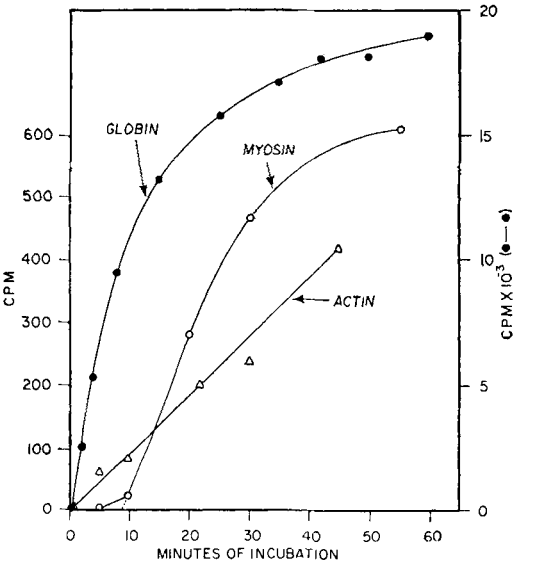

Figure 2. The kinetics of myosin synthesis in a heterogeneous cell-free amino acid incorporating system. The conditions for amino acid incorporation using chicken erythroblast ribosomes were as previously described (Rourke and Heywood, 1972). Three different incubation mixtures were used: (1) to measure globin system without added mRNA (●—●); (2) with added 26S myosin mRNA to measure myosin synthesis (○—○)—in this reaction mixture, globin synthesis was similar to the incubation mixture without added mRNA; and (3) with added 10–17S muscle mRNA to measure actin synthesis. Globin synthesis was taken as the total incorporation due to endogenous mRNA of the system. Myosin synthesis was determined as previously described by acrylamide gel electrophoresis (Rourke and Heywood, 1972), and the actin-like protein was measured from the radioactivity which was extracted from an acetone powder (0.2 mM ATP, 1 mM dithiothreitol, pH 7.2) of the incorporation system and migrating with carrier actin on SDS acrylamide gels. The radioisotope used was 30 μCi of a mixture of ^{14}C-amino acids (New England Nuclear, NEC-445). Radioactivity was measured on a low background, gas flow counter.

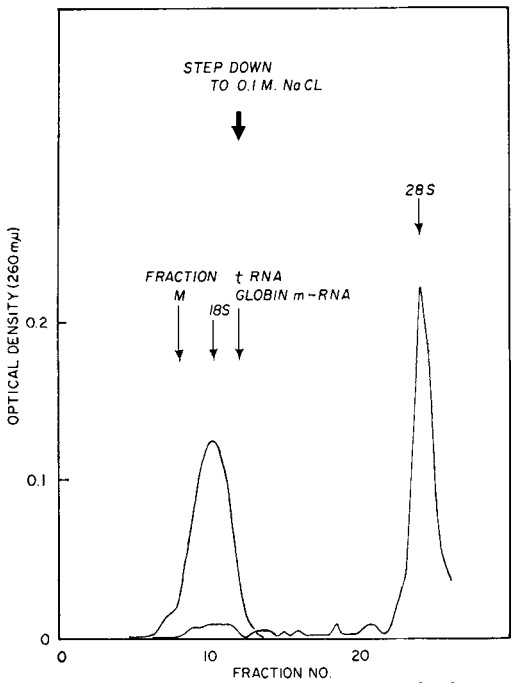

Figure 3. Separation of RNA species by a Sepharose 2B column. 18S and 28S RNA were prepared separately from chick embryonic leg muscle ribosomes by sucrose density gradient centrifugation (see Fig. 5). Chick erythroblast globin mRNA was prepared as described by Heywood (1970) and chick muscle tRNA as described by von Ehrenstein (1968). Fraction *M* refers to the radioactivity from polysomes which we identify as myosin mRNA. The column dimensions were 50 × 1 cm and 120 drop fractions (approx. 3 ml) were collected. The elution buffer was 500 mM NaCl, 20 mM Tris, 2.5 mM EDTA, 0.5% SDS, pH 7.5. To elute 28S rRNA, the salt concentration was reduced to 100 mM as indicated.

of 4–5 amino acids per second, which is comparable to the rate of 4–6 amino acids per second estimated for globin on the basis of observed translation times of the order of half a minute (Lamfrom and Knopf, 1965; Hunt et al., 1969).

The synthesis of an actin-like protein, responsive to the addition of 10–17S muscle mRNA, was also followed in the amino acid incorporating system (Fig. 2). Some radioactivity is observed to be incorporated into this protein in the absence of added mRNA, and the synthesis of the actin-like protein did not require the addition of muscle initiation factors as does the synthesis of myosin (Rourke and Heywood, 1972). Although preliminary, these results suggest that chicken erythroblasts may synthesize a small amount of an actin-like protein in addition to their main synthetic product hemoglobin.

The myosin mRNA fraction obtained by sucrose density gradient centrifugation contains a large contaminant of 28S rRNA. In order to investigate the details of myosin mRNA synthesis and appearance into polysomes during muscle differentiation, we have sought a method of purifying intact myosin mRNA.

We have taken advantage of the observation that 28S rRNA is bound to Sepharose 2B at high ionic strength (Petrovic et al., 1971), and have found that mRNA is not bound in this way. Figure 3 shows the behavior of several RNA species on a Sepharose 2B column. More than 98% of added 28S RNA is retained by the Sepharose at high ionic strength and released when the ionic strength is reduced. When a pulse-labeled polysomal RNA fraction expected to contain 28S RNA and myosin mRNA is subjected to Sepharose 2B chromatography, a considerable proportion (fraction M) is not retained by the Sepharose and elutes slightly ahead of an 18S marker. In the same way a preparation of 9S globin mRNA was not bound to the Sepharose, but because of its smaller size, was considerably retarded relative to 18S RNA.

Our procedure for isolating fraction M and partially characterizing it as myosin mRNA is shown in Table 2. A gradient of polysomes obtained from cultured embryonic chick thigh muscle cells

Table 2. Procedure for Isolation of Myosin mRNA

Five 4–5-day cultures of embryonic chick thigh muscle cells, labeled for 60 min with [^{3}H]uridine (100 μCi per culture)

↓

Lyse cells with 0.5% Triton X-100 in buffer. Centrifuge at 10,000 *g* for 10 min

↓

Isolate myosin-synthesizing polysomes (MSP) from polysome gradient (Fig. 4)

↓

Collect polysomes on Millipore filters and treat with 0.5% SDS

↓

Isolate 24–30S RNA fraction (containing myosin mRNA and 28S ribosomal RNA) on RNA gradients (Fig. 5)

↓

Precipitate with ethanol

↓

Remove 28S rRNA using Sepharose 2B column (Fig. 6)

↓

Precipitate with ethanol

mRNA fraction		rRNA fraction	
Rerun on RNA gradients (Fig. 7A)	Gel electrophoresis (Fig. 7C)	Rerun on RNA gradients (Fig. 7B)	Gel electrophoresis (Fig. 7D)

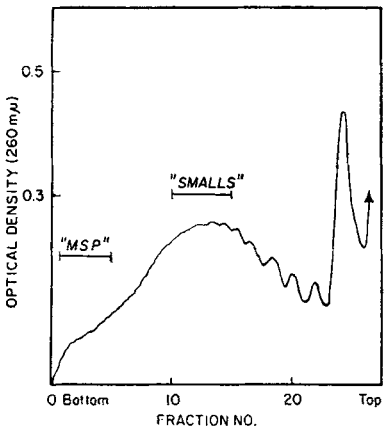

Figure 4. Sucrose density gradient of polysomes from cultured chick muscle cells. 0.2–0.3 ml of packed cells were lysed in 1 ml of TAM buffer (10 mM Tris, 250 mM NH_4Cl, 10 mM $MgCl_2$, pH 7.4) containing 0.5% Triton X-100. The 10,000 *g* supernatant was placed on a 15–40% sucrose gradient in TAM buffer and centrifuged at 25,000 rpm for 84 min at 2°C.

(Morse et al., 1971) is shown in Fig. 4. No changes in the pattern of the optical density profiles have been observed between one and five days in culture. Hosick and Strohman (1971) observed no change in the ratio of polysomes to monomers over this period. The myosin-synthesizing polysome fraction was collected on Millipore filters (Heywood and Nwagwu, 1969) and the RNA from it was analyzed by gradient centrifugation (Fig. 5). Fraction A (24–30S RNA) from the gradient was then subjected to Sepharose 2B chromatography (Fig. 6).

Both the mRNA and rRNA fractions were divided into two and analyzed on sucrose gradients and polyacrylamide gels as shown in Fig. 7. On sucrose gradients all the radioactivity in the mRNA fraction sediments as a symmetrical peak at about 26S (Fig. 7A), whereas the radioactivity from the rRNA fraction sediments with the carrier 28S rRNA (Fig. 7B). The absence of lower molecular weight material indicates that the RNA is not degraded during Sepharose column chromatography.

When the other half of the mRNA fraction is analyzed on polyacrylamide gels (Fig. 7C), it migrates as a symmetrical peak of very high apparent molecular weight. The second peak in Fig. 7C is not always present. The 28S RNA fraction on gels migrated as a well-defined peak (Fig. 7D). The aggregated material near the top of the gel does not correspond to the mRNA peak of Fig. 7C but may be related to aggregated material seen on the sucrose gradient (Fig. 7B). To emphasize that the mRNA peaks on gradients and gels are the same material, 628 cpm were recovered from the gels and 606 cpm from the gradients. The corresponding figures for the RNA fraction were 6600 and 7600 cpm, respectively.

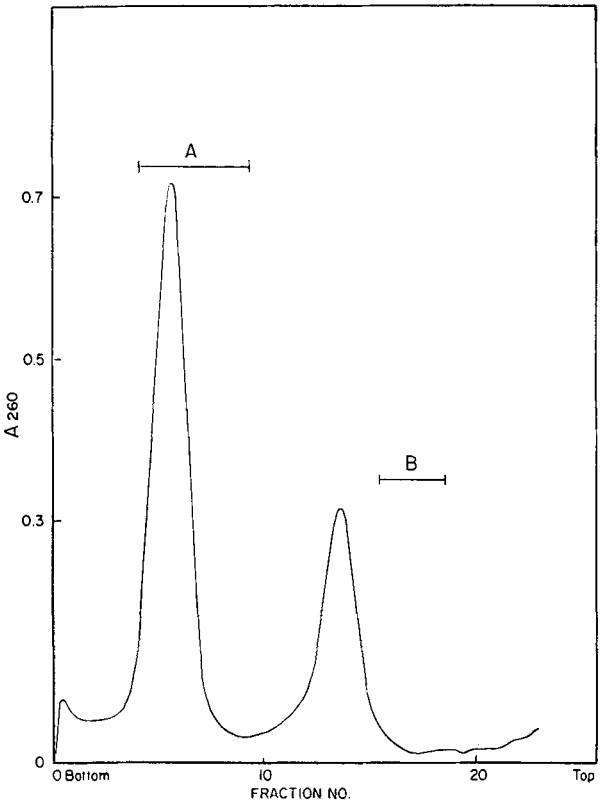

Figure 5. Sucrose density gradient of RNA from SDS-treated polysome fractions. 10–30% sucrose gradients in buffer (50 mM Tris-acetate, 5 mM EDTA, 0.5% SDS, pH 7.4) were centrifuged at 22,000 rpm for 16 hr at 2°C. Embryonic chick muscle ribosomes were added to the sample as carrier if necessary.

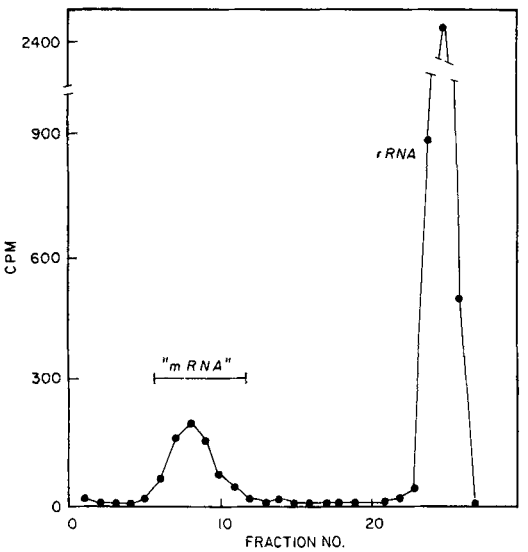

Figure 6. Sepharose 2B elution profile (Fig. 3) of fraction A RNA (24–30S; Fig. 5) from myosin-synthesizing polysomes (MSP; Fig. 4), showing the proposed separation of 26S myosin mRNA from 28S rRNA. Samples were taken up in Triton scintillation fluid (0.5% PPO and 30% Triton X-100 in toluene) and counted in a Nuclear Chicago liquid scintillation counter at 30% efficiency.

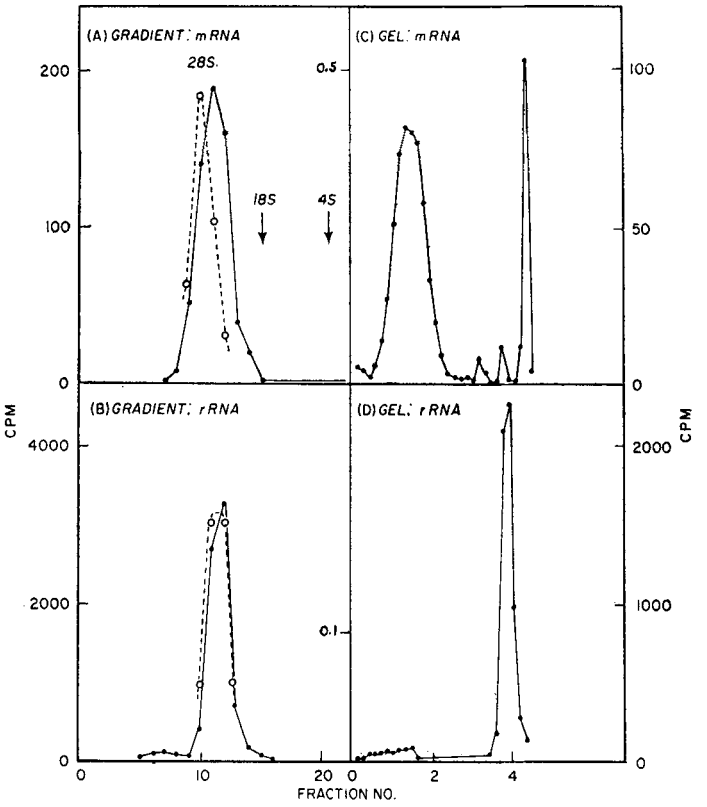

Figure 7. Analysis of RNA fractions from Sepharose 2B columns by sucrose density centrifugation and polyacrylamide gel electrophoresis. The two fractions from Sepharose 2B (mRNA and rRNA Fig. 6) were made 0.25 M in Tris-HCl pH 7.4 and precipitated with ethanol. Fifty μg of tRNA were added as carrier to the mRNA fraction. Half of each fraction was analyzed on gradients and the other half on gels. Analysis by gradient centrifugation was performed as in Fig. 5. One-ml fractions, after A_{260} determination (○—○) were taken up in Aquasol (New England Nuclear) and radioactivity was determined by liquid scintillation. Gels were 10 cm, 2.5% acrylamide/0.125% bisacrylamide in 40 mM Tris-acetate, 1 mM EDTA, 0.5% SDS, pH 7.6 and electrophoresis was at 4°C with 5 mA/tube. Only the first 4 cm of gel is shown for all the radioactivity is in this area of the gel. Gels were frozen and cut into 3 × 0.5 mm fractions, which were shaken for 6 hr in 1 ml of 0.6 M sodium acetate, 0.5% SDS, pH 6, at 37°C. Radioactivity was determined in Aquasol.

Myosin mRNA, coding for a protein of 200,000 daltons mol wt, should itself have a mol wt of the order of 2×10^6 daltons compared with a value of 1.6×10^6 estimated for chick 28S RNA (Loening, 1968). The relatively low apparent S value as determined by sucrose density gradients and the relatively low electrophoretic mobility on acrylamide gels suggest that myosin mRNA may have a more extended configuration than 28S rRNA under the conditions used. Additional studies, utilizing conditions assuring the complete unfolding of RNA molecules, will be required before a precise determination of the molecular weight of myosin mRNA can be obtained.

Further properties of the myosin mRNA fraction are shown in Table 3. The ratio of mRNA to rRNA from the Sepharose column is somewhat variable between experiments, probably because of varying degrees of resolution of MSP on the polysome gradients. The proportion of mRNA falls off drastically with longer labeling periods, which may reflect a delay in the appearance of newly synthesized rRNA in the heavy polysomes. Myosin-synthesizing polysomes (MSP) contain a high proportion of the mRNA fraction relative to smaller polysomes, indicating that the RNA is not merely nonspecific RNA cosedimenting with the polysomes.

The absolute requirement for a proposed mRNA species is that it direct the synthesis of its corresponding protein in a heterologous cell-free system. Sepharose column RNA fractions were prepared as

Table 3. Analysis of Labeled Polysomal-bound mRNA from In Vivo and In Vitro Labeled Muscle

Source of RNA	Labeling Time (hr)	Polysome[a] Fraction	RNA Gradient Fraction[b]	Percent mRNA[c] (cpm)
^{32}P-labeled 14-day embryos	1.5	MSP	A	10
	5	MSP	A	2
	1.5	Smalls	B	90
[^{3}H]uridine-labeled 3–4-day cultured muscle cells	1	MSP	A	48
	1	Smalls	A	10

[a] See Fig. 4. [b] See Fig. 5. [c] The radioactivity in the mRNA fraction of Fig. 6 is expressed as a percentage of the total radioactivity eluted from the Sepharose 2B column.

Table 4. Cell-free Synthesis of Myosin from mRNA Fractionated on Sepharose 2B Column

RNA	Experiment 1	2	3
—	4	0	1
Unfractionated	78	36	—
mRNA fraction	62	37	446
rRNA fraction	4	2	8

cpm incorporated into the 200,000 mol wt subunit of myosin.

The 24–30S RNA fraction from myosin-synthesizing polysomes was prepared from 20–25 14-day, chick embryo leg muscle as previously described (Heywood and Nwagwa, 1969). The cell-free synthesis and acrylamide gel analysis of myosin heavy chain synthesis was performed as previously described (Rourke and Heywood, 1972). In Experiment 1 rRNA (28S) was added to the incubation mixture to an equal concentration as that eluted from the Sepharose 2B column. Radioactivity was measured on a low backgound (2 cpm) gas flow counter.

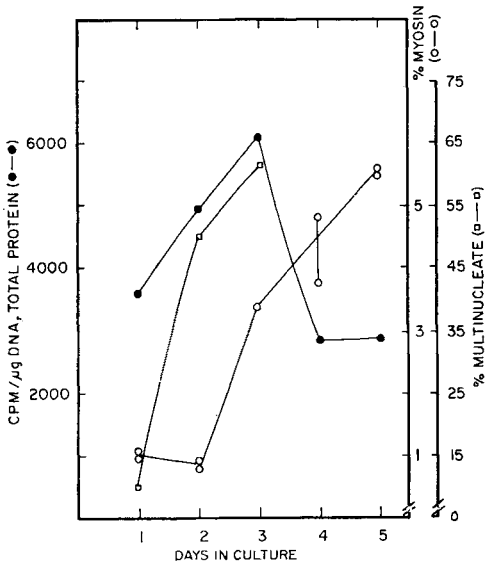

Figure 8. Relationship between myosin synthesis and the appearance of multinucleate muscle cells in culture. Muscle cells were prepared by a mechanical dissociation of leg muscle from 11-day chick embryos. Gels were plated at 5×10^6 cells per 15 cm plate. The cultures were assayed at 24 hr intervals. Four hours prior to analysis the cultures were fed and labeled with 5 μCi ^{3}H-amino acid mixture (New England Nuclear, NET-445) per plate. The number of plates per assay was 5 plates for 1 day, 5 on day 2, 5 on day 3, 3 on day 4, and 2 on day 5. Cells were harvested and lysed as previously described (Morse et al., 1970) except the buffer used was 0.47 M KCl, 0.01 M KH_2PO_4, 0.01 M $K_4P_2O_7$, pH 6.2. Myosin was extracted, purified, and analyzed by acrylamide gel electrophoresis as previously described (Rourke and Heywood, 1972). Radioactivity of myosin heavy chain was determined after electrophoresis by slicing the gel in 1-mm slices, solubilizing in NCS (Nuclear Chicago) and counting by liquid scintillation. Total readioactivity was determined from an aliquot of the whole lysate. DNA was determined by the method of Burton (1956). The percent multinucleated cells was determined from representative cultures at 24 hr intervals after fixing in methanol. Binucleate cells were not scored. (●—●) Total cpm incorporated per μg DNA; (□—□) percent nuclei scored in multinucleated cells; (○—○) percent of total cpm incorporated into myosin heavy chain.

described in Table 2 using RNA prepared from 14-day embryonic leg muscle. The RNA fractions were tested in a cell-free system derived from chicken erythroblasts. As shown in Table 4, over 98% of the messenger activity is found in the mRNA fraction and, when this activity is compared with that of an equivalent amount of unfractionated RNA, little or no mRNA activity appears to be lost during chromatography.

To summarize, our isolation procedure produces an apparently homogeneous RNA species with many of the expected properties of an active, undegraded myosin mRNA. We hope it will allow for an analysis of the synthesis of this specific transcription product during muscle cell differentiation. As a beginning in the attempt to understand the molecular events occurring during the terminal differentiation of muscle, an analysis of myosin synthesis occurring in differentiating cultured cells was undertaken preliminary to looking for the appearance of myosin mRNA during differentiation. As shown in Fig. 8, a low but significant amount of myosin synthesis is occurring in one-day muscle cell cultures. As cell fusion occurs between days 1 and 3 there is an apparent lag between fusion and increase in myosin synthesis (up to 6% of total protein synthesis), suggesting that the increase in myosin synthesis is not closely associated with cell fusion. The rate of incorporation into total protein per plate slowly decreases after three days of culture (not shown). The relatively rapid drop in cpm per μg DNA is due to an increase in DNA per plate. These results suggest the presence of active myosin mRNA in mononucleated myogenic cells. In addition, analysis of radioactive polysomal mRNA on Sepharose 2B as described in this paper suggests the possibility that myosin mRNA synthesis is occurring in the one-day cell cultures.

Acknowledgments

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