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Poly(riboadenylate)-Containing Messenger Ribonucleoprotein Particles of Chick Embryonic Muscles†

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ABSTRACT: Poly(A)-containing cytoplasmic messenger ribonucleoprotein (mRNP) particles were isolated from subcellular fractions of 12–14-day-old chick embryonic muscles by chromatography on oligo(dT)-cellulose and elution of the bound mRNP with 50% formamide. Two types of mRNP, free or nonpolysomal and polysome-derived, were obtained from the postpolysomal supernatant fraction and EDTA-dissociated polysomes, respectively. The mRNP were characterized by the absence of ribosomal RNAs and typical ribosomal proteins, by the presence of a limited number of characteristic proteins, and by the polydisperse sedimentation of the particles and their RNA moieties in sucrose gradients. Although the two types of particles show many similarities, their buoyant densities and $NaDodSO_4$–gel electrophoretograms of their protein moieties indicate that they represent two distinct types of macromolecular complexes. The free mRNP are relatively protein rich and contain 10 distinct proteins in the 40 000–100 000 molecular weight range. The polysome-derived mRNP are comparatively protein deficient and show two major proteins of about 52 000 and 78 000 daltons, which appear to be common to both classes of mRNP. The gel patterns of the poly(A)-associated proteins in both classes of mRNP are indistinguishable, suggesting that the complex protein patterns of the free mRNP are due to the presence of a set of additional proteins which are associated with the nonpoly(A) regions of their mRNA moieties. The presence of a major protein of 78 000 molecular weight in the poly(A)-protein fragments of both classes of mRNP suggests that the association of this protein to the poly(A) tracts is not influenced by translation. The distribution of pulse-labeled total poly(A)$^+$-RNA and two muscle-specific mRNAs, myosin heavy-chain mRNA and actin mRNA, between free mRNP and polysome fractions was about 35:65, respectively, and did not change during embryonic development between 11 and 17 days, suggesting that the two types of mRNP may exist in equilibrium in embryonic muscle cells. The possible relevance of these findings to a mechanism of translational control involving mRNP particles is discussed.

In eukaryotic cells mRNAs are complexed with proteins to form messenger ribonucleoprotein (mRNP)[1] particles (Perry & Kelley, 1968; Infante & Nemer, 1968; Henshaw, 1968; Kafatos, 1968; Cartouzou et al., 1969; Spohr et al., 1970, 1972; Olsnes, 1970; Lebleu et al., 1971; Jacobs-Lorena & Baglioni, 1972; Blobel, 1972, 1973; Bryan & Hayashi, 1973; Barrieux et al., 1975; Bag & Sarkar, 1975, 1976; Liautard et al., 1976; Jeffery, 1977; for a review see Spirin, 1969; Williamson, 1973; Greenberg, 1975). Two types of mRNP particles, polysome-derived mRNP, which are released by the dissociation of polyribosomes, and nonpolysomal free mRNP (also referred to as informosomes), have been reported in the literature. The polysomal mRNP particles derived from a large number of species and tissues contain two major proteins of about 52 000 and 78 000 molecular weight and a large number of additional polypeptides ranging usually from two to thirteen in the 16 000–150 000-dalton range (Lebleu et al., 1971; Blobel, 1973; Bryan & Hayashi, 1973; Barrieux et al., 1975; Burns & Williamson, 1975; Chen et al., 1976; Gander et al., 1973; Gedamu et al., 1977; Irwin et al., 1975; Lindberg & Sundquist, 1974; Kumar & Pederson, 1975; Morel et al., 1973; for a review see Greenberg, 1975). Although the reported variations in the size and number of the protein components of eukaryotic polysomal mRNP particles remain a matter of considerable dispute (Williamson, 1973; Greenberg, 1975), the widespread occurrence of the two major proteins of molecular weights 52 000 and 78 000 strongly suggests that they may be specifically associated with nucleotide sequences which are common to most mRNA molecules. With regard to the interrelationship of the protein moieties of free and polysomal mRNP particles, the few published reports in the literature have led to highly conflicting conclusions. Some of these studies indicate that the protein components of the two classes of mRNP, as judged by sodium dodecyl sulfate

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[1] Abbreviations used: mRNP, messenger ribonucleoprotein; poly(A), poly(riboadenylate); 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; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; hnRNP, heterogeneous nuclear ribonucleoprotein; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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($NaDodSO_4$)-polyacrylamide gel electrophoresis, are identical or essentially similar (Baglioni, 1974; Barrieux et al., 1975; van Venrooij et al., 1977), while according to other reports their protein patterns are different (Gander et al., 1973; Liautard et al., 1976). Furthermore, low molecular weight proteins in the 15 000–30 000-dalton range, which resemble the typical ribosomal and/or cellular proteins in size, have been found in variable amounts and numbers in mRNP particles in many of these reports (Gander et al., 1973; Liautard et al., 1976; for a review see Williamson, 1973; Greenberg, 1975).

Among the mRNA-associated proteins found in eukaryotic systems, the 78 000-dalton protein is currently believed to be bound specifically to the 3′-poly(A) segment of mRNA moieties of both intact polysomes and polysomal mRNP particles (Blobel, 1973; Kumar & Pederson, 1975; Kish & Pederson, 1976; Barrieux et al., 1975; Schwartz & Darnell, 1976; Janssen et al., 1976; for a review see Greenberg, 1975). It has been suggested that the stoichiometry of eukaryotic polysomal mRNP particles consists of one molecule of mRNA and one molecule of each of the two major proteins of 78 000 and 52 000 daltons (Barrieux et al., 1976; Chen et al., 1976). However, the presence of multiple poly(A)-bound proteins other than the 78 000-dalton polypeptide in polysomal mRNP particles of ascites tumor cells (Jeffrey, 1977) and HeLa cells (Schwartz & Darnell, 1976) suggests that the nucleoprotein structure of the poly(A)-protein segments may be very complex. The poly(A)-bound proteins of the HeLa cell mRNAs have been reported to be exchanged in vivo, the 78 000-dalton protein being associated with the newly synthesized mRNA species having the largest poly(A) tracts (Schwartz & Darnell, 1976). According to another report (van Venrooij et al., 1977) this protein is not present in the free mRNP particles of ascites tumor cells and rabbit reticulocytes, and the authors have suggested that it remains attached to mRNAs only during the translation phase.

Several studies from a number of laboratories have suggested that in addition to the transcriptional control (Strohman et al., 1977; John et al., 1977), translational control involving mRNP particles (Heywood et al., 1975; Buckingham et al., 1974, 1976; Bag & Sarkar, 1975, 1976) may also regulate myogenesis in a subtle manner during muscle growth and development. In order to investigate the regulation of mRNA metabolism in chick embryonic muscle cells we have undertaken a detailed study of the isolation and characterization of muscle mRNP particles by utilizing properties of the poly(A)-protein segments of their mRNA moieties. Our results indicate that the free and polysome-bound mRNP particles of chick embryonic muscles differ considerably in their nucleoprotein organization. Furthermore, the poly(A)-bound proteins of both types of particles are essentially the same, suggesting that the major difference between the nucleoprotein nature of the two types of particles is due to a set of additional proteins associated with the nonpoly(A) regions of mRNA moieties of the free particles. Finally, the distribution of mRNAs between the free mRNP and polysomal fraction as a function of embryonic muscle development suggests that the two types of mRNP particles may be in equilibrium—a feature which may be involved in the regulation of cellular translation.

Experimental Procedure

Isolation of Subcellular Fractions. Leg and breast muscles of chick embryos (12–14-day-old unless otherwise specified) were homogenized in buffer A [10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.25 M KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT)] containing 0.25 M sucrose, and postmitochondrial supernatant was prepared from the homogenate as previously described (Bag & Sarkar, 1975, 1976). After supplementation with heparin (100 μg/mL) and phenylmethanesulfonyl fluoride (0.50 mM) used as nuclease and protease inhibitor, respectively, the postmitochondrial supernatant was centrifuged at 105000*g* for 1 h to pellet the total polysomes. The resulting supernatant was then adjusted to 0.5 M KCl and centrifuged at 255000*g* for 4 h to obtain a pellet of postpolysomal particles. For the isolation of poly(A)-containing free mRNP particles, the postpolysomal pellets were gently resuspended in buffer B (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5M NaCl, 100 μg/mL of heparin, and 0.50 mM phenylmethanesulfonyl fluoride) at a concentration of 10–15 A_{260} units/mL. After a low-speed centrifugation at 5000*g* for 10 min to remove any aggregated material, the clear supernatant was saved as the source of free mRNP for oligo(dT)-cellulose chromatography.

The polysomal pellets were resuspended in buffer C (10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 M KCl, 0.1 mM EDTA, and 0.1 mM DTT) at a concentration of about 10 A_{260} units/mL. The polysomes were then centrifuged through a discontinuous sucrose gradient consisting of three 5-mL layers of 1.35, 1.6, and 2.0 M sucrose in buffer C at 177000*g* for 16 h in a Ti 60 Spinco rotor to give a transparent pellet of KCl-washed free polysomes. In order to dissociate the KCl-washed polysomes, the pellets were gently resuspended in buffer D (10 mM Tris-HCl, pH 7.6, 0.25M NaCl, and 20 mM EDTA) to a concentration of 10 A_{260} units/mL and stirred at 0 °C for 30 min. After centrifugation at 8000*g* for 10 min to remove any aggregated material, the clear supernatant was dialyzed against buffer B and was then used for the isolation of polysomal mRNP by oligo(dT)-cellulose chromatography.

Isolation of mRNP. Chromatography of poly(A)-containing mRNP on oligo(dT)-cellulose was carried out as described in the literature (Lindberg & Sundquist, 1974; Kumar & Pederson, 1975) with some modifications. Oligo(dT)-cellulose (Type T_3; Collaborative Research, Inc.) was washed with 0.1% sarkosyl and 0.1 N NaOH and then was equilibrated with buffer B. About 500 A_{260} units of postpolysomal particles or dissociated polysomes suspended in buffer B, as described in the previous section, were stirred with 0.5–0.6 g of oligo(dT)-cellulose for 4–5 h at 4 °C. The oligo(dT)-cellulose was collected by centrifugation and then packed into a small column (5.0 × 0.6 cm) at 4 °C. After the supernatant was passed through the columns, the columns were washed extensively at 4 °C with buffer B until the UV absorbance of the fractions was reduced to the background level. The bound materials were sequentially eluted at 4 °C with buffer E (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 μg/mL of heparin, and 0.5 mM phenylmethanesulfonyl fluoride) and then at 25 °C with 50% formamide in buffer E. Prior to use, the formamide (Baker Analytical Reagent Grade) was treated with 40 g of Bio-Rad resin AG-501-X8 (D) and 10 g of activated charcoal per L of formamide, filtered, and stored in brown bottles.

The formamide-eluted fractions were dialyzed against 10 mM Tris-HCl, pH 7.6, and 1 mM EDTA and then centrifuged at 255000*g* for 16 h to pellet the mRNP.

RNA Extraction. Samples of mRNP were suspended in 100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 1 mM EDTA, and 1% sodium dodecyl sulfate ($NaDodSO_4$) at a concentration of 5–10 A_{260} units/mL and RNA was isolated by three successive extractions with phenol–chloroform–isoamyl alcohol (50:50:1) as described by Aviv & Leder (1972). The final

aqueous phase was adjusted to 0.2 M potassium acetate, pH 5.0, and RNA was precipitated with 2.5 volumes of ethanol at −20 °C. The RNA samples were dissolved in autoclaved distilled water, and the precipitation was repeated four times to remove traces of heparin and $NaDodSO_4$.

For the preparation of poly(A)-containing in vivo pulse-labeled RNA, the procedure of Boedtker et al. (1974) was used with some modification. Minced leg and breast muscles obtained from three to five embryos were incubated in 20–25 mL of Dulbecco's modified Eagle's medium [10 g of Gibco solid medium and 30 mL of 1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5, made to 1L] supplemented with 2–5 mCi of carrier-free $H_3{}^{32}PO_4$ (New England Nuclear) for 60 min. The tissues were then homogenized and RNA was isolated from the free and polysomal mRNP as described in the previous section.

Sucrose Density-Gradient Centrifugation. The mRNP particles were suspended in 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.25 M KCl at a concentration of 8–10 A_{260} units/mL. About 2–3 A_{260} units of mRNP were centrifuged through a 13.5-mL 15–40% linear sucrose gradient in the same buffer at 39 000 rpm for 7 h in a Spinco SW 40 Ti rotor. The gradients were fractionated using an ISCO gradient fractionator and the fractions were monitored for UV absorbance at 260 nm.

The RNA samples were dissolved in 0.1 M LiCl, 5 mM EDTA, 0.2% $NaDodSO_4$, 1 mM Tris-HCl, pH 7.6, and 50% formamide (Anderson et al., 1974) at a concentration of 10–15 A_{260} units/mL. About 2–3 A_{260} units of the samples were centrifuged through a 5–20% linear sucrose gradient in the same buffer at 39 000 rpm for 24 h in a Spinco SW 40 Ti rotor.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of mRNP Proteins. The mRNP samples were dissolved in 50 mM Tris-HCl, pH 8.0, containing 8 M urea and 0.01 M β-mercaptoethanol. After the mixture was stirred at room temperature for 45 min in a sealed flask, solid α-iodoacetamide was added to a concentration of 0.2 M, and the samples were stirred for 90 min at room temperature. The alkylated samples were then dialyzed against 1% $NaDodSO_4$ and 1% β-mercaptoethanol. $NaDodSO_4$–polyacrylamide gel electrophoresis of protein samples was carried out as previously described (Bag & Sarkar, 1976).

CsCl Density-Gradient Analysis. The mRNP samples were dissolved in 20 mM triethanolamine hydrochloride, pH 7.0, 25 mM KCl, 2 mM magnesium acetate, 0.1 mM EDTA, and 0.5 mM DTT to a concentration of about 5–10 A_{260} units/mL. The samples were fixed with 4% freshly neutralized formaldehyde in the same buffer and about 3 A_{260} units of the samples were then layered on 4.5 mL of preformed CsCl gradients of densities 1.2–1.7 g/cm^3 prepared in the same buffer and containing 2% formaldehyde. The gradients were centrifuged at 35 000 rpm for 60 h in a Spinco SW 50.1 rotor. After the gradients were fractionated in an ISCO density-gradient fractionator, the fractions (0.1 mL each) were monitored for UV absorbance at 260 nm. The density of the fractions was estimated from the refractive indices measured in a Bausch & Lomb refractometer.

Messenger Activity of RNA Samples. The preincubated wheat germ embryo S-30 system of Roberts & Paterson (1973) was used for the estimation of actin mRNA contents (Bag & Sarkar, 1975) of RNA fractions isolated from mRNP samples. The in vitro translated products were copurified with chicken muscle actin added as carrier by three cycles of salt-dependent polymerization and depolymerization, and the purified products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as previously described (Bag & Sarkar, 1975).

The myosin heavy-chain mRNA contents of RNA samples were estimated by using the rabbit reticulocyte lysate programmed with the RNA fractions as described previously (Bag & Sarkar, 1976). After addition of embryonic myosin as carrier and copurification by precipitation at low ionic strength and DEAE-cellulose chromatography, the in vitro products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Bag & Sarkar, 1976). In both translation systems the assay conditions were maintained such that the radioactivities recovered in the $NaDodSO_4$–gel bands of the polypeptide chain purified with carrier muscle proteins—the 200 000-dalton myosin heavy-chain and the 42 000-dalton actin—were linearly dependent on the amount of mRNA added (Bag & Sarkar, 1975, 1976).

Isolation of Poly(A)-Protein Fragments. Samples of mRNP were suspended in 20 mM Tris-HCl, pH 7.5, 0.5M NaCl, and 2 mM EDTA at a concentration of 5 A_{260} units/mL. The samples were incubated with RNase T_1 (1 μg/mL) and pancreatic RNase (1 μg/mL) for 30 min at 10 °C. After incubation, the reaction mixture was diluted with 10 volumes of buffer B. The samples were then stirred with oligo(dT)-cellulose and chromatography of the poly(A)-protein complexes was carried out by the same procedure as described for the isolation of intact mRNP in the previous section. Column fractions eluted with 50% formamide were dialyzed against 10 mM Tris-HCl, pH 7.6, and 1 mM EDTA and then lyophilized. The samples were then processed for $NaDodSO_4$–polyacrylamide gel electrophoresis.

Materials. Phenylmethanesulfonyl fluoride, unlabeled amino acids, creatine phosphate, creatine kinase, hemin, dithiothreitol, and nucleoside triphosphates were purchased from the Sigma Chemical Co. L-[^{35}S]Methionine (sp act. 320 Ci/mmol) and uniformly labeled [^{14}C]amino acid mixture were purchased from the New England Nuclear Inc. Cesium chloride (optical grade) was obtained from Schwarz/Mann.

All preparative operations were carried out at 0–4 °C unless otherwise specified. Standard precautions to prevent nuclease activity, viz., use of acid-washed glassware and sterilized solutions, were strictly maintained throughout all operations.

Results

Isolation of Poly(A)-Containing mRNP Particles. Poly(A)-containing mRNP particles were isolated by oligo(dT)-cellulose chromatography of subcellular fractions of chick embryonic muscles (Figure 1). The postpolysomal particles and 0.5 M KCl-washed EDTA-dissociated free polysomes (for details see Experimental Procedure) were used as the sources of free and polysomal mRNP particles, respectively. In both cases about 97–98% of the UV-absorbing material applied to the column was recovered in an unadsorbed early peak obtained with the binding buffer (panels A and B). After washing the column with the binding buffer (indicated by arrow 1), elution with a low-salt buffer at 2 °C (arrow 2) released a small amount of UV-absorbing material. As judged by A_{260}/A_{280} ratio of about 2.0, this fraction presumably represents the small amount of protein-free mRNA present in the preparations. Elution with 50% formamide in the low-salt buffer (arrow 3) released quantitatively the bound mRNP particles as a sharp peak (indicated by bars). When formamide was omitted in the elution buffer, the bound material was not released (results not shown here), indicating that the peaks eluted with formamide were not due to the presence of protein-free poly(A)-containing mRNAs, since the latter are routinely eluted from such columns with low-salt

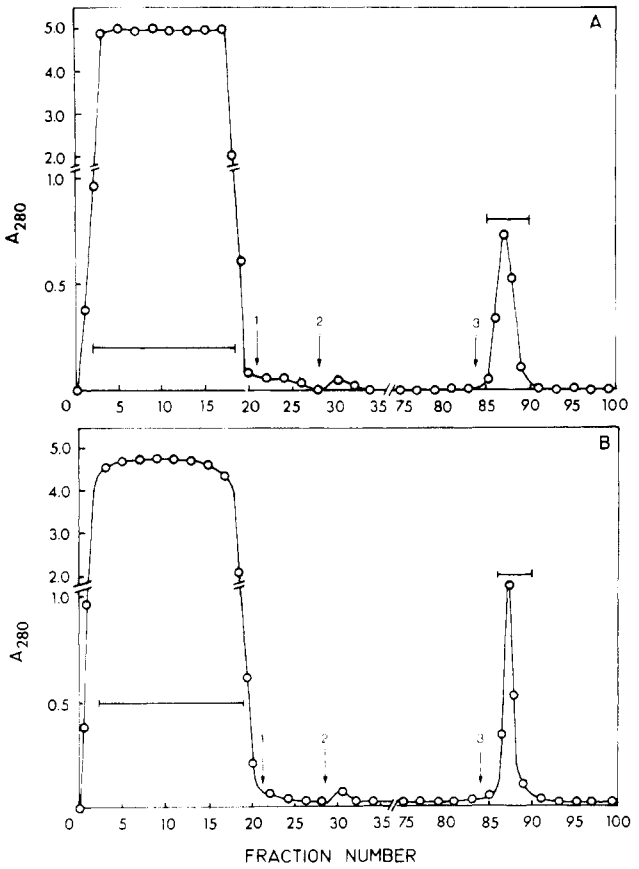

FIGURE 1: Isolation of mRNP by oligo(dT)-cellulose chromatography of subcellular fractions of chick embryonic muscles. For details see Experimental Procedure. Panel A: chromatography of postpolysomal particles. Panel B: chromatography of EDTA-dissociated polysomes. The arrows 1, 2, and 3 indicate the positions where the column was washed with buffer B at 4 °C, buffer E at 4 °C, and buffer E containing 50% formamide at 25 °C, respectively. The bars indicate the fractions which were pooled and processed for further analysis.

buffer alone (see also Figure 2).

In order to test the possibility that the particles eluted from oligo(dT)-cellulose column with formamide are formed due to nonspecific association of cytoplasmic proteins with mRNA during the preparative procedures (Spirin, 1969; Baltimore & Huang, 1970), we have incubated deproteinized poly(A)-containing mRNA isolated from chick embryonic muscle polysomes with 100-fold excess of a cytosol fraction from which the mRNP particles were removed by oligo(dT)-cellulose chromatography (unbound fraction in Figure 1, panel A). The reaction mixture was then applied to a column of oligo(dT)-cellulose and chromatography was carried out as described above. As shown in Figure 2, poly(A)-containing RNA samples when chromatographed alone (closed circle and dotted line) or after incubation with the cytosol (open circle and solid line) were eluted quantitatively with low-salt buffer at 2 °C. The A_{260}/A_{280} ratios of the eluted fractions ranged from 1.97 to 2.04. Subsequent elutions with 50% formamide did not give any significant amount of UV-absorbing material in contrast to the results obtained with both EDTA-dissociated polysomes and postpolysomal particles (Figure 1). These results strongly suggest that the oligo(dT)-cellulose bound and formamide-eluted material present in the subcellular fractions of chick embryonic muscles is not formed due to nonspecific association of cytoplasmic proteins with the mRNA under these experimental conditions.

Properties of mRNP Particles. The size distribution of the two classes of mRNP and deproteinized mRNA samples

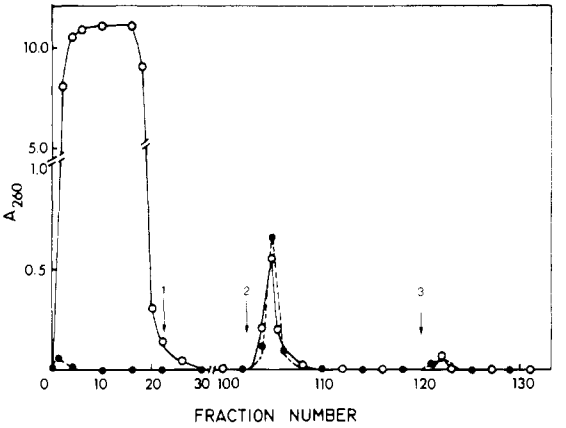

FIGURE 2: Chromatography of poly(A)-containing RNA moieties of free mRNP (formamide-eluted peak; Figure 1, panel A) on oligo(dT)-cellulose. For details see Experimental Procedure and legends to Figure 1. (●----●) Two A_{260} units of RNA obtained by deproteinization of free mRNP were dissolved in buffer B and were applied on oligo(dT)-cellulose column and chromatography was carried out as described for the isolation of mRNP (Figure 1). In a separate experiment (○–○) two A_{260} units of the same RNA sample were incubated with 100-fold excess of cytosol (the unbound fraction obtained by oligo(dT)-cellulose chromatography of postpolysomal supernatant; panel A, Figure 1) in buffer A containing 0.5 M KCl for 1 h at 25 °C. The mixture was then chromatographed on oligo(dT)-cellulose, and the bound and unbound material was separated as described under Experimental Procedure and legends to Figure 1.

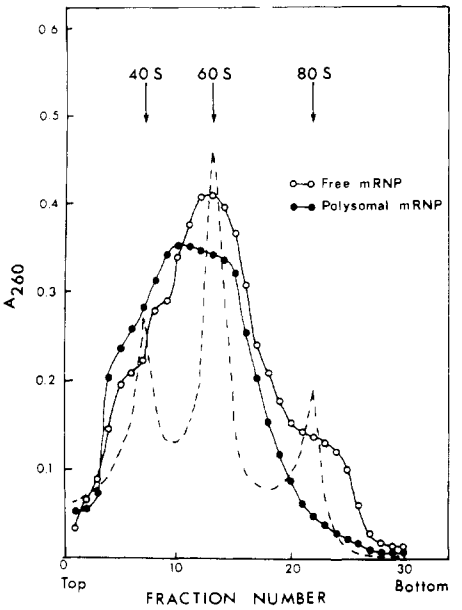

FIGURE 3: Sucrose density-gradient sedimentation of mRNP. For details see Experimental Procedure. Five to six A_{260} units of mRNP particles (Figure 1, panels A and B) were centrifuged through a 13.5-mL 15–40% linear sucrose gradient in buffer containing 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 0.25 M KCl for 7 h at 39 000 rpm in a Beckman SW 40 Ti rotor. The arrows and the plot with dashed line indicate the positions of the 40 S, 60 S, and 80 S chick embryonic muscle ribosomes and ribosomal subunits run as markers in parallel gradients. (○–○) Free mRNP; (●----●) polysomal mRNP.

derived from the particles was examined by sucrose gradient centrifugation. Both free and polysomal mRNP gave very similar and heterogeneous distribution (Figure 3) with the majority of the particles sedimenting between 20 and 80 S. No detectable peaks of UV-absorbing material cosedimenting with ribosomal subunits or 80S monosomes were observed in the gradient profiles. When examined in a denaturing formamide–sucrose gradient, the deproteinized RNA samples

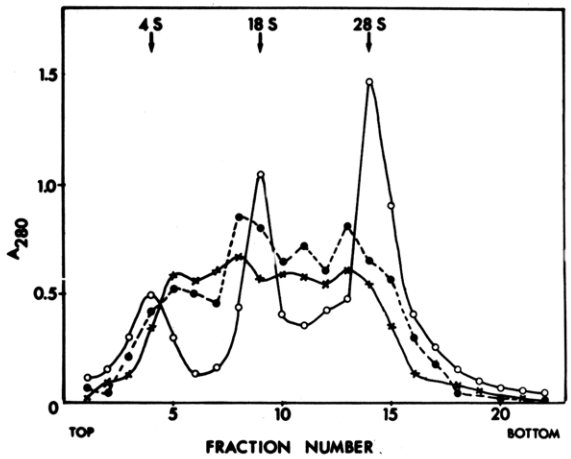

FIGURE 4: Sucrose density-gradient sedimentation of RNA fractions obtained from mRNP (Figure 1, panels A and B). For details see also Experimental Procedure. Centrifugation was carried out in a linear 5–20% sucrose gradient made in 0.1 M LiCl, 5 mM EDTA, 0.2% $NaDodSO_4$, 1 mM Tris-HCl, pH 7.5, and 50% formamide at 39 000 rpm in a Spinco SW 40 rotor. (x–x) RNA isolated from free mRNP; (●----●) RNA isolated from polysomal mRNP; (O–O) mixture of chick embryonic muscle 4S, 18S, and 28S RNAs run as markers.

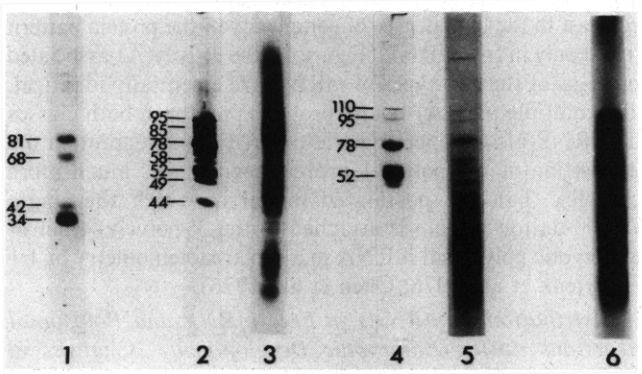

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of the protein components of mRNP. For details see Experimental Procedure. Gel 1: a mixed sample of markers containing 12 μg of transferrin, 10 μg of bovine serum albumin, 5 μg of actin, and 14 μg of tropomyosin. Gel 2: Sixty micrograms of free mRNP (panel A, Figure 1). Gel 3: One hundred micrograms of unbound fraction obtained from the chromatography of free mRNP (panel A, Figure 1). Gel 4: Forty micrograms of polysomal mRNP (panel B, Figure 1). Gel 5: One hundred micrograms of unbound fraction obtained from the chromatography of EDTA-dissociated polysomes (panel B, Figure 1). Gel 6: One hundred micrograms of native 40S muscle ribosomal subunit.

obtained from both classes of mRNP showed a very similar and heterogeneous distribution with about 85% of the RNA sedimenting between 8 and 30 S (Figure 4). Again, no discrete peaks cosedimenting with the peaks of marker 28-, 18-, and 4S RNAs were observed. These results are in agreement with the expected size of most eukaryotic mRNAs (Brawerman, 1974). The similarities in the sedimentation profiles shown in Figures 3 and 4 also rule out the possibility that mRNA species of any particular size class are selectively enriched in either the free or polysomal mRNP.

Protein Components of mRNP Particles. The protein moieties of the two classes of mRNP particles were analyzed by $NaDodSO_4$–gel electrophoresis. Free mRNP gave a complex protein pattern consisting of about 10 polypeptides in the 35 000–95 000 molecular weight range (Figure 5, gel 2) with six major bands of molecular weights of 44 000, 52 000, 58 000, 64 000, 78 000 and 95 000, respectively. The unbound fraction obtained from the chromatography of postpolysomal

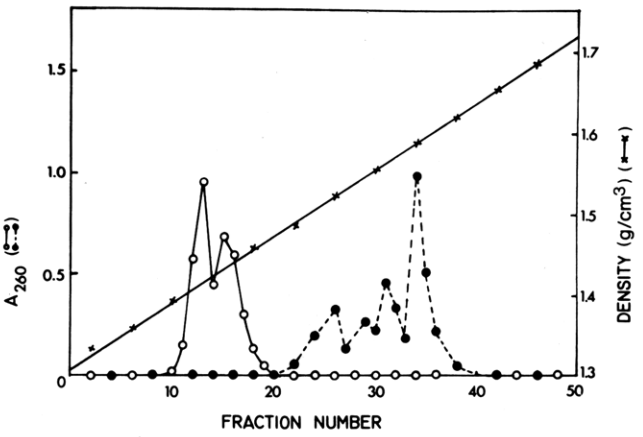

FIGURE 6: Determination of buoyant densities of mRNP. For details see Experimental Procedure. (O–O) Free mRNP; (●----●) polysomal mRNP.

particles (Figure 1, panel A) showed the presence of a large number of proteins which varied widely in size from 10 000 to about 180 000 (gel 3). Polysomal mRNP, on the other hand, gave a relatively simpler electrophoretogram consisting of two major bands of 52 000 and 78 000 daltons (gel 4). In addition, another less intense band of about 60 000, which was not always well resolved from the 52 000-dalton component, and two minor bands of molecular weights of about 95 000 and 110 000 were also present. The unbound fraction obtained from the oligo(dT)-cellulose chromatography of polysomal mRNP (Figure 1, panel B) contained mainly proteins in the 15 000–30 000-dalton range (gel 5). As judged by comparison with the electrophoretogram of 40S ribosomal subunit (gel 6), these polypeptides appear to be typical ribosomal proteins. This is in agreement with the view that the unbound fraction contains dissociated ribosomal subunits (Lindberg & Sundquist, 1974; Kumar & Pederson, 1975). From an analysis of eight gel scans using four different mRNP preparations it was estimated that the major bands of 52 000 and 78 000 daltons constituted on the average about 85 and 40% of the total proteins present in polysomal and free mRNP particles, respectively. These two proteins were found to coelectrophorese when a mixed sample of free and polysomal mRNP was run together (data not shown), suggesting that they may be common to both classes of mRNP. Using subcellular fractions obtained from embryos labeled separately with [^{3}H]adenosine and [^{14}C]amino acids, we observed that about 90% of the pulse-labeled poly(A)$^+$-RNA is recovered in the free and polysomal mRNP particles in an approximate ratio of about 30–40:70–60, respectively (see also Table I). In contrast, only a total of about 4–5% of the labeled proteins was recovered in the free and polysomal mRNP fractions. Thus, the oligo(dT)-cellulose purified particles contain only a small fraction of the cellular RNA and proteins, which is also in agreement with the mRNP nature of the particles. The large spectrum of proteins found in the unbound fraction of the postpolysomal particles (Figure 5, gel 3) and the relatively inordinate amount of UV absorbance present in this fraction (Figure 1, panel A) strongly suggest that, in addition to the bulk of the cytoplasmic proteins, some cellular RNA species such as tRNA and poly(A)$^-$-mRNP are also most likely to be present in this fraction.

The nucleoprotein nature of the two classes of mRNP was further confirmed by estimating their buoyant densities. Samples of mRNP particles were first fixed with formaldehyde, layered onto preformed CsCl gradients, and centrifuged to equilibrium. As indicated by the UV-absorbance

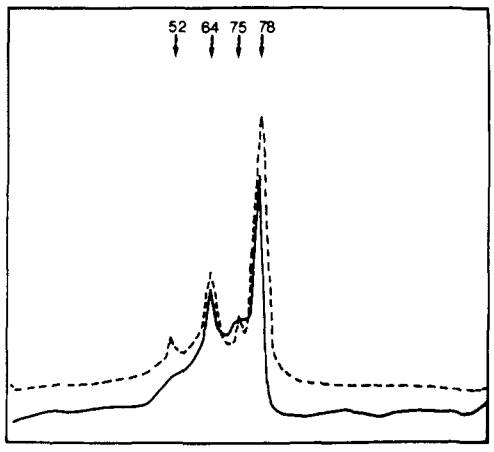

FIGURE 7: $NaDodSO_4$–polyacrylamide gel electrophoresis of poly(A)-associated proteins of mRNP. For details see Experimental Procedure and legend to Figure 1. The Coomassie Blue stained gels were scanned at 590 nm in a Joyce-Loebel microdensitometer. (----) Free mRNP; (–) polysomal mRNP. The arrows indicate the molecular weights $\times 10^{-3}$.

profiles in Figure 6, free mRNP particles banded as two distinct peaks of densities of 1.41 and 1.43 g/cm^3 (solid line and open circles). The polysomal mRNP particles on the other hand gave four peaks which were distributed more widely in the density range of 1.49–1.62 g/cm^3 (closed circles and dotted lines). The buoyant densities shown in Figure 6 are characteristic of RNA–protein complexes and are different from those of pure RNA or proteins (Spirin, 1969). Furthermore, the multiple peaks of different buoyant densities shown by both free and polysomal mRNP indicate the presence of subpopulations which differ in their relative protein contents. The average protein contents of free and polysomal mRNP particles calculated from their buoyant densities (Spirin, 1969) were about 72 and 40%, respectively. It should be noted that the buoyant densities of free mRNP particles containing actin and myosin heavy-chain mRNAs, previously isolated by us by repeated cycles of sucrose density-gradient centrifugation (Bag & Sarkar, 1975, 1976), are very similar to those of the free poly(A)$^+$-mRNP purified by oligo(dT)-cellulose chromatography (Figure 6). Furthermore, isopycnic centrifugation of oligo(dT)-cellulose purified mRNP particles, labeled in protein moieties, indicates that about 95–96% of the protein counts was recovered in the mRNP fractions. These observations rule out the possibility that the protein composition of mRNP particles, as reported here, is altered by oligo(dT)-cellulose chromatography or CsCl gradient centrifugation.

In order to gain some insight into the possible differences in the nucleoprotein nature of free and polysomal mRNP particles, we next examined the poly(A)-associated proteins of these particles. The mRNP samples were incubated with T_1 and pancreatic RNase by use of conditions in which the poly(A)–protein segments are resistant to nucleolytic cleavage (Jeffery & Brawerman, 1975; Kumar & Pederson, 1975). The poly(A)-protein complexes were isolated from the incubation mixtures by oligo(dT)-cellulose chromatography using the same procedure as described for the isolation of intact mRNP particles (see also Experimental Procedure). $NaDodSO_4$–polyacrylamide gel electrophoresis of the resulting poly(A)–protein segments showed that two major proteins of about 78 000 and 64 000 daltons were present in both free and polysomal mRNP particles, the 78 000 molecular weight component being the most prominent species (Figure 7). In addition, two other proteins of about 52 000 and 75 000 molecular weights were also present in minor quantities. Furthermore, densitometric scans of the gel runs indicate that the relative ratio of the protein moieties was found to be very similar in both types of mRNP. A protein of molecular weight of about 64 000 has been previously reported to be attached to the 3′-poly(A) segment of HeLa cell polysomal mRNA (Kish & Pederson, 1976). These results indicate that in contrast to the high degree of complexity of the protein pattern found only in free mRNP (Figure 5), the 3′-poly(A)-associated proteins of the two types of mRNP are essentially identical. The multiple poly(A)-bound proteins present in both classes of mRNP also support the view that the nucleoprotein organization of the poly(A)–protein segments is much more complex than the postulated model in which the single 78 000-dalton protein is attached to the 3′-poly(A) tract of eukaryotic polysomal mRNA in a molar stoichiometry of 1:1 (Barrieux et al., 1976; Chen et al., 1976).

Table I: Distribution of Poly(A)-Containing RNA between Free Ribonucleoprotein and Polysomal Cytoplasmic Fractions during Embryonic Development

age of embryonic development (days)	distribution of mRNAs[a]					
	total poly(A)-containing pulsed RNA		myosin heavy-chain mRNA		actin mRNA	
	polysomes (%)	free mRNP (%)	polysomes (%)	free mRNP (%)	polysomes (%)	free mRNP (%)
11	60	40	66	34	73	27
14	65	35	71	29	69	31
17	59	41	62	38	66	34

[a] For details on the isolation and assay of mRNAs see Experimental Procedure. The values are expressed as percent of total present in the free mRNP and polysomes.

Distribution of mRNAs in Free mRNP and Polysomal Fractions during Embryonic Development. Changes in patterns of different size groups of muscle polysomes during different stages of embryonic muscle development (Heywood & Rich, 1968), as well as changes in the distribution of pulse-labeled RNA fractions between free mRNP and polysomal fractions in cultured muscle cells (Buckingham et al., 1974, 1976) have been previously reported in the literature. These results have suggested that posttranscriptional controls, presumably operating at the level of loading of preformed mRNAs to form polysomes, may play a subtle regulatory role during embryonic muscle cell growth and differentiation, although other interpretations of these observations are also possible. We have examined the distribution of total poly(A)-containing pulse-labeled RNA and mRNAs coding for two myofibrillar proteins, actin and myosin heavy-chain, in order to study the possible function of mRNP particles in the regulation of mRNA metabolism in muscle cells. The results are shown in Table I. Two features become clear from these analyses. After a 2-h [^{3}H]adenosine pulse, poly(A)-containing labeled RNA species are distributed in a ratio which ranged from about 25–40% for free mRNP and 75–60% for polysomal fractions. Furthermore, this pattern of distribution (average 35:65 partition) was not significantly altered during the stages of embryonic development. Studies on the myosin heavy-chain mRNA and actin mRNA distribution indicate that these two mRNA species also gave a similar distribution (Table I)—about 30–40% for free mRNP and 70–60% for polysomes. This pattern remained about the same during the developmental stages studied, indicating that this characteristic distribution is shared by most, if not all, of the mRNA

populations in embryonic muscle cells.

Discussion

In the present study we have isolated and partially characterized poly(A)-containing free and polysomal mRNP particles of chick embryonic muscles using affinity chromatography on oligo(dT)-cellulose. In making a meaningful comparison of the properties of the two types of mRNP particles, we have maintained certain precautions and very similar, almost identical conditions during the isolation procedure. These are as follows: protease and nuclease inhibitors to prevent fragmentation of the mRNP particles; and the use of high-salt buffers containing 0.5 M KCl or 0.5 M NaCl and EDTA, which are known to prevent or minimize nonspecific association of cytoplasmic proteins with mRNAs (Baltimore & Huang, 1970; Blobel, 1973; Kumar & Pederson, 1975). Finally, prior to the oligo(dT)-cellulose chromatography step, the subcellular fractions, used as the sources of the two types of mRNP particles, were equilibrated against the same high ionic strength buffer. In many earlier reports dealing with the nature of the protein moieties of eukaryotic mRNP particles these conditions were not strictly followed (for a review see Greenberg, 1975; Van der Marel et al., 1975), and in several instances the mRNP particles were isolated following limited RNase treatment of subcellular fractions, e.g., polysomes and postpolysomal particles (Auerbach & Pederson, 1975; Liautard et al., 1976)—a procedure which does not leave the mRNP particles fully intact.

Although it can not be strictly excluded that nonspecific RNA–protein interaction takes place during our preparative procedures, two points argue against this possibility. The two types of particles show the presence of only a limited number of characteristic proteins in the 40 000–100 000-dalton range (Figure 4). These protein patterns are in sharp contrast to the large number of cellular proteins having a wide range of molecular weights (15 000–150 000), which are present in the unbound fractions obtained by oligo(dT)-cellulose chromatography of subcellular fractions. Secondly, as judged by the inability of deproteinized poly(A)-containing RNA after incubation with the cytosol fraction to form oligo(dT)-cellulose-bound and formamide-eluted particles (Figure 2), it appears that the particles isolated by us (Figure 1) are not artifacts of cell fractionation and oligo(dT)-cellulose chromatography and thus represent true cellular entities. However, it should be pointed out that these results do not strictly rule out the possibility of the loss of some loosely bound protein components which may be present in mRNP in the in vivo state and which are preferentially dissociated during the purification step, particularly by treatment with 0.5 M KCl. The question whether or not the mRNP particles purified by us represent the true in vivo state remains to be settled by defining the biological role of the mRNP particles and finally by reconstitution of the particles from the dissociated components.

Among the several properties, which are shared by both free and polysomal mRNP particles of chick embryonic muscles, are the absence of ribosomal RNAs (Figure 4) and typical ribosomal proteins (Figure 5) and the similarities in the polydisperse sedimentation of the particles and their RNA moieties in sucrose gradients (Figures 3 and 4). In contrast to the complete absence of low molecular weight proteins in the 15 000–30 000-dalton range, which may be the typical ribosomal or the bulk of cellular proteins, in our mRNP preparations (Figure 5), several previous reports have shown that free mRNP particles isolated from duck reticulocytes (Gander et al., 1973), mouse plasmocytes (Egly et al., 1974), and HeLa cells (Liautard et al., 1976), either by sucrose density-gradient centrifugation or by limited RNase digestion, contain considerable amounts of proteins in the low molecular weight range mentioned above. We have recently shown that the free and polysomal mRNP particles isolated by oligo(dT)-cellulose chromatography from a large number of representative eukaryotic cells such as rabbit reticulocytes, rat liver, and Ehrlich ascites tumor cells have protein patterns very similar to those obtained from embryonic muscle cells (unpublished results). Although it has been speculated that the variation and diversity of some of the mRNA-associated proteins in various eukaryotic cells are due to species specificity (Liautard et al., 1976), these conflicting results may be due to differences in the methodology used. Our results, which represent the first reported isolation of poly(A)-containing free mRNP particles by oligo(dT)-cellulose chromatography, indicate that this method is ideally suited for such purposes, since it eliminates the need for lengthy sucrose gradient fractionation routinely used in the literature (Williamson, 1973; Baglioni, 1974; Gander et al., 1973; Bag & Sarkar, 1975, 1976).

Previous reports in the literature have suggested that eukaryotic mRNP particles, in general, are characterized by a relatively high protein content (for a review see Williamson, 1973; Greenberg, 1975; Jeffery, 1977). However, our results showing the buoyant densities of the free and polysomal mRNP (Figure 6) indicate that their nucleoprotein natures are not identical. While the presence of multiple peaks of different buoyant densities show that within each class of mRNP there are subpopulations having different RNA and protein contents, the free particles are comparatively protein rich with an average protein content of about 72%. In contrast, the polysomal particles contain only about 40% protein. Further difference between the two classes of mRNP is also reflected in the electrophoretic patterns of their protein moieties. The polysomal mRNP exhibit a relatively simple protein pattern containing two major bands of 52 000 and 78 000 daltons, which compares favorably with the published reports on polysomal mRNA-associated proteins found in a wide variety of cell types (Blobel, 1973; Bryan & Hayashi, 1973; Lindberg & Sundquist, 1974; for a review see Greenberg, 1975). In contrast, the free mRNP reveal a more complex pattern consisting of about 10 characteristic polypeptides among which are also the above-mentioned 52 000- and 78 000-dalton components. Since the 3′-poly(A)-associated proteins in both classes of mRNP are strikingly similar, if not identical (Figure 7), the proteins present only in free mRNP must be restricted to the nonpoly(A) regions of their mRNA moieties. Thus, our results not only demonstrate that the nucleoprotein organization of the two classes of mRNP is different but also suggest that this difference is mainly due to the association of a set of free mRNP-specific proteins to the nonpoly(A) regions of the mRNA moieties.

The presence of multiple and similar poly(A)-bound proteins in both free and polysomal mRNP, as reported here, raises a number of interesting points. The stoichiometry of eukaryotic mRNP previously postulated as consisting of 1 mol of mRNA and 1 mol of each of the 52 000- and 78 000-dalton proteins (Barrieux et al., 1976; Chen et al., 1976), according to our results, does not appear to be true. Two poly(A)-bound proteins of 78 000 and 64 000 molecular weights have been previously reported in HeLa cell polysomal mRNA (Kish & Pederson, 1976). The presence of two proteins of similar molecular weights, as the major poly(A)-associated components in both free and polysomal mRNP, as reported here,

strongly suggests that their association to the 3′-poly(A) tracts is not influenced by translation. It is quite likely that distinct subpopulations of mRNP, both free and polysomal, and containing either the 78 000- or the 64 000-dalton component as the major poly(A)-associated protein, exist in embryonic muscle cells. On the other hand, it should also be pointed out that the nonequimolar amounts of the 78 000- and 64 000-dalton poly(A)-proteins, as shown in Figure 7, may be due to partial loss of one of the proteins during the isolation procedure and do not necessarily reflect the intrinsic heterogeneity of the poly(A)-protein segments of the mRNP populations.

Several previous reports have shown that a 78 000-dalton protein or proteins of similar size are specifically associated with the 3′-poly(A) tracts of hnRNP in a number of eukaryotic cells (Kish & Pederson, 1975; Quinlan et al., 1974; Kumar & Pederson, 1975). The present results, in conjunction with these reports, strongly suggest that the 78 000-dalton protein, or similar proteins, always remains bound to the 3′-poly(A) tracts of primary transcripts and the processed mRNAs. Furthermore, the association of this protein to the poly(A) segment seems to be independent of whether or not the mRNAs remain free or polysome-bound in the cytoplasm. The electrophoretic prominence of some of the proteins, e.g., the 52 000-dalton protein in free and polysomal mRNP (Figure 5), suggests that these proteins are bound as either single or multiple copies to nucleotide sequences which are common to most mRNA species.

To what extent are the two classes of mRNP reported here involved in the regulation of translation in the embryonic muscle cell? The results on the cytoplasmic distribution of total pulse-labeled poly(A)-containing RNAs, actin mRNA, and myosin heavy-chain mRNA during embryonic muscle development demonstrate that these RNA species are partitioned approximately 35:65% between the free mRNP and polysomal fractions, respectively (Table I), and this distribution pattern remains unaltered between 11 and 17 days—a period when intensive synthesis of myofibrillar proteins takes place in the terminally differentiated muscle cell (Heywood & Rich, 1968). In view of these results it appears that the free and polysomal mRNP do not represent a simple model of precursor–product in embryonic muscles. The most likely explanation of these observations is that the two types of mRNP exist in dynamic equilibrium. Although the distribution pattern of pulse-labeled poly(A)$^+$-RNA between the free mRNP and polysomal fractions strongly suggests that newly synthesized mRNA also equilibrates between these two cytoplasmic compartments, it should be noted with caution that this distribution may also be a coincidence due to the time of labeling used. It is quite likely that the association of the specific polypeptides, which we have found only in free mRNP, with eukaryotic mRNA moieties to form the characteristic protein-rich free mRNP particles provides a regulatory mechanism of translational control involving relatively stable mRNA species. Whether the protein moieties of free mRNP regulate in vivo the entry of the mRNAs from the nonpolysomal pool to the polysomes or whether they act as a stabilization mechanism against cleavage of mRNAs by intracellular nucleases, particularly when the mRNAs are not protected by binding to ribosomes, remains to be studied. The mRNA-associated proteins may also exist as a pool, and the size and availability of this pool may act as a subtle regulatory parameter in controlling translation in embryonic muscle cell.

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Measurement of Macromolecular Equilibrium Binding Constants by a Sucrose Gradient Band Sedimentation Method. Application to Protein–Nucleic Acid Interactions†

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ABSTRACT: A method is described for determining equilibrium binding constants using sucrose gradient band sedimentation data. The method is particularly well suited to measuring the binding of small ligands or proteins to much more rapidly sedimenting macromolecules or macromolecular complexes (e.g., large DNA molecules or ribosomes). Only very small quantities of radioactively labeled binding proteins are needed for each determination, and association constants (K) as large as 10^{11} M^{-1} can be measured. Over a wide range of sedimentation conditions the only information necessary for calculation of the association constant is the initial concentration of potential binding sites for protein, the distance of sedimentation of the fast-sedimenting peak, and the fraction of total binding protein migrating with the peak. Results are presented demonstrating the applicability of this method to three different systems: (1) the interaction of bovine pancreatic ribonuclease with double-stranded DNA, (2) the interaction of *Escherichia coli* ribosomal protein S1 with single-stranded DNA, and (3) the interaction of ribosomal protein S1 with 30S ribosomal subunits. In each case the binding constant obtained reproduces (within a factor of two) that measured on the same system by a completely independent method. Common errors or misinterpretations in the application of this technique are described, as well as the extension of this approach to proteins with multiple binding sites, to cooperatively binding proteins, and to the determination of binding site stoichiometry.

The determination of binding parameters for interacting macromolecules (for example, in protein–nucleic acid complex formation) is often of central importance to the quantitative analysis of a biological control system. However this can be an experimentally difficult problem and one for which straightforward procedures of general utility and interpretability are still largely not available.

Standard equilibrium dialysis techniques generally cannot be used for such systems because both macromolecular components may be too large to dialyze; in addition binding constants are often so large ($K > 10^7$ M^{-1}) that concentrations required for the reasonably accurate determination of optical changes upon complex formation (at least 10^{-7} M components) often cannot be employed. Futhermore, for many systems only small quantities of material are available.

For protein–nucleic acid interactions, nitrocellulose filter binding assays have been successfully used (Riggs et al., 1970; Yarus & Berg, 1970; Hinkle & Chamberlin, 1972). This assay depends on the preferential retention of protein and protein–nucleic acid complexes by the filter; artifacts can arise if the filter is rinsed too thoroughly after trapping the complex or if the complex has a short half-life relative to the time of filtering (Hinkle & Chamberlin, 1970). In addition a number of factors affect the retention of complexes by filters, including temperature, ionic strength, and presence of divalent metal ions; this often complicates a survey of binding conditions by this technique. Furthermore, protein–protein interactions cannot, of course, be measured by the filter assay technique.

A boundary sedimentation method worked out by Jensen & von Hippel (1977; see also Revzin & von Hippel, 1977) can be used to study a variety of interactions. However it generally cannot be applied to systems with binding constants greater

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