Cytoplasmic Nonpolysomal Messenger Ribonucleoprotein Containing Actin Messenger RNA in Chicken Embryonic Muscles†

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ABSTRACT: Cytoplasmic nonpolysomal mRNAs have been isolated in the form of 16–40S ribonucleoprotein particles from the postribosomal supernatant of 14-day-old chick embryonic muscles. An 8–20S RNA fraction isolated from these particles directs the synthesis of actin in a wheat germ embryo S-30 system, as judged by copurification of the products with chicken muscle actin by repeated cycles of G- to F-actin transformation; mobilities of the purified product on sodium dodecyl sulfate–polyacrylamide gels and urea gels; and analysis of the CNBr-cleaved peptides. The 16–40S particles have a buoyant density of 1.4 g/cm^3 which corresponds to an RNA/protein ratio of 1:3. They do not contain detectable levels of ribosomal subunits, as judged by the absence of typical ribosomal proteins in the range of 15,000–30,000. They contain at least eight distinct polypeptide species in the molecular weight range of 44,000–100,000, including a prominent 44,000 species. The presence of these particles suggests that they may have a role in the regulation of translation in developing muscles.

It was first proposed by Spirin (1969) that in eukaryotic cells mRNAs are complexed with specific proteins to form cytoplasmic messenger ribonucleoprotein particles (mRNP) or informosomes. Such particles have been reported in extracts of sea urchin (Infante and Nemer, 1968); fish (Spirin, 1969); silk worm (Kafatos, 1968); L cells (Perry and Kelly, 1968); liver (Henshaw and Lowenstein, 1970); HeLa cells (Spohr et al., 1970); rabbit reticulocytes (Jacobs-Lorena and Baglioni, 1972); and duck erythrocytes (Spohr et al., 1972) (for a review see Spirin, 1972). Recently mRNP particles containing specific mRNAs have been demonstrated in two eukaryotic systems. A 20S particle containing globin mRNAs has been isolated from the postribosomal supernatant of rabbit reticulocytes (Jacobs-Lorena and Baglioni, 1972) and duck erythrocytes (Spohr et al., 1972). Pretranscribed histone mRNA has been found to be stored as "maternal" mRNA in a 20S mRNP particle in unfertilized sea urchin eggs (Gross et al., 1973). Although it has been suggested that mRNP particles may play a role in the regulation of mRNA translation (Spirin, 1969, 1972; Spohr et al., 1972; Baglioni, 1974), very little is known about the biochemical events that might be involved in such regulations.

It is now believed that the contractile proteins actin and myosin are also present in a large variety of nonmuscle eukaryotic cells (for a review see Pollard and Weihing, 1974). It has been suggested that they may play important roles in many cellular processes such as motility, cytokinesis, and adhesion (Pollard and Weihing, 1974). The regulation of biosynthesis of these proteins is, therefore, an important aspect in the physiology of many eukaryotic cells. Several studies on the appearance of mRNAs in cultured muscle cells have concluded that myogenesis is regulated by transcriptional control (Przybyla and Strohman, 1974; Paterson et al., 1974). On the other hand, the requirement of muscle-specific initiation factors for in vitro translation of muscle mRNAs (Heywood et al., 1974), the stability of mRNAs in cultured muscle cells (Buckingham et al., 1974), and the effect of actinomycin D on the appearance of muscle-specific proteins (Yaffe and Dym, 1972) suggest that cytoplasmic post-transcriptional control may operate during the growth and differentiation of skeletal muscle fibers.

The biosynthesis of muscle proteins and its regulation in developing muscle are currently being studied in a number of laboratories. It has been previously shown that the different subunits of the heteropolymeric muscle protein, myosin, are translated independently from separate monocistronic mRNAs (Sarkar and Cooke, 1970; Low et al., 1971; Rourke and Heywood, 1972). More recently the mRNA coding for the myosin heavy chain (Heywood and Nwagwu, 1969; Thompson et al., 1973) has been purified and characterized in our laboratory (Sarkar et al., 1973; Mondal et al., 1974; Sarkar, 1974, 1975). In order to elucidate the regulatory mechanisms involved in the biogenesis of contractile proteins, we have searched for nonpolysomal forms of specific mRNAs in developing muscles. We report here the isolation and characterization of mRNP particles from chicken embryonic muscles. In vitro translation of an mRNA fraction isolated from these particles in a heterologous cell-free system shows that actin is one of the products. The significance of these observations in relation to regulation of translation in muscle cells is discussed.

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[1] Abbreviations used are: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; G-actin, globular actin; F-actin, fibrillar actin; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; mRNP, messenger ribonucleoprotein; poly(A), ribopoly(adenylate); A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm when measured in a cell of 1-cm path length.

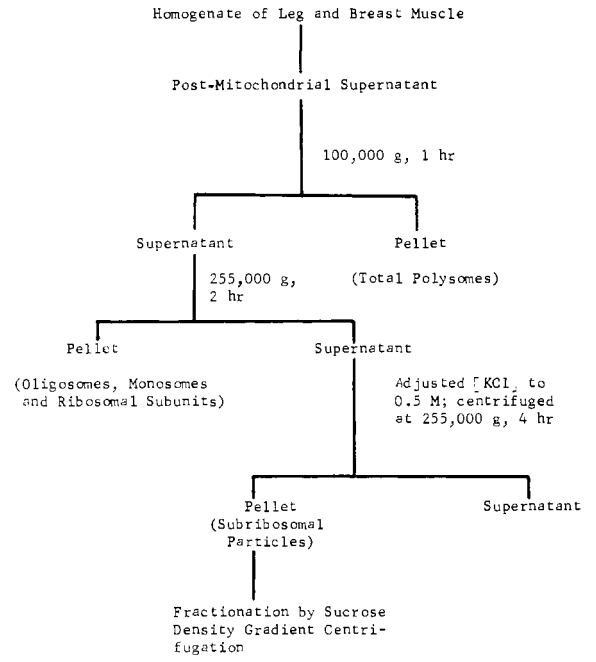

FIGURE 1: Scheme of isolation of mRNP particles from embryonic chick muscles.

Experimental Section

Isolation of Subribosomal Particles. The scheme used for the isolation of subribosomal particles is shown in Figure 1. Leg and breast muscles (about 140 g) obtained from 120 14-day old chick embryos were homogenized according to the previously published procedure (Sarkar et al., 1973) in equal volume of buffer I, which contains 0.01 *M* Tris-HCl (pH 7.6)-0.25 *M* KCl-0.01 *M* $MgCl_2$-0.001 *M* EDTA-0.1 m*M* dithiothreitol-0.25 *M* sucrose (RNase free; Schwarz/Mann)-500 μg/ml of heparin (used as RNase inhibitor)-0.5 m*M* phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$, used as a protease inhibitor). The postmitochondrial supernatant fraction, obtained by centrifugation of the homogenate at 12,000*g* for 15 min (Sarkar and Cooke, 1970; Sarkar et al., 1973), was further centrifuged at 100,000*g* for 1 hr to pellet the polysomes. The supernatant, thus obtained, was diluted with two volumes of buffer II (same as buffer I minus sucrose) and was centrifuged at 255,000*g* for 120 min to sediment monosome and ribosomal subunits. The KCl concentration was then adjusted to 0.5 *M* and the material was centrifuged at 255,000*g* for 4 hr to obtain a pellet of crude subribosomal particles. The use of 0.5 *M* KCl in the centrifugation step is believed to minimize the nonspecific binding of cytoplasmic proteins to the mRNP particles (Blobel, 1972). The pellet was gently resuspended in 5-6 ml of buffer III (0.01 *M* Tris-HCl (pH 7.6)-0.5 *M* KCl-0.002 *M* EDTA-500 μg/ml of heparin-0.5 m*M* $PhCH_2SO_2F$) and the suspension was centrifuged at 10,000*g* for 10 min to remove any aggregated material. About 25-30 A_{260} units of subribosomal particles suspended in 0.5-1 ml were layered on 28 ml of linear 5-20% sucrose gradient in buffer III and were centrifuged at 24,000 rpm for 17 hr in a SW 25.1 Beckman-Spinco rotor at 2°. Gradient fractions were collected in an ISCO gradient fractionator and were analyzed for uv absorbance.

RNA Extraction and Fractionation. Samples of subribosomal particles or fractions pooled from the sucrose gradient runs were dialyzed against buffer IV (0.1 *M* Tris-HCl (pH 7.6)-0.1 *M* NaCl-0.001 *M* EDTA) to remove sucrose. The samples containing 2-10 A_{260} units/ml were then adjusted to pH 9.0 and 1% sodium dodecyl sulfate and RNA were isolated from the particles by extraction twice with equal volume of phenol-chloroform-isoamyl alcohol, 50:50:1, as described by Aviv and Leder (1972). The aqueous phases obtained by centrifugation at 20,000*g* for 20 min (Aviv and Leder, 1972) were combined and adjusted to 0.2 *M* potassium acetate (pH 5.0). The RNA was precipitated with 2.5 volumes of ethanol at −20°. The precipitation step was repeated three times to remove all traces of dodecyl sulfate and heparin, as previously described (Sarkar et al., 1973). The RNA samples were dissolved in buffer V (0.01 *M* sodium acetate (pH 5.0)-0.1 *M* NaCl-0.001 *M* EDTA); 1 ml of the RNA solution (10-25 A_{260} units) was layered on a 28 ml of 5-2 linear sucrose gradient in buffer V and centrifuged at 24,000 rpm for 17 hr in a SW 25.1 Beckman-Spinco rotor at 2°. The gradients were fractionated and the profile for uv absorbance was obtained, as described above. Fractions were pooled, concentrated by precipitation with ethanol, dissolved in autoclaved distilled water, and were immediately assayed for mRNA activity.

Table I: Stimulation of Amino Acid Incorporation by RNA Fractions Isolated from mRNP Particles in Wheat Germ Embryo Cell-Free[a] System.

Source of RNA	μg of RNA Used	[^{35}S]Met Incorporated (cpm/assay)	Degree of Stimulation
Minus RNA		2,400	Control
Rabbit globin mRNA	2.5	30,000	12.5-fold
Chicken 4S RNA	80	2,600	
Chicken 28S rRNA	10	2,300	
Total RNA from pooled tubes 1-8 (Figure 3)	10	2,900	
Total RNA from pooled tubes 9-25 (Figure 3)	12	29,000	12-fold
8-20S RNA fraction (Figure 4)	8	36,000	15-fold

[a] Assays were performed as described in the text. For details, see Experimental Section.

Assay for mRNA Activity of RNA Fractions. The preincubated wheat germ embryo S-30 system was prepared and assays for mRNA activity were carried out by the method of Roberts and Paterson (1973). Incubation mixtures contained in a total volume of 100 μl: 40 μl of wheat germ S-30 fraction, 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.6), 2 m*M* dithiothreitol, 1 m*M* ATP, 0.1 m*M* GTP, 8 m*M* creatine phosphate, 5 μg of creatine phosphokinase, 80 m*M* KCl, 3 m*M* magnesium acetate, 2-3 nmol of each of 20 amino acids, indicated amounts of RNA fractions, and 5 μCi of [^{35}S]methionine (specific activity of 110 Ci/mmol; New England Nuclear Corporation) as the single radioactive amino acid. In some experiments 5 μCi of a mixture of uniformly labeled ^{14}C amino acids (New England Nuclear Corporation) was used. Globin mRNA, used as a standard, was prepared from polysomes of rabbit reticulocytes by extraction with phenol-$CHCl_3$-isoamyl alcohol (Aviv and Leder, 1972) and was further purified on a column of cellulose (Sigma cell type 38, microcrystalline, particle size 38 μ, Sigma Chemical Company) (Kitos et al., 1972), as described by Sarkar et al. (1973) for the purification of myosin heavy chain mRNA. Initially the assays were optimized for concentrations of

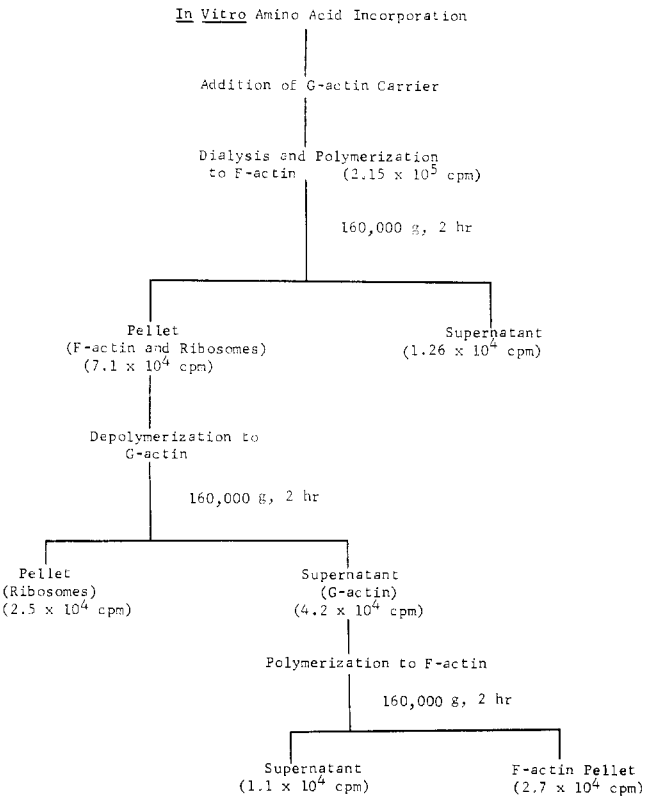

FIGURE 2: Scheme of copurification of the in vitro translation products with actin. For details see Experimental Section. The numbers in parentheses indicate the CCl_3COOH-insoluble radioactivity recovered in the purification step.

mRNA samples which ranged from 6 to 10 μg for RNA fractions and 2-3 μg for globin mRNA. These concentrations were routinely used in incubation mixtures which were subsequently processed for the identification of products of cell-free translation. Incubations were carried out at 25° for 90 min. The reaction mixtures were then processed for CCl_3COOH-insoluble (95°) radioactivity.

Identification of Products of Translation. The protocol used to identify actin as one of the products of cell-free translation programmed by the RNA fraction isolated from the mRNP particles is shown in Figure 2. 2-3 mg of highly purified chicken actin, prepared by the method of Spudich and Watt (1971) and suspended in a depolymerizing medium, 5 m*M* Hepes (pH 7.0)-0.2 m*M* $CaCl_2$-0.2 m*M* ATP, was added to reaction mixtures pooled from eight to ten incubations. The products were then subjected to three cycles of reversible salt-dependent transformation of globular (G) to fibrillar (F) actin. The material was first dialyzed overnight at 4° against 200 volumes of a polymerizing medium, 5 m*M* Hepes (pH 7.0)-0.1 *M* KCl-0.003 *M* $MgCl_2$. The volume of the sample was then adjusted to 2 ml with the polymerizing medium. The ribosomes and carrier F-actin were pelleted by centrifugation at 160,000*g* for 2 hr using a 2-ml adapter (No. 303376; Beckman-Spinco) in a Spinco 65 rotor. The pellet was resuspended in 2 ml of the depolymerizing medium mentioned above and was dialyzed overnight at 4° against 100 volumes of this solution. The material was then centrifuged at 160,000*g* for 2 hr to separate the ribosomes as a pellet from the soluble G-actin which remained in the supernatant. The cycle was repeated once more to give a final pellet of ribosome-free F-actin. The concentration of actin was determined in each step of the isolation procedure by uv absorbance according to the method of Lehrer and Kerwar (1972). About 50-60% of the F-actin was recovered in the final pellet after three cycles of polymerization and depolymerization.

Polyacrylamide Gel Electrophoresis of the Purified Products. Electrophoresis in dodecyl sulfate gels was carried out using 10% gels as described by Sarkar et al. (1973). The gels were stained with Coomassie Brilliant Blue, destained, and then scanned on a Joyce-Lobel microdensitometer (Model no. S/N 1040) using a 595-nm filter (Sarkar, 1972; Sarkar et al., 1973). The gels were then sliced into 1.5-cm slices using a Canalco lateral gel slicer and the radioactivity of the gel slices was determined as previously described (Sarkar and Cooke, 1970; Sarkar et al., 1973). Electrophoresis of the purified products was also carried out in 8 *M* urea and in the absence of dodecyl sulfate by a modification of the method of Williams and Resifeld (1964). Samples were dialyzed against 8 *M* urea containing 0.1 *M* Tris-acetate (pH 8.5) and 1% β-mercaptoethanol. The samples were then electrophoresed using 10% polyacrylamide gels. The buffer used for electrophoresis and polymerization of gels contained 0.1 *M* Tris-acetate (pH 8.5) and 8 *M* urea.

Analysis of CNBr Peptides of the Purified Products. Reaction mixtures in which incubation was carried out with a mixture of ^{14}C-labeled amino acids were processed for the copurification of the products with actin, as described above. The products were dissolved in 2 ml of 5 m*M* Hepes (pH 7.0)-0.1 m*M* $CaCl_2$-0.1 m*M* ATP and were made to 8 *M* urea by the addition of solid urea. β-Mercaptoethanol was added to a final concentration of 0.08 *M* and the solution was stirred for 30 min at 25° in a sealed flask. The pH was then adjusted to 9.0 with 1 *N* KOH and ethyleneimine was added to a final concentration 0.1 *M*. The material was then aminoethylated (Raftery and Cole, 1966) by stirring for 1 hr at 25° as previously described by Sarkar et al. (1973) for the aminoethylation of myosin heavy chain. The sample was then exhaustively dialyzed against 6 l. of 10 m*M* Tris-HCl (pH 7.6) and lyophilized. The material was dissolved in 2 ml of 70% HCOOH and cleaved with CNBr as previously described (Sarkar et al., 1973). The resulting peptides were diluted with 15 ml of distilled water and evaporated to dryness in a rotary evaporator. The peptides were then analyzed by polyacrylamide gel electrophoresis in the presence of 8 *M* urea and 1% dodecyl sulfate using 12.5% high cross-linked gels (bisacrylamide ratio of 1:10), as described by Swank and Munkres (1971).

CsCl Density Gradient Analysis. The buoyant density of mRNP particles and 80S monoribosomes was determined by a modification of the methods of Spirin et al. (1965) and Hirsch et al. (1973). The pooled fractions from sucrose gradient runs of mRNP particles were dialyzed against buffer VI (20 m*M* triethanolamine-HCl (pH 7.0)-25 m*M* KCl-2 m*M* magnesium acetate-0.1 m*M* EDTA-0.5 m*M* dithiothreitol) to remove sucrose and then concentrated by ultrafiltration on PM 10 membrane filters (25 mm diameter; Amicon Corporation) to give a density of 5-10 A_{260} units/ml. Monoribosomes obtained by ultracentrifugation of sucrose gradient fractions of postmitochondrial supernatant of embryonic leg muscles (Sarkar and Cooke, 1970) were directly dissolved in buffer VI to a concentration of 5-10 A_{260} units/ml. Formaldehyde freshly neutralized to pH 7.0 with 2 *N* KOH was added to the samples to give a final concentration of 4% and the material was kept at 0° for 1 hr. About 1-2 A_{260} units of the samples were then layered on

4.5 ml of preformed CsCl gradients of densities 1.3–1.7 g/ml prepared in buffer VI and containing 2% HCHO. After centrifugation at 35,000 rpm for 40 hr in a Beckman SW 50.1 rotor, the gradients were fractionated in an ISCO density gradient fractionator using Fluorinert (ISCO) to push the liquid from the bottom of the gradient. The fractions, 0.2 ml each, were monitored for uv absorbance at 260 nm using ISCO recorder and density by measuring the refractive indices in a Bausch-Lomb refractometer.

Carboxymethylation of Protein Moieties of mRNP Particles and Ribosomal Subunits. Fractions pooled from sucrose gradient runs of mRNP particles were dialyzed against 10 m*M* Tris-HCl (pH 7.6) and 50 m*M* NaCl to remove sucrose. The samples were then adjusted to 0.1 *M* NaCl and precipitated by adding 2 volumes of ethanol at −20°. The precipitates were collected by centrifugation at 20,000*g* for 20 min, washed twice with 66% ethanol, and dissolved in 50 m*M* Tris-HCl (pH 8.0) containing 8 *M* urea and 0.04 *M* β-mercaptoethanol. After the mixture was stirred at room temperature for 60 min in a sealed flask, solid α-iodoacetamide was added to the solution to a concentration of 0.25 *M* and the material was stirred for another 2 hr at room temperature. The reaction mixture was then adjusted to 0.1 *M* sodium acetate (pH 6.5) to terminate the alkylation reaction. The carboxymethylated samples were dialyzed against 0.1 *M* Tris-acetate (pH 8.5), 1% β-mercaptoethanol, and 1% dodecyl sulfate for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The pellet (255,000 g, 2 hr) of oligosomes, monosomes, and ribosomal subunits (Figure 1) was used for the preparation of 60S and 40S embryonic muscle ribosomal subunits using a modification of the method of Burke et al. (1973). The pellet was gently suspended in buffer VII (0.025 *M* Tris-HCl (pH 7.4)–0.5 *M* KCl–0.005 *M* magnesium acetate). About 70–75 A_{260} units were layered on 28 ml of 15–30% linear sucrose gradients in buffer VII. The gradients were centrifuged for 16 hr in the Beckman-Spinco SW 25.1 rotor at 21,000 rpm. The gradients were fractionated and analyzed with an ISCO density gradient fractionator. Authentic rabbit reticulocyte subunits, prepared by the method of Blobel and Sabatini (1971), were used as markers. The peak fractions of the 60S and 40S muscle ribosomes were pooled and processed for carboxymethylation as described above.

In order to prevent nucleolytic activity during the preparation of mRNP particles and mRNAs, special precautions such as the use of acid-washed glassware, RNase-free sucrose (Schwarz/Mann), heparin (Sigma Chemical Co.), and sterile pipets were taken. All buffers and reagents were made fresh in autoclaved distilled water.

Results

Isolation of Heterogeneous Subribosomal Particles. Starting with 120 14-day old chicken embryos about 100 A_{260} units of crude subribosomal particles were obtained by the procedure described in Figure 1. When these particles were analyzed by sucrose gradient centrifugation, about 60% of the uv-absorbing material sedimented as a broad peak about 8–10 S and the remaining material sedimenting between 16 S and 40 S showed considerable heterogeneity (Figure 3). Since a large fraction of cellular mRNAs which code for 15,000–35,000 polypeptides have *s* values in the range 9–20 S (for a review see Brawerman, 1974), the expected size of mRNP particles containing such mRNAs

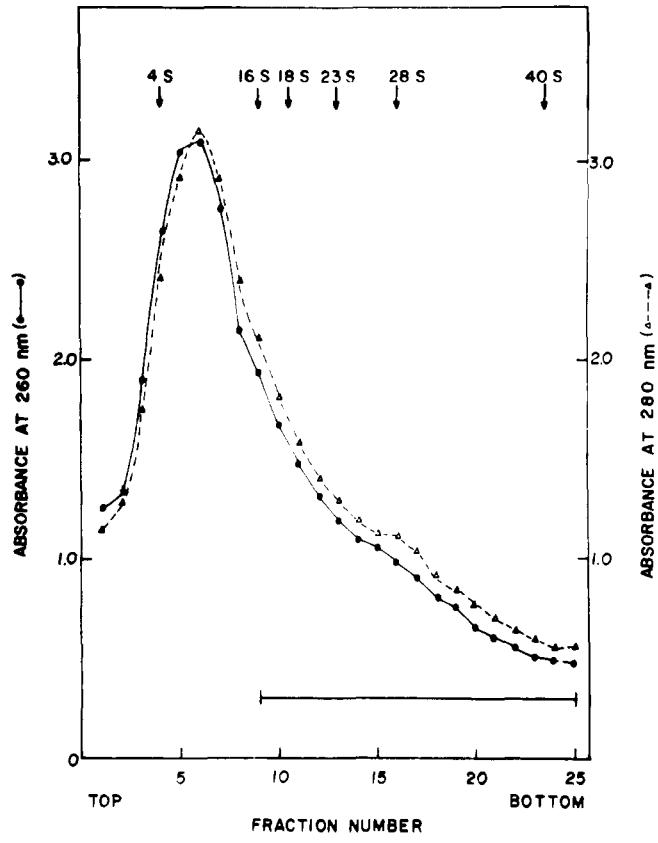

FIGURE 3: Sedimentation profile of subribosomal particles in sucrose gradients. For details see Experimental Section. Centrifugation was done for 17 hr at 24,000 rpm at 2° in a Beckman SW 25.1 rotor. RNAs from *E. coli* and embryonic muscles and 40S muscle ribosomal subunit were used as markers. Fractions indicated by the bar were pooled for the isolation of RNA fractions with messenger activity.

should be in the 20–40S range (Spirin, 1969, 1972; Jacobs-Lorena and Baglioni, 1972; Spohr et al., 1972; Gross et al., 1973). We, therefore, selected the 16–40S region of the gradients (tubes 9–25 and indicated by the bar) to look for the presence of cytoplasmic mRNP particles. A comparison of the A_{260}/A_{280} ratio, which usually varied from 0.8 to 1.1 throughout the gradient, indicates that the 16–40S region had a lower ratio than the slowly sedimenting particles (tubes 1–8). This also suggests that mRNP particles which are known to contain a relatively larger amount of proteins than other ribonucleoprotein particles, e.g., ribosomes (Spirin, 1969, 1972), may sediment in this region.

RNA Fractions Isolated from Subribosomal Particles. To define the approximate size of the RNA species present in the 16–40 S particles (Figure 3), the RNA isolated from the particles was analyzed by sucrose density gradient centrifugation. As shown in Figure 4, the bulk of the uv-absorbing material showed a broad peak sedimenting about 18–20 S. However, a considerable amount of RNA showed sedimentation values equal to and larger than that of 28S rRNA, run as a marker, indicating a high degree of heterogeneity in size. In contrast, when samples of RNA isolated from the slowly sedimenting particles (tubes 1–8 in Figure 3) were similarly analyzed, the bulk of the preparation sedimented as a major peak of about 4–5 S (data not shown in Figure 4). The heterogeneity characteristic of RNA present in 16–40S particles was hardly detectable in this fraction. Since Morris et al. (1972) have previously shown that a 10–17S fraction, obtained from muscle polysomal RNA,

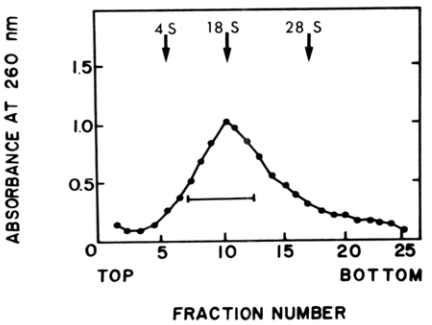

FIGURE 4: Sucrose gradient sedimentation profile of RNA fractions isolated from the pooled subribosomal particles (Figure 3). For details see Experimental Section. Centrifugation was done at 2° for 17 hr at 24,000 rpm in a Beckman SW 25.1 rotor. The arrows indicate the position of the peak tubes when samples of chicken embryonic 4S, 18S, and 28S RNAs were used as markers. The bar indicates the pooled fractions (8–20S, used for in vitro translation and identification of products.

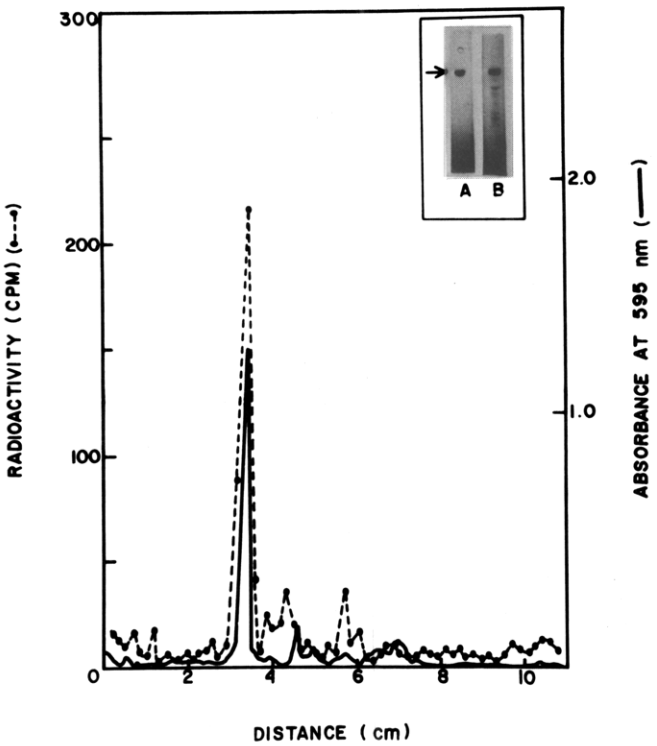

FIGURE 5: Electrophoresis of purified labeled products in sodium dodecyl sulfate polyacrylamide gels. For details see Experimental Section. Portions of the F-actin samples containing 1200–1400 cpm (specific activity 25,000–30,000 cpm/mg) were analyzed using 10.5 cm of 10% gels at 4 mA/gel for 4 hr. After staining with Coomassie Brilliant Blue the gels were scanned, sliced, and processed for radioactivity as described in the text. The insert shows electrophoretograms of gel runs of purified actin (gel A) and the labeled product (gel B).

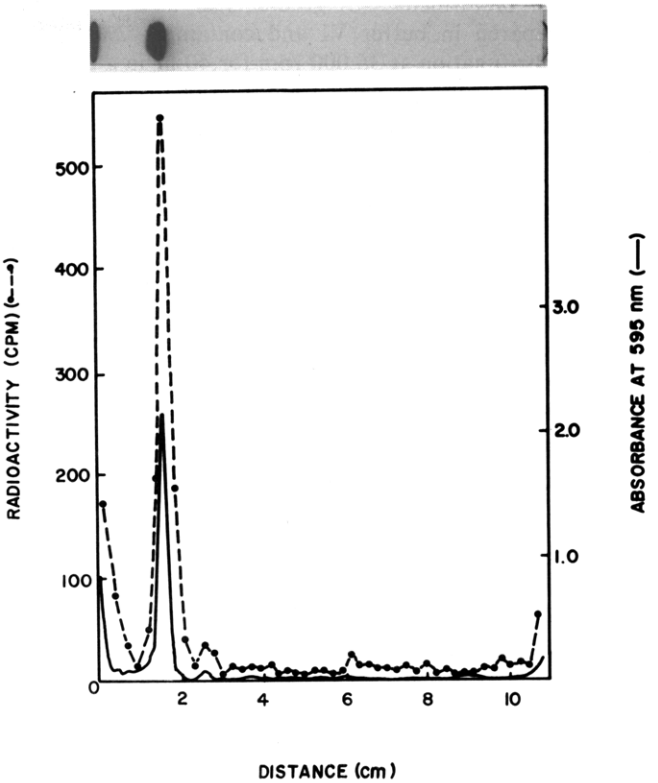

FIGURE 6: Electrophoresis of purified labeled products in 8 *M* urea. For details see Experimental Section. Portions of the purified products containing 2000–2200 cpm were electrophoresed using 10.5 cm of 10% gels at 4 mA/gel for 4 hr. Top: electrophoretogram of a sample of 75 μg of purified chicken actin run simultaneously in a separate gel.

can program the synthesis of actin in a heterologous cell-free system, we selected an 8–20S fraction (indicated by the bar in Figure 4) in order to test whether this nonpolysomal RNA fraction indeed codes for any myofibrillar protein in the 40,000–50,000 range.

Messenger Activity of RNA Fractions. Both the heterogeneous RNA isolated from the 16–40S particles (tubes 9–25, Figure 3) and 8–20S RNA fraction strongly stimulated [^{35}S]methionine incorporation into total proteins in the wheat germ embryo cell-free system (Table I). The degree of stimulation was comparable to that obtained with a preparation of partially purified rabbit globin mRNA. The 4–5S RNA fraction isolated from the slowly sedimenting particles (tubes 1–8 in Figure 1) did not show any significant mRNA activity. These results indicate that the messenger activity was predominantly associated with the 16–40S fraction of subribosomal particles.

Identification of Actin. In order to test if the 8–20S RNA fraction directs the synthesis of actin, the products, labeled with [^{35}S]methionine, after copurification with carrier actin (see Experimental Section), were further analyzed by using two types of gel systems. Approximately 10% of the nondialyzable radioactivity incorporated in the cell-free system was recovered in the final F-actin pellet after three cycles of G- to F-actin copolymerization (Figure 2). Upon electrophoresis in the presence of dodecyl sulfate about 70% of the radioactivity applied to the gel comigrated with the densitometric peak of the actin band (Figure 5). The radioactivity profile also indicates a number of minor peaks. These may be due to other proteins of the thin filaments such as tropomyosin and troponin(s) which migrate in these regions in dodecyl sulfate gel runs (Potter, 1974) and are copurified with F-actin due to strong mutual interactions (Potter and Gergely, 1974).

When the purified in vitro products were electrophoresed in the presence of 8 *M* urea, the only major radioactive peak obtained in the gel run comigrated with the actin band (Figure 6). The radioactive and dye-stained material at the top of the gel is presumably due to denatured or aggregated proteins which failed to enter the gel or some non-actin proteins present in the system. The radioactivity recovered in this region amounted to about 20% of that present in the actin band. These results indicate that the translated products, judged by mobilities in two different electrophoretic

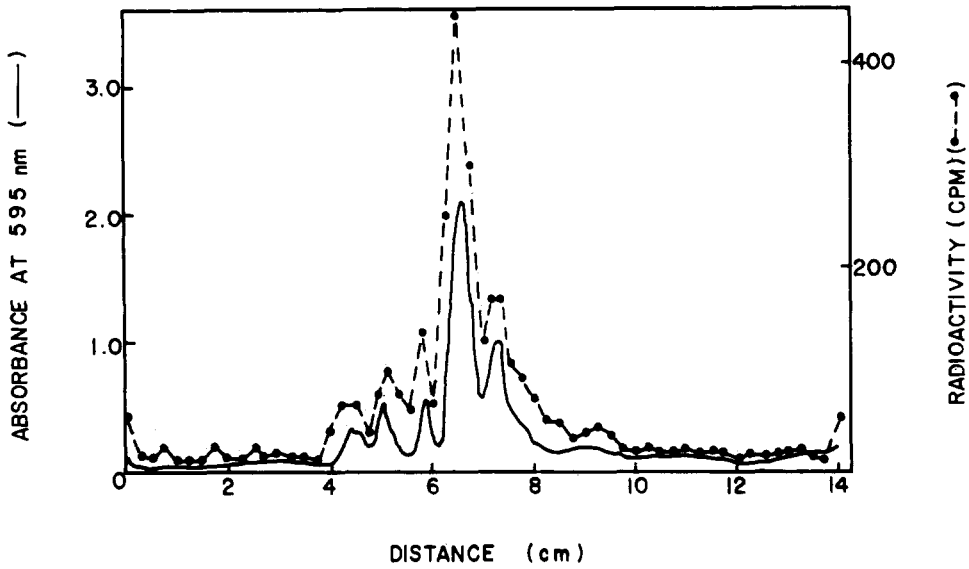

FIGURE 7: Polyacrylamide gel electrophoresis of CNBr peptides of purified products labeled with ^{14}C amino acids. For details see Experimental Section. Samples containing 3000–3500 cpm were electrophoresed using 14-cm 12.5% polyacrylamide high cross-linked gels (bisacrylamide ratio of 1:10) in the presence of 8 *M* urea and 1% dodecyl sulfate. Gels were run at 2 mA/gel for 20 hr, and then stained, scanned at 595 nm, sliced, and counted as described in the text.

systems, one based on size and the other based on charge, showed properties similar to actin.

In order to test the fidelity of the translation programmed by the 8–20S RNA fraction, the in vitro products were labeled with a mixture of ^{14}C-labeled amino acids and copurified with actin as described above. The products were aminoethylated and cleaved with CNBr and the resulting peptides were analyzed by dodecyl sulfate gel electrophoresis using high cross-linked gels (see Experimental Section). As shown in Figure 7, one major and five minor peaks were resolved in the gel run. The major peak probably represents a large group of CNBr peptides of actin containing 20–30 amino acid residues (Elzinga et al., 1973). Some of these peaks are mixtures of incompletely resolved peptides and on further analysis would undoubtedly separate into a large number of resolved peptides (Elzinga, 1970). Each radioactive peak from the in vitro translated products corresponded to a densitometric peak. About 85% of the radioactivity was recovered in the peptides. When the 8–20S RNA was omitted, only about 1% of the radioactivity incorporated in the incubation mixtures were recovered in the copolymerized F-actin pellet. Subsequent analysis by dodecyl sulfate gel electrophoresis gave essentially no significant amount of radioactivity in the actin band. These results indicate that the 8–20S fraction indeed directs the de novo synthesis of actin.

Protein Components of 16–40S Particles. When formaldehyde-fixed 16–40S particles were centrifuged to equilibrium in preformed CsCl gradients, the absorbance profile gave a peak at a density of 1.40 g/cm^3 (Figure 8). This value corresponds to a protein content of about 75% (Spirin, 1969, 1972). The 80S single ribosomes from embryonic chicken muscles, on the other hand, gave a peak at a density of 1.57 g/cm^3, which is in agreement with the values reported in the literature (Spirin et al., 1965; Spirin, 1969, 1972; Hirsch et al., 1973). Furthermore, the absorbance profiles indicate that the 16–40S particles were free of any detectable amounts of ribosomes. However, as judged by the broadening of the peak, some of the 16–40S particles may have densities higher than the peak density of 1.4. The density of the 16–40S particles is in good agreement with those reported by other workers who have used mainly ra-

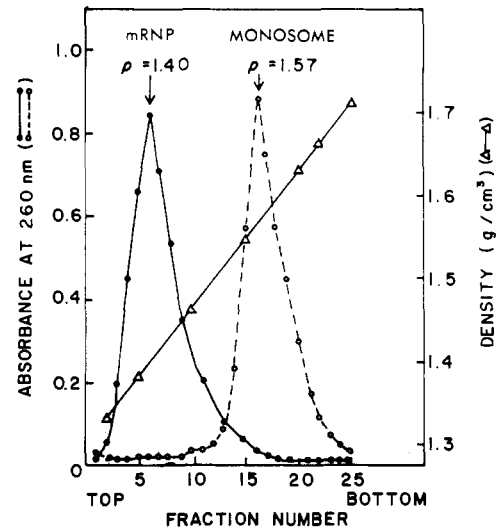

FIGURE 8: Determination of buoyant densities of mRNP particles and 80S chick embryonic muscle monosomes. For details see Experimental Section. Centrifugation was done at 35,000 cpm for 40 hr in a Beckman SW 50.1 rotor at 20°. (●—●) Absorbance of mRNP particles; (○—○) absorbance of monosomes; (Δ—Δ) density of gradient fractions. The absorbance plotted for each fraction is calculated from the ISCO uv recorder.

dioactivity after pulse-labeling as a probe to identify cytoplasmic free mRNP in a number of eukaryotic systems (Spirin, 1969, 1972). Since free RNA is pelleted in CsCl runs and we were unable to detect any pellet in the CsCl gradients, it is unlikely that the messenger activity reported in the previous sections is due to free RNA species present in the 16–40S particles.

When samples of carboxymethylated proteins of 16–40S particles and muscle ribosomal subunits were analyzed by dodecyl sulfate gel electrophoresis, the electrophoretograms indicated that the protein patterns of these two types of particles are quite distinct (Figure 9). The typical ribosomal proteins (ranging from 15,000 to 30,000) could not be detected in the 16–40S particles. At least eight polypeptides in the molecular weight range of 44,000–100,000, which did

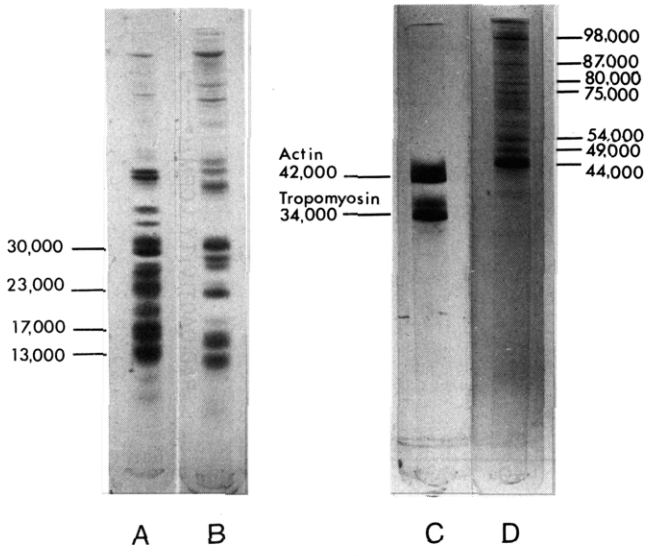

FIGURE 9: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of mRNP particles and chick embryonic muscle ribosomal subunits. For details see Experimental Section. Electrophoresis of carboxymethylated samples of mRNP particles and ribosomal subunits were performed as described in the legend to Figure 5. The amounts of sample applied to the gels are: (A) 0.4 A_{280} unit of 60S ribosomal subunit; (B) 0.4 A_{280} unit of 40S ribosomal subunit; (C) 15 μg of chicken actin and 10 μg of tropomyosin run as markers; (D) 0.05 A_{280} unit of mRNP particles. The numbers corresponding to the bands indicate the molecular weight of the polypeptides.

not appear to represent the typical ribosomal proteins, were present in the mRNA-associated particles. The major band of these proteins is a 44,000 component which is also absent in the ribosomal subunits.

Discussion

The results presented here show that mRNA isolated from 16S to 40S subribosomal (mRNP) particles of embryonic chick muscles directs the synthesis of a polypeptide in a heterologous cell-free system, which is identical with skeletal muscle actin. The ability of this RNA fraction to program the synthesis of actin in an mRNA-dependent system suggests that it is present in a form which can be translated without extensive further processing. The mRNP particles are distinct entities unrelated to ribosomes, as shown by the absence of typical ribosomal proteins and by their characteristic buoyant density, much lower than that of ribosomes. Two previous reports have shown the presence of specific mRNP particles in eukaryotic systems: maternal histone mRNA stored as a ribonucleoprotein particle in unfertilized sea urchin eggs (Gross et al., 1973) and globin mRNPs in rabbit reticulocytes (Jacobs-Lorena and Baglioni, 1972) and duck erythrocytes (Spohr et al., 1972). While the sea urchin egg may be considered as an undifferentiated cell, the reticulocyte represents a special type of cell in which predominantly only one protein, viz., globin, is made. In contrast, embryonic muscle used in our studies represents a terminally differentiated eukaryotic tissue which is characterized by a relatively high degree of cellular and functional complexity. A preliminary estimate by us indicates that approximately 10% of the total actin mRNA present in 14-day old embryonic muscles is located in the mRNP particles (unpublished results). These particles, therefore, may play an important role in the translation of actin, a protein which is a unique component of the fibrillar as well as the cytoplasmic contractile systems in muscle and non-muscle cells, respectively.

There are conflicting reports concerning the nature of the proteins associated with free vs. polysomal mRNP (i.e., mRNP released from polysomes by treatment with EDTA or puromycin and high salt). Gander et al. (1973) have reported that the free 20S globin mRNP present in duck erythroblasts contains seven polypeptides ranging in molecular weight from 15,000 to 24,000 and one polypeptide with a molecular weight of 51,000. None of these proteins was identical with the eight polypeptides in the molecular weight range 49,000–120,000 present in the 15S globin polysomal mRNP. On the other hand, using a similar system derived from rabbit reticulocytes, Baglioni (1974) has recently reported that the protein patterns of cytoplasmic and polysomal globin mRNP are identical, both containing only two components of molecular weights 52,000 and 78,000. Polysomal mRNP particles derived from a number of species and tissues such as rabbit reticulocytes, mouse L cells, and rat hepatocytes have been reported to contain, as judged by dodecyl sulfate gel electrophoresis, bands of proteins of molecular weights 52,000 and 78,000 and a large number of minor bands ranging usually from 6 to 13 (for a review see Blobel, 1973). These results support the view that polysome-derived mRNAs in a wide variety of eukaryotic cells are specifically associated with the two proteins mentioned above and possibly others. The specific function of these proteins in the function and metabolism of mRNA remains to be understood. The results presented here indicate that cytoplasmic 16–40S mRNP particles present in embryonic muscle tissue contain at least eight distinctly identifiable protein bands and a number of other minor bands. The most intense band consistently observed in preparations of mRNPs has a molecular weight of 44,000. Among the other distinct protein bands present are those with mobilities similar to those of the previously mentioned 52,000 and 78,000 components. Although our preparative procedure involves gradient centrifugation in the presence of 0.5 *M* KCl, which is known to minimize nonspecific association of mRNAs with cytoplasmic proteins (Blobel, 1973), we cannot exclude the possibility that other cytoplasmic large protein particles or aggregates may cosediment with the 16–40S mRNP particles. We are currently engaged in obtaining highly purified preparations of both cytoplasmic and polysomal mRNPs from embryonic muscles and analyzing their protein components.

Recent studies from a number of laboratories have suggested that critical control(s) of the translation process may operate during myogenesis of the skeletal muscle fiber. Buckingham et al. (1974) have reported that the transition of mononucleated myoblast cells to the multinucleated myotube in culture is accompanied by an increase in the half-lives of rapidly labeled poly(A)-containing RNA species, presumably mRNAs. This stage, namely the time of cell fusion and the formation of the myotube, is correlated with the onset of intensive synthesis of myosin and a number of muscle-specific enzymes (for a review see Yaffe and Dym, 1972; Buckingham et al., 1974). It has been suggested that the 26S poly(A)-containing putative myosin heavy chain mRNA is stabilized during this period, perhaps as a ribonucleoprotein particle (Buckingham et al., 1974). It is also at this stage that treatment of cultured muscle cells with actinomycin D has no effect on the subsequent appearance of muscle-specific proteins (Yaffe and Dym, 1972). The present results, which show that mRNA coding for an im-

portant muscle protein such as actin exists as a nonpolysomal complex with proteins, strongly suggest that posttranscriptional controls may operate at a cytoplasmic level during growth and terminal differentiation of the muscle cells. The mRNP particles which we have described here may have one of several possible functions. They may be a storage form of mRNA, as in the case of unfertilized eggs (Gross et al., 1973), may represent intermediate species of mRNA during the transit from nucleus to polysomes, or may contain mRNAs whose entry into polysomes is regulated by some translational control. Present work in our laboratory is now directed toward answering some of these questions.

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