Partial Characterization of Presumptive Myosin Messenger Ribonucleic Acid*

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ABSTRACT: A procedure is described for obtaining high molecular weight ribonucleic acid from myosin-synthesizing polysomes.

Analysis of this ribonucleic acid by ^{32}P labeling and sucrose density gradient centrifugation reveals, in addition to the characteristic ribosomal ribonucleic acid (18 and 28 S), a unique species with a sedimentation coefficient of approximately 26 S. This species of ribonucleic acid is not found to be associated with nonmyosin-synthesizing polysomes or single ribosomes. The 26S ribonucleic acid was judged as presumptive messenger ribonucleic acid for the large 200,000 molecular weight subunit of myosin by the following criteria. When added to a cell-free system, it caused the formation of polysomes; it caused the synthesis of a protein which both precipitated with antimyosin and cochromatographed with myosin on DEAE-cellulose; and it specifically resulted in the synthesis of the large, major component of myosin distinguished by acrylamide gel electrophoresis.

A major concern of molecular biology is the manner by which genetic information is transcribed into mRNA and how this is further translated into the sequence of amino acids making up a polypeptide chain. Many facets of this information transfer have been revealed by studies with bacteria. Although the general models for control of protein synthesis derived from microbial genetics appear to be universally applicable, the complexity and lack of well-defined system have hindered progress in this area in higher organisms.

The isolation and characterization of mRNA which can be shown to direct the synthesis of a specific polypeptide chain is therefore of great interest. With such a mRNA questions may be asked about its synthesis, nuclear processing, transportation to the cytoplasm, binding to ribosomes, and initiation of protein synthesis. Recent success has been achieved in isolating hemoglobin mRNA from reticulocytes (Chantrenne *et al.*, 1967; Scherrer and Marcaud, 1968). A cell-free system from *Escherichia coli* primed with this RNA has been shown to produce a material having the properties of globin (Laycock and Hunt, 1969). Nevertheless, hemoglobin mRNA must be a mixture of at least two different mRNAs—that coding for the α chain and that coding for the β chain. It therefore has not yet been feasible to use this system for the isolation and characterization of an individual species of mRNA.

Techniques have recently been developed for obtaining intact polysomes from embryonic chick muscle (Heywood *et al.*, 1968). These polysomes can be separated into different size classes that can be shown to synthesize different cell-specific proteins (Heywood and Rich, 1968). The largest class, those containing 55–65 ribosomes, has been shown to be responsible for the synthesis of myosin (Heywood *et al.*, 1967). Subsequent studies demonstrated that total RNA extracted from myosin-synthesizing polysomes could direct the synthesis of myosin when added to a cell-free system, while RNA from other polysomes or single ribosomes was not effective (Heywood and Nwagwu, 1968). These experiments indicated the possibility of isolating and characterizing myosin mRNA. The results reported here suggest that a 26S RNA, extracted from myosin-synthesizing polysomes, is myosin mRNA. This RNA species appears to code for the large subunits of myosin, which have a molecular weight of approximately 200,000.

Materials and Methods

Preparation of Polysomes and RNA. Polysomes were obtained from 14-day-old chick embryo leg muscle as previously described (Heywood *et al.*, 1968). Polysomes of two size classes (A and B, Figure 1), as well as single ribosomes (C, Figure 1), were collected. Normally, six sucrose density gradients were used to collect fraction A polysomes and three to collect fractions B and C. The polysomes were collected on membrane filters (0.45 μ pore size, Millipore Filter Corp.) at 2°. The filters had been washed previously in sodium dodecyl sulfate, rinsed in H_2O, and finally washed with 0.25 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4). As the polysomes were filtered, the filtrate was collected into two volumes of cold ethanol and 100 μg of tRNA was added. After 12 hr at −20° the precipitate was collected and analyzed by sucrose density centrifugation (see below). After obtaining the polysomes on membrane filters, the filters were washed rapidly with 0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4). The RNA was then removed by washing the filters in 1 ml of buffer containing 0.5% sodium dodecyl sulfate, 0.005 M EDTA, 0.02 M sodium acetate, and 0.04 M Tris-HCl (pH 7.8) for 30 min. The filters were removed and allowed to drain. Both the wash, containing RNA, and the ethanol-precipitable material from the polysome filtrates were layered on 27 ml, 10–30% sucrose density gradients containing the sodium dodecyl sulfate, EDTA buffer, and centrifuged at 23,500 rpm for 18 hr in a Spinco No. 25.1 rotor. While fractions of the gradient were collected, the optical density at 260 mμ was recorded continuously with a Gilford spectrophotometer; 0.1 mg of tRNA was added to each 2-ml RNA fraction that was to be tested in a cell-free protein-

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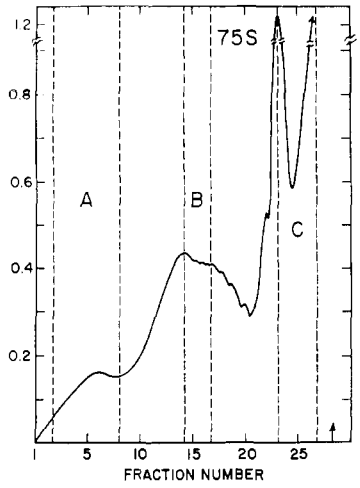

FIGURE 1: Sucrose density gradient profile of cytoplasmic extract of embryonic chick muscle. Muscle (6 g) was homogenized in 6 ml of cold buffer (0.25 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl, pH 7.4) and centrifuged at 10,000*g* for 10 min to obtain the cytoplasmic extract. The cytoplasmic extract from 0.7–0.8 g of muscle was layered on a 27 ml, 15–40% linear sucrose density gradient containing the homogenizing buffer and centrifuged at 25,000 rpm for 2 hr in a Spinco No. 25.1 rotor. The arrow at the lower right indicates the last fraction at the top of the gradient; 75 S indicates the peak of single ribosomes. Material from fractions A–C were collected on membrane filters as described in Materials and Methods.

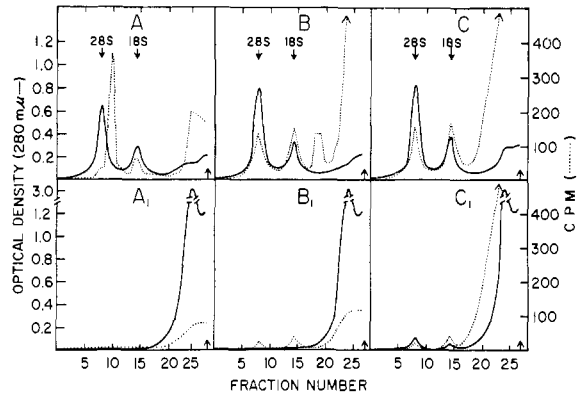

FIGURE 2: Sucrose density gradient patterns of RNA from polysomes and ribosomes. Embryos (14-days old) were injected with 750 μCi of [^{32}P]phosphoric acid and incubated for 75 min prior to preparation of the polymoses. Two 2.5-cm membrane filters were used together to filter the material from each gradient (Figure 1A–C). The filtrates (A_1–C_1) were collected, 100 μg of tRNA was added and precipitated with two volumes of ethanol. Polysome and filtrate RNA were layered on 27-ml 10–30% sucrose density gradients and centrifuged for 18 hr at 23,500 rpm in a Spinco No. 25.1 rotor; 1.0-ml fractions were collected and assayed for radioactivity after precipitation in cold trichloroacetic acid.

synthesizing system. The sample was then made 0.2 M with respect to KCl, chilled rapidly in an ice bath, and centrifuged to remove the potassium dodecyl sulfate precipitate. Finally two volumes of ethanol were added to the supernatant and the RNA was allowed to precipitate for 12–24 hr at −20°. Before use, the RNA was centrifuged from the ethanol and the tube was allowed to drain for 1 hr at 2°. In order to obtain active RNA preparations, great care is required to avoid introducing ribonuclease during the preparation of RNA. To this end, all glassware was acid washed, and sterile procedures were used in pipetting.

tRNA was prepared from adult chicken muscle by the method described by Von Ehrenstein (1968). RNA sedimentation coefficients were estimated using the method of Martin and Ames (1961) and are used only as an approximation for descriptive purposes.

Preparation of Ribosomes and S-150 Fraction. Ribosomes for cell-free amino acid incorporation were prepared from 14-day-old embryonic chick leg muscle; 20 g of muscle was dissected and homogenized with a Dounce-type homogenizer in 10 ml of B buffer (0.005 M $MgCl_2$–0.15 M KCl–0.02 M Tris-HCl, pH 7.6). The total homogenate was then made 0.015 M with respect to NaF and incubated for 20 min at 35°. Following incubation, the homogenate was centrifuged at 30,000*g* for 30 min; 8 ml of the resulting supernatant was layered on 2 ml of B buffer containing 10% glycerol and centrifuged for 2 hr at 150,000*g*. The ribosomal pellet was resuspended in B buffer containing 10% glycerol and 0.006 M 2-mercaptoethanol and dialyzed against the same solution for 12 hr and stored at −20°.

The enzymes for *in vitro* amino acid incorporation (S-150 fraction) were prepared from 14-day-old embryonic chick muscle homogenized in B buffer containing 10% glycerol and 0.006 M 2-mercaptoethanol. The lower two-thirds of the 150,000*g* supernatant was dialyzed against 25 volumes of 0.01 M Tris-HCl (pH 7.6) containing 10% glycerol and 0.006 M 2-mercaptoethanol for 3–4 hr at 0°. The resulting myosin precipitate was cleared from the solution by centrifuging at 150,000*g* for 1 hr. The supernatant was then dialyzed against a large volume of B buffer containing 10% glycerol and 0.006 M 2-mercaptoethanol for 12–14 hr. The resulting S-150 fraction was stored in 50% glycerol at −20°.

Cell-Free Amino Acid Incorporating System. In vitro amino acid incorporation was carried out in B buffer containing 0.003 M dithiothreitol. The following were added to each milliliter of final volume: 0.5 mg of ribosomes, 0.6 mg of S-150 fraction, 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate, 0.5 μmole of GTP, 50 μg of pyruvate kinase, and 0.1 mμmole each of 20 amino acids. This mixture was preincubated for 10 min at 37° in order to lower endogenous incorporation. Although longer preincubations lowered endogenous incorporation further, the efficiency of the system was also greatly reduced. After preincubation, the mixture was chilled and 0.5 μCi of a uniformly labeled L-[^{14}C]amino acid mixture (0.25 mμmole each) was added per ml; 0.5 ml of the mixture was added directly to the ethanol-precipitated RNA fractions and mixed gently for 5 min at 0°. This was followed by incubating the reaction mixtures for 30 min at 37°. After incubation, KCl was added to a final concentration of 0.5 M and the mixtures were centrifuged at 150,000*g* for 1 hr. The supernatants were treated with 20 μg of pancreatic ribonuclease and dialyzed for 16 hr against 0.02 M $K_4P_2O_7$ (pH 8.5) containing a 10^3–10^4-fold excess of L-[^{12}C]amino acids.

Assay of Products of Cell-Free System. The products of amino acid incorporation were analyzed by acrylamide gel electrophoresis, by DEAE-cellulose chromatography, and by antigen–antibody precipitin reaction. Electrophoresis was performed as previously described (Heywood *et al.*, 1967) except that the samples were first treated with 5 M guanidine

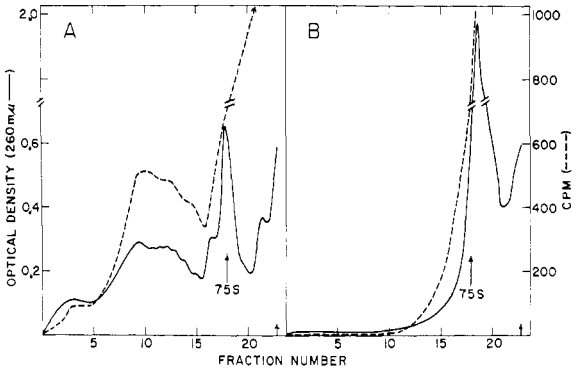

FIGURE 3: Sucrose density gradients of ^{32}P-labeled muscle polysomes before and after EDTA treatment. Chicks (14-days old) were injected with 750 μCi of [^{32}P]phosphoric acid and incubated for 90 min. The cytoplasmic extract was divided into two equal portions and the polysomes precipitated by lowering the ionic strength (Heywood *et al.*, 1968). Polysomes (A) were resuspended in 0.25 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4) and centrifuged on a 27-ml 15–40% sucrose density gradient for 2 hr at 25,000 rpm. Polysomes (B) were resuspended in 0.25 M KCl–0.005 M EDTA–0.01 M Tris (pH 7.4) and centrifuged on a similar gradient containing the EDTA buffer; 1.0-ml fractions were collected and assayed for radioactivity after precipitation in cold trichloroacetic acid. Gradient A contained 10,200 cpm and B contained 9800 cpm; 75 S refers to the peak of single ribosomes in part A and indicates the similar area of the gradient in part B.

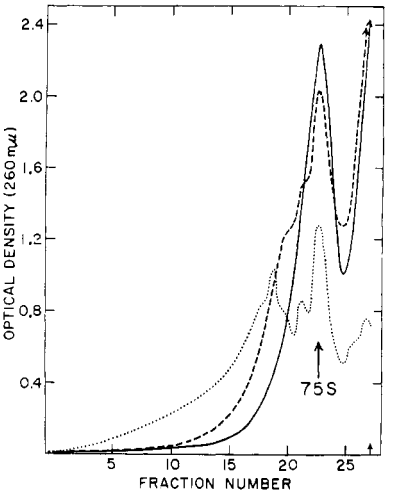

FIGURE 4: NaF ribosomes. Ribosomes (0.25 mg) were layered on sucrose density gradients and centrifuged as in Figure 1. (– – – –) Freshly prepared ribosomes, (· · · · · ·) ribosomes kept at 2° for 16 hr, and (———) ribosomes after preincubation in cell-free amino acid incorporating system for 10 min.

hydrochloride and 0.001 M *n*-ethylmaleimide. They were then dialyzed against 0.005 M citric acid in 0.075 M Tris (pH 8.6) to remove guanidine hydrochloride followed by dialysis against the same buffer at 45° containing 12 M urea and 20% sucrose. The antigen–antibody precipitin reaction followed the method of Baril and Herrmann (1967). Antimyosin, prepared against adult chicken myosin, was a gift of Dr. Robert Dowben at Brown University. DEAE-cellulose cochromatography of radioactive products of the cell-free system was performed as previously described (Heywood and Nwagwu, 1968) with the exception that 1.0 × 20.0 cm columns were used and the elution volume of each buffer was 50 ml.

Myosin used as a marker was prepared from chicken leg muscle by the method of Baril *et al.* (1966).

Radioactivity was measured after hot trichloroacetic acid and ethanol–chloroform extractions with a Nuclear-Chicago low-background counter (less than 2-cpm background).

Chemicals. Chemicals for *in vitro* protein synthesis were obtained from Sigma Chemical Co. and Boehringer Mannheim Corp. Radioactive amino acids were obtained from New England Nuclear Corp. Ribonuclease-free sucrose for density gradients was purchased from Mann Research Laboratories.

Results

Analysis of Polysome RNA. A typical sucrose density gradient polysome profile from 14-day-old embryonic chick muscle is shown in Figure 1. Fraction A contains myosin-synthesizing polysomes, B contains smaller polysomes, and C is a fraction containing single ribosomes. To analyze the RNA associated with these three fractions, 14-day-old chick embryos were injected (intravenously) with 750 μCi of ^{32}P-labeled phosphoric acid and incubated for 75 min. After this period of incubation, the rRNA (18 and 28 S) from A polysomes has only a small amount of radioactivity associated with it, while a peak of radioactivity is associated with the RNA that sediments at approximately 26 S (Figure 2A). No radioactive peak is found with 26S RNA from B polysomes or C ribosomes (Figure 2B,C). The 18S and 28S RNA from fractions B and C of Figure 1 have more radioactivity associated with it in comparison with the RNA from A polysomes (Figure 2). Characteristic of B polysomes is a radioactive peak in the material sedimenting at 10–12 S (Figure 2B).

To examine the extent of retention of polysome RNA on membrane filters under the conditions used, the filtrates of A–C (Figure 2) were analyzed by sucrose density gradient centrifugation. In all three filtrates, radioactivity was observed sedimenting with low molecular weight RNA, while a small amount was observed sedimenting with 18S and 28S RNA from B and C filtrates (Figure 2, A_1–C_1). The degree of retention of polysome material by membrane filters is similar to that reported by Infante and Nemer (1968). This procedure therefore offers a rapid and efficient way of obtaining high molecular weight polysome RNA.

Penman *et al.* (1968) have demonstrated that cytoplasmic, nonpolysomal RNA may cosediment with polysomes during sucrose density gradient centrifugation. Before any clear relationship can be established between myosin-synthesizing polysomes and the 26S RNA that is uniquely found in this fraction, it is necessary to demonstrate that the 26S RNA is functionally associated with the polysomes; 14-day-old chick embryos were injected with 750 μCi of ^{32}P-labeled phosphoric acid and incubated for 90 min. A fraction of the muscle cytoplasmic extract was examined by sucrose density gradient centrifugation. Figure 3A shows the ^{32}P radioactivity associated with the polysomes. The myosin-synthesizing polysomes (fractions 1–5, Figure 3A) have little radioactivity compared with the other polysomes on the gradient. This result agrees well with the pattern of rRNA labeling in Figure 2. EDTA treatment of polysomes has been demonstrated to dissociate them into ribosomal subunits (Penman *et al.*, 1968). Therefore, an equal volume of the same ^{32}P-labeled cytoplas-

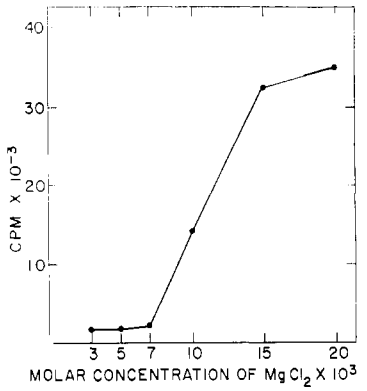

FIGURE 5: Poly U stimulation of L-[^{14}C]phenylalanine incorporation. Poly U (0.05 mg) was added to the reaction mixtures described in Materials and Methods at various Mg^{2+} concentrations. After adding 0.25 μCi of L-[^{14}C]phenylalanine, the samples were incubated for 30 min. Each sample was run in triplicate. The reactions were stopped by the addition of hot trichloroacetic acid.

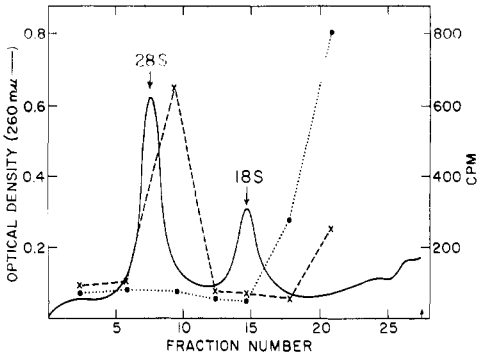

FIGURE 6: Stimulation of amino acid incorporation by different RNA fractions from A and B polysomes. Fractions of 2.0 ml of RNA were collected from the sucrose density gradients and tested in the cell-free system. The optical density profiles of A and B polysome RNA have been superimposed since they are identical. (———) Radioactivity of cell-free systems incubated with RNA fractions from A polysomes. (· · · · · ·) Radioactivity of cell-free systems incubated with RNA from B polysomes. A background incorporation of 380 cpm has been subtracted from each value.

mic supernatant was treated with EDTA and centrifuged on a sucrose density gradient containing EDTA (Figure 3B). All of the polysomes were completely dissociated by this treatment. No radioactivity is found in the area of the gradient normally containing the myosin-synthesizing polysomes. Instead, the radioactivity now shifts to the top of the gradient. This suggests that the 26S RNA is associated with the polysomes; however, additional evidence is required to firmly establish this point.

Cell-Free Amino Acid Incorporation System. Since NaF has been used in reticulocyte systems to free ribosomes of endogenous mRNA (Grossbard *et al.*, 1968), a similar procedure was used to prepare muscle ribosomes. An analysis of these ribosomes is shown in Figure 4. Freshly prepared ribosomes sediment mostly as single ribosomes (75 S); some small faster sedimenting aggregates of two to three ribosomes are present. After the ribosomes are kept at 2° for 16 hr, a shift to larger aggregates with a predominant tetramer peak is found. This cold induced aggregation of chick ribosomes into tetramers has been reported previously (Byers, 1966; Humphreys and Bell, 1967). After preincubation in a cell-free amino acid incorporating system for 10 min, all of the ribosomes are found in the 75S peak (Figure 4).

Even after NaF treatment and preincubation, some endogenous messenger activity remained. Depending upon the ribosomal preparation, this endogenous activity resulted in a background level of 500–1500 cpm/mg of ribosomes in the absence of added RNA.

The cell-free amino acid incorporating system was tested for poly U stimulation of L-[^{14}C]phenylalanine incorporation at different Mg^{2+} concentrations (Figure 5). Since a Mg^{2+} concentration above 0.007 M resulted in high incorporation, indicating improper initiation (Lamfrom and Grunberg-Manago, 1967), a concentration of 0.005 M Mg^{2+} was chosen to assay the various RNA fractions for messenger activity.

Test of Polysome RNA for Messenger Activity. RNA from both A and B polysomes was tested for its capacity to stimulate amino acid incorporation in the cell-free system. The RNA from A polysomes, sedimenting from 25 to 27 S, was found to be particularly active in stimulating amino acid incorporation (fractions 8–10, Figure 6). A 10–12S RNA from A polysomes was also found to stimulate amino acid incorporation (fractions 20–22, Figure 6). The activity of this fraction was variable and is presumably derived from smaller polysomes contaminating A polysomes, since polysomes sediment in a diffuse pattern (Filson and Bloomfield, 1968). This is supported by the fact that the same RNA fraction from B polysomes was found to cause a marked increase in amino acid incorporation (Figure 6). No increased activity was observed when 25–27S RNA (fractions 8–10, Figure 6) from B polysomes was added to the cell-free system.

RNA fractions from A polysomes were assayed for their capacity to promote polysome formation (Figure 7). Although all the RNA fractions were found to increase the endogenous activity of the single ribosomes above that of the control (no added RNA), RNA sedimenting from 25 to 27 S caused the greatest increase in amino acid incorporation when added to the cell-free system. Moreover, addition of the 25–27S RNA resulted in the formation of polysomes as determined by the radioactivity sedimenting ahead of the ribosome peak. By comparison with known polysome profiles on similar sucrose density gradients, these polysomes formed *in vitro* are estimated to contain up to six ribosomes. No polysome formation was observed when 25–27S RNA from B polysomes was tested.

Test for Myosin mRNA. The products of the cell-free synthesis of proteins under the direction of different RNA fractions from both A and B polysomes were tested for myosin using an antigen–antibody precipitin reaction. The 150,000*g* supernatants of the incubation mixtures were dialyzed against excess [^{12}C]amino acids and allowed to react with antimyosin. Of the RNA fractions tested, only the addition of 25–27S RNA from A polysomes resulted in a significant increase in radioactivity above the control level in the antimyosin-precipitable protein (Table I). The 10–12S RNA from B polysomes as well as the 25–27S RNA from A polysomes caused an increase in total radioactivity with an accompanying increase in ribosomes-released radioactivity.

The products of the RNA-directed amino acid incorporation

TABLE I: Antimyosin-Precipitable Radioactivity (cpm).[a]

Polysomal RNA Fraction (S)	Total	Released	Antimyosin Precipitable
A, 28–32	400	150	74
A, 25–27	780	310	185
A, 17–19	370	160	50
A, 10–12	450	140	32
B, 25–27	360	155	57
B, 10–12	850	270	48
None	375	150	52

[a] The polysome RNA fractions are denoted by their approximate sedimentation coefficient. (A) RNA from A polysomes; (B) RNA from B polysomes. Released radioactivity was taken as the radioactivity not sedimenting at 150,000*g*; 20 μg of carrier myosin was added to the supernatants of the incubation mixtures before antimyosin was added. The antigen–antibody precipitate was washed in buffer and precipitated with hot trichloroacetic acid.

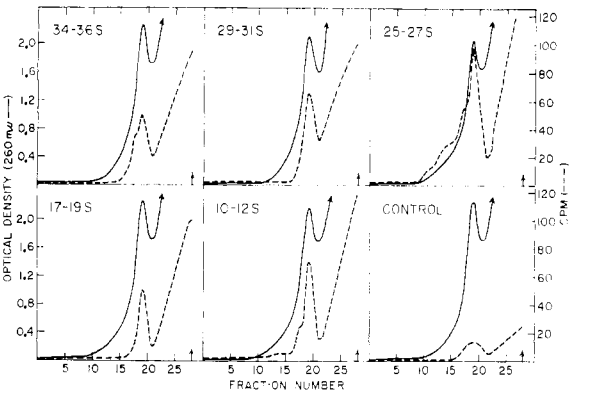

FIGURE 7: Polysome formation in cell-free system. Cell-free amino acid incorporating systems with RNA fractions from A polysomes were incubated for 7.5 min. The reactions were stopped by the addition of 1 mg of cyclohexamide. The incubation mixtures were then centrifuged for 2.5 hr at 25,000 rpm on 27 ml, 15–30% sucrose density gradients containing the same buffer as the incubation mixture; 1.0-ml fractions were collected and assayed for radioactivity after precipitation in hot trichloroacetic acid. This radioactivity represents [^{14}C]amino acids incorporated into nascent polypeptide chains. The single optical density peak represents single ribosomes. The numbers associated with each figure represent the estimated range of the sedimentation coefficients of the RNA fraction added to the cell-free system. The control is a cell-free system with no added RNA.

were examined further by chromatography on DEAE-cellulose; 100 μg of myosin was added to the dialyzed, 150,000*g* supernatant of the incubation mixtures. Elution with 0.02 M $K_4P_2O_7$ (pH 8.5) releases soluble proteins. These appear in fractions 2–4 in Figure 8. Elution with 0.36 M KCl–0.02 M $K_4P_2O_7$ (pH 8.5), which was added after fraction 10 was collected releases myosin which is found in fractions 12–14. The curves in Figure 8 represent the radioactivity of the proteins synthesized after the addition of different fractions of RNA from A polysomes. The control, containing no added RNA, has a low level of radioactivity with a small peak cochromatographing with myosin. The addition of 28–31S RNA to the incubation mixture resulted in a small increase over that of the control of radioactivity which cochromatographed with myosin. The 10–12S RNA caused an increase in the radioactivity eluting with the soluble proteins. However, the 25–27S RNA, when added to the cell-free system, resulted in a marked increase, over the control value, of radioactivity eluting with myosin. In other experiments, addition of different RNA fractions from A polysomes and similar ones from B polysomes to the incubation mixtures caused no increase, over the control values, of radioactivity eluting with myosin. RNA (10–12 S) from B polysomes, however, did cause an increase in radioactivity of the soluble proteins. These results are in agreement with those reported previously in which total RNA from A and B polysomes was used (Heywood and Nwagwu, 1968).

Myosin is known to contain subunits of molecular weight of approximately 200,000 (Kielley and Harrington, 1960; Dreizen *et al.*, 1966). Several recent reports have suggested that myosin, in addition to the large subunits, may contain several smaller peptides (Dreizen *et al.*, 1966; Frederikson and Holtzer, 1968). Electrophoretic analysis has demonstrated that myosin migrates as a monodisperse single band (Heywood *et al.*, 1967). However, if treated with guanidine hydrochloride and *n*-ethylmaleimide and then examined by acrylamide gel electrophoresis, a large major component and a faster moving smaller component are observed (Figure 9). The major component migrates with the same mobility as has been reported for total myosin (Heywood *et al.*, 1967). It is not known if the smaller component is an integral part of the myosin molecule or a contaminant of the myosin preparation. Nevertheless, the altered electrophoretic analysis permitted us to determine if the 26S RNA associated with myosin-synthesizing polysomes was directing the synthesis of the large subunit, the smaller component, or both. After incubation with 26S RNA, the supernatant was chromatographed on DEAE-cellulose. A 4-ml portion of fraction 12 (Figure 8) was collected and concentrated to 0.8 ml with Sephadex G-200.

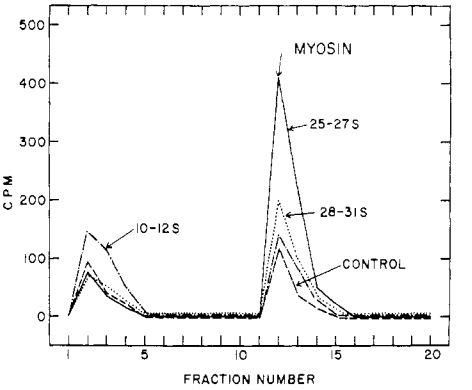

FIGURE 8: DEAE-cellulose chromatography of radioactive products of RNA fractions from A polysomes. After centrifuging, the supernatants of the reaction mixtures were dialyzed against excess [^{12}C]-amino acids. Then, 100 μg of myosin was added to each sample. The elution of myosin was followed by absorbance at 280 mμ (fractions 12–14); 5-ml fractions were collected and assayed for radioactivity. Numbers represent approximate sedimentation coefficients of the different RNA fractions. The control has no RNA added to the reaction mixture.

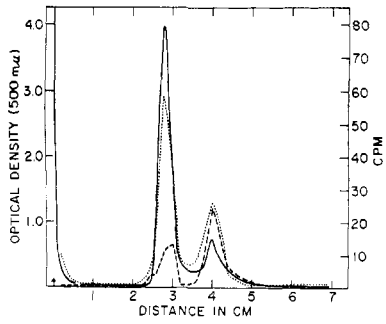

FIGURE 9: Acrylamide gel electrophoresis of fraction 12 from DEAE-cellulose chromatography of reaction mixtures (see Figure 8). A 0.2-ml sample was layered on each gel and run for 2 hr at 4 mA/tube. Direction of migration is to the right. A myosin marker was run in parallel. This gel was stained with Naphthol Blue Black and scanned at 500 mμ on a Gilford spectrophotometer gel scanner. The solid line denotes the position of the myosin marker; (———) radioactivity of control, no RNA added; (· · · · · ·) radioactivity when 25–27S RNA from A polysomes (Figure 1) was added to reaction mixture. The gels were sectioned into 1-mm slices after freezing. Radioactivity was measured by placing two adjacent slices on the same planchet and counting with a low-background counter.

Dialysis against the 12 M urea buffer containing 20% sucrose further reduced the volume to approximately 0.2 ml. This was then layered on a polyacrylamide gel. A myosin marker was run in parallel on a separate gel. The results of the gel analysis are shown in Figure 9. When no RNA was added to the cell-free system (control, Figure 9), some radioactivity was found to migrate with the large subunit of myosin; however, about 70% of total background incorporation, which is presumably due to the presence of endogenous mRNA, migrated with the smaller component. The protein synthesized by added 25–27S RNA from A polysomes and eluting with myosin on DEAE-cellulose migrated with both the major and minor peaks of the myosin marker. However, the radioactivity migrating with the major component of myosin showed a marked increase over the control, while that migrating with the minor component was virtually identical with the control values. These results suggest that the 26S RNA, associated with the A polysomes, is the mRNA that directs the synthesis of the 200,000 mol wt subunit of myosin. However, the results do not exclude the possibility that the 26S RNA specifically stimulates or protects the mRNA coding for the large subunit of myosin. That the latter possibility is unlikely is demonstrated by the fact that the 26S RNA was found to direct the synthesis of myosin using chicken reticulocyte ribosomes (S. M. Heywood, manuscript in preparation).

Discussion

mRNA represents but a small portion of the total cellular RNA, and is itself a mixed population of molecules of varying sizes and half-lives. The isolation of a mRNA is therefore a more difficult task than that of isolating a major species of cellular RNA. If the synthesis and properties of mRNA are to be studied, it is desirable to isolate a specific species of mRNA responsible for the synthesis of a defined polypeptide chain. In only a few cell types such as the reticulocyte and muscle has it been possible to isolate polysomes responsible for the synthesis of one protein, thereby presenting the opportunity to isolate the mRNA associated with these polysomes (Chantrenne *et al.*, 1967); Heywood *et al.*, 1967).

Embryonic muscle is particularly well suited for this. (1) It has a population of large polysomes, consisting of 55–65 ribosomes, which can be isolated from the rest of the polysomes. These large polysomes have geen shown to be responsible for the synthesis of myosin (Heywood *et al.*, 1967). (2) Cell-free protein-synthesizing systems derived from embryonic muscle are highly active and essentially free of ribonuclease activity (Heywood *et al.*, 1968). Using such a system, it is possible to demonstrate the RNA-directed *de novo* synthesis of myosin (Heywood and Nwagwu, 1968). (3) Because of the large size of the myosin subunit, initiations of relatively few molecules will produce levels of radioactivity that are detectable. This then increases the sensitivity of assaying for myosin mRNA. (4) A period of time during embryonic development exists (14–15 days) when it is possible to radioactively label mRNA associated with myosin-synthesizing polysomes while a very small amount of radioactivity is incorporated into the rRNA of these polysomes. This is likely a result of the fact that ribosome synthesis is considerably reduced after the fusion of mononucleated myoblasts (Marchok, 1966), while in chick embryos myosin synthesis apparently occurs only after this fusion (Okazaki and Holtzer, 1966). The higher degree of radioactive labeling of rRNA in smaller polysomes may reflect contamination of the myosin-synthesizing cells by myoblasts or fibroblasts which constitute an appreciable volume of the muscle used in these experiments (Marchok and Herrmann, 1967).

The 26S RNA, found to be associated only with the myosin-synthesizing polysomes, was tested by a number of criteria for messenger activity. The fact that it stimulated amino acid incorporation and caused the formation of polysomes may be a result of some property of the molecule other than its ability to direct the proper sequencing of amino acids into protein. For example, it may be particularly effective in stimulating protecting endogenous mRNA. The stimulating effect of high molecular weight RNAs has been previously reported (Hunt and Wilkinson, 1967; Heywood and Nwagwu, 1968). However, when this 26S RNA was added to the cell-free system, radioactive protein was synthesized in amounts significantly above the background. This protein, precipitated in the presence of antimyosin, cochromatographed on DEAE-cellulose with myosin, and migrated with the major 200,000 molecular weight component of myosin on acrylamide gels. If, in fact, the smaller component observed by gel electrophoresis is an integral part of the myosin molecule (Frederiksen and Holtzer, 1968) and the 26S RNA is myosin mRNA, it must be concluded that the 26S RNA is not polycistronic, *i.e.*, it does not direct the synthesis of both the large and small subunits of myosin. Taken together, these results suggest that the 26S RNA carries the information for directing the proper sequence of amino acids into the large subunit of myosin. Nevertheless, final characterization of 26S RNA as myosin mRNA will require peptide analysis of the synthesized product.

An mRNA coding for a 200,000 molecular weight polypeptide would have about 6000 nucleotides, thereby having a molecular weight of approximately 1.8×10^6. A polynucleotide of this molecular weight would be expected to sediment close to 28S rRNA which has a molecular weight of approximately 1.6×10^6 (Hamilton, 1967). The fact that myosin mRNA sediments at a slower rate than 28S rRNA may be due

to the fact that it has a more extended configuration than rRNA under the conditions used. Nevertheless, a general agreement exists between the size of the presumptive mRNA and the polypeptide for which it codes.

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