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Polypeptides of Nonpolyribosomal Messenger Ribonucleoprotein Complexes of Sea Urchin Eggs†

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ABSTRACT: RNA competent in directing protein synthesis is sequestered in unfertilized sea urchin eggs as translationally quiescent, nonpolyribosomal, messenger ribonucleoprotein complexes (mRNPs). Following fertilization, these mRNPs are derepressed and actively translated, presumably due to changes in the mRNA-associated proteins and their interaction with the mRNA. We have isolated poly(A)-containing egg mRNPs free of contaminating monoribosomes and ribosomal subunits by chromatography on oligo(dT)–cellulose and identified their constituent proteins. Egg mRNPs isolated by using near physiological ionic conditions have 15–20 major proteins, most of which are in the molecular weight range of 40 000–100 000, and ~15–23 minor proteins in the 22 000–190 000 molecular weight range. The association of the proteins with poly(A)-containing mRNA is indicated by their greatly reduced retention on oligo(dT)–cellulose after pretreatment of the crude mRNP fraction with saturating amounts of poly(uridylic acid). Three of the proteins present in poly(A)-containing mRNPs from eggs, with molecular weights of 48 000, 67 000, and 140 000, were not detected in poly(A)-containing mRNPs derived from polyribosomes of hatched blastula-stage embryos. In addition, stoichiometric differences were found between some of the proteins associated with the two types of mRNP. The potential regulatory role of these proteins is discussed.

As a consequence of the number and complexity of the components involved in translation, there are numerous potential mechanisms for regulating the total rate of protein synthesis as well as the synthesis of specific proteins. Since eucaryotic mRNA exists in association with specific proteins from the time of its nuclear synthesis until its involvement in polyribosome formation [reviewed by Spirin (1979)], and probably until its degradation, it is likely that these proteins affect the utilization of mRNA. Generally less than 15 proteins have been found in both messenger ribonucleoprotein complexes (mRNPs)[1] that are free in the cytosol and mRNPs derived from polyribosomes (Cardelli & Pitot, 1977; Grubman

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[1] Abbreviations used: mRNPs, messenger ribonucleoprotein complexes; hnRNPs, heterogeneous nuclear ribonucleoprotein complexes; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; A_{260nm} unit, the amount of material per 1 mL having an absorbance of 1 at 260 nm in a 1-cm path length.

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& Shafritz, 1977; Mirkes, 1977; Mueller et al., 1977; Slegers et al., 1979; Bag & Sells, 1979; Jain & Sarkar, 1979). Proteins with molecular weights of ~52 000 and ~78 000 have been identified in mRNPs from several cell types. Many other mRNP proteins, which range in molecular weight from 15 000 to 150 000, may be species specific [reviewed by Jain & Sarkar (1979)].

While significant differences in the proteins associated with free mRNPs and polyribosomal mRNPs have been reported, others have concluded that the differences are slight [reviewed by Jain & Sarkar (1979)]. These conflicting findings, as well as the variations in the number and molecular weights of mRNP proteins isolated from different sources, may in part be attributable to the isolation of mRNPs under nonphysiological ionic conditions. For prevention of the artifactual binding of proteins, mRNPs containing poly(A) have been isolated by chromatography on oligo(dT)–cellulose in the presence of either 0.5 M NaCl or 0.5 M KCl [e.g., Jain & Sarkar (1979), Jeffery (1977), and van Venrooij et al. (1977)]. However, these concentrations of salts have also been shown to remove many proteins from bound mRNPs during chromatography (Cardelli & Pitot, 1977; Huynh-Van-Tan & Schapira, 1978). Additionally, removal of Mg^{2+} from ribosomes with EDTA in the presence of 0.5 M NaCl or KCl results in the loss of up to 63% of the proteins normally associated with the ribosomes (Spitnik-Elson & Atsmon, 1969) and also results in the loss of proteins from mRNPs of KB cells (Kumar & Lindberg, 1972). Furthermore, mRNPs from sea urchin eggs isolated with 0.35 M NaCl and 5 mM EDTA have a higher buoyant density in Cs_2SO_4 than those isolated with 0.35 M NaCl or 0.35 M KCl and 5 mM Mg^{2+}, indicative of the loss of proteins (Kaumeyer et al., 1978). The use of nonphysiologically high concentrations of Cl^- may also destabilize protein–nucleic acid and protein–protein interactions (Weber et al., 1977).

Protein synthesis in sea urchin eggs proceeds at a very low rate despite the large amount of cytoplasmic ribosomes and preformed mRNA in the eggs (Denny & Tyler, 1964). After fertilization, protein synthesis increases dramatically (~30-fold) by the two-cell stage (Humphreys, 1971; A. S. Goustin, personal communication). The rapid rise in the rate of protein synthesis may be partially attributed to the increased availability of active template, derived from a pool of free mRNPs that are translationally inefficient. Sea urchin egg mRNPs isolated in KCl are poor templates in in vitro cell-free translating systems. On the other hand, mRNPs isolated in high concentrations of NaCl and $MgCl_2$ as well as RNAs purified from crude RNP fractions are active in directing protein synthesis (Jenkins et al., 1978; Ilan & Ilan, 1978). This suggests that the template activity of sea urchin egg mRNPs may be modulated by their constituent proteins.

As an initial step to understanding how the proteins of repressed mRNPs may regulate the template activity of the mRNP, we have purified sea urchin egg free poly(A)-containing mRNPs from cosedimenting ribosomes and ribosomal subunits using physiological ionic conditions. Differences were found between the proteins of free egg mRNPs and the major proteins purified from polyribosomal poly(A)-containing mRNPs isolated from blastula-stage embryos.

Materials and Methods

Solutions. Buffers had the following designations and compositions unless otherwise specified: isolation buffer (IB), 220 mM K^+, 5 mM Mg^{2+}, 80 mM Cl^-, 140 mM OAc^-, 20 mM Pipes (Calbiochem), 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride (Calbiochem), pH 6.8 (0 °C); phosphate isolation buffer (PIB-20 and PIB-67), 20 and 67 mM PO_4^{2-}, respectively, plus 220 mM K^+, 40 mM Na^+, 5 mM Mg^{2+}, 80 mM Cl^-, 180 mM OAc^-, 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.8 (0 °C); column binding buffer (CBB), 220 mM K^+, 80 mM Cl^-, 140 mM OAc^-, 10 mM EDTA, and 20 mM Hepes (Calbiochem), pH 7.4 (0 °C); elution buffer (EB), 15 mM KCl, 5 mM $Mg(OAc)_2$, and 20 mM Pipes, pH 6.8 (45 °C); formamide elution buffer (FEB), 50% (v/v) formamide (BDH Chemicals Ltd., England) in 50% (v/v) IB, pH 7.2 (0 °C). All solutions and sea water were purified by filtration through nitrocellulose filters (0.45-μm pore size). Contamination with ribonuclease was further reduced by baking all glassware at 180 °C for 12 h and treating solutions with 0.2% diethyl pyrocarbonate, followed by autoclaving.

Isolation of Total RNPs from Eggs. Eggs were collected from the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* by intracoelomic injection of 0.55 M KCl. Eggs were washed twice by centrifugation at $2500g_{max}$ for 10 s in 30 volumes of sea water (12 °C for *S. purpuratus*; 16 °C for *L. pictus*) and twice in 30 volumes of IB, PIB-20, or PIB-67 at −4 °C. Washed eggs were suspended in 5–10 volumes of buffer at −4 °C and homogenized by gentle extrusion through either a 20- or 22-gauge needle. Nuclei and residual intact eggs were pelleted from the homogenate by centrifugation at $5000g_{max}$ for 5 min (−2 °C). Recovery of intact nuclei was confirmed by phase microscopy. Mitochondria and large membranes were pelleted at $27000g_{max}$ for 10 min (−2 °C). The upper two-thirds of the supernatant were layered in 4-mL quantities onto 33-mL 10–30% (w/v) linear sucrose gradients made to the final concentration and pH of the homogenization buffer and centrifuged in a Beckman SW27 rotor at $93000g_{av}$ for 3.5 h (1 °C). The absorbance of the gradients at 254 nm was monitored during fractionation by pumping gradients through an ISCO Model UA-5 absorbance fluorescence monitor equipped with a 5-mm path length flow cell. Total RNPs sedimenting from 40 to 90 S were collected and pooled.

Preparation of Embryo Polyribosomes. Embryos of *S. purpuratus* and *L. pictus* were stirred continuously in sea water with 100 mg/L streptomycin at 12 and 16 °C, respectively, at a concentration of 10 mL of packed eggs per 2 L of sea water. Upon hatching of 90% of the embryos, the blastulae were concentrated in a handmade zonal rotor. To allow reformation of polyribosomes, we reoxygenated concentrated embryos by rotating 1 mL of packed embryos in 10 mL of sea water for 30 min before plunging them into 20 volumes of −4 °C PIB-20 (pH 7.2). After collection by centrifugation at $2500g_{max}$ for 10 s, the embryos were resuspended and centrifuged in another 10 volumes of PIB-20 and then homogenized as described for eggs. The homogenate was centrifuged once at $5000g_{max}$ and then once at $14000g_{max}$ for 10 min each (−2 °C). Up to 4 mL of the resulting supernatant was layered over 3 mL of 1.0 M sucrose made to the concentration of PIB-20 (pH 7.2). Polyribosomes were selectively pelleted by centrifugation in a Beckman SW40 rotor at $190000g_{av}$ for 2.4 h (5 °C) (M. V. Danilchik, personal communication), leaving most monoribosomes, ribosomal subunits, and free mRNPs in the sucrose. The pellet was gently rinsed with PIB-20 to remove traces of supernatant, dissolved in PIB-20 (pH 7.2), and centrifuged to remove undissolved material. The final concentration of polyribosomes was 66 A_{260nm} units/mL. The intact nature of the polyribosomes, and their enrichment from monoribosomes, was determined by sucrose gradient analysis in 15–40% (w/w) linear sucrose gradients.

Oligo(dT)–Cellulose Chromatography. Poly(A)-containing mRNPs were purified by binding to oligo(dT)–cellulose. Up to 500 A_{260nm} units of total egg RNPs sedimenting from 40 to 90 S in preparative sucrose gradients was pooled (~125 mL) and mixed on a rotary shaker at 25 rpm for 3 h (4 °C) with 0.5–1.0 g of oligo(dT)–cellulose (P-L Biochemicals type 7) preequilibrated with the homogenization buffer. The oligo(dT)–cellulose was then allowed to settle through three 20-volume changes of either IB, PIB-20, PIB-67, or CBB. Washed oligo(dT)–cellulose was transferred to a 15 × 1 cm jacketed column and washed with an additional 100 mL of the same buffer at a flow rate of 1.0–1.25 mL/min. The absorbance of the eluted material was monitored at 254 nm with an ISCO monitor. Bound poly(A)-containing mRNPs were then eluted at 45 °C with 10–15 mL of EB, followed by 10 mL of FEB at 6 °C (Jain et al., 1979). Eluted material was concentrated by negative-pressure dialysis against PIB-20 for 24 h and then either analyzed on Cs_2SO_4 and sucrose gradients or precipitated as a magnesium–phosphate complex with ethanol (Dessev & Grancharov, 1973).

Poly(A)-containing mRNPs were isolated from polyribosomes as follows. Two hundred A_{260nm} units of embryo polyribosomes in PIB-20 was diluted with 30 volumes of CBB and mixed with 0.5 g of oligo(dT)–cellulose on a rotary shaker for 3 h (4 °C). Unbound material was removed by washing with CBB and then mRNPs were eluted as described.

To investigate the specificity of binding of the poly(A)-containing mRNPs to oligo(dT)–cellulose, we mixed 400 A_{260nm} units of egg total RNP in 60 mL from several sucrose gradients on a rotary shaker for 2 h at 4 °C with 8 mg of poly(U) (P-L Biochemicals) prior to mixing them with 0.5 g of oligo(dT)–cellulose as described above. An identical sample was simultaneously mixed in the absence of poly(U) for 2 h and then mixed with 0.5 g of oligo(dT)–cellulose as described above.

Density Analyses. Cs_2SO_4 gradients in 100 mM KCl, 20 mM PO_4^{2-}, and 1% gluteraldehyde (Tousimis, EM grade), pH 6.8, were preformed according to Kaumeyer et al. (1978) by using 2.7 mL of a 1.2 g/cm^3 Cs_2SO_4 solution and 2.0 mL of a 1.7 g/cm^3 Cs_2SO_4 solution. Samples (0.5 mL) were dialyzed against PIB-67 and then fixed with 5% gluteraldehyde (adjusted to pH 6.8 with $NaHCO_3$) for 1 h (0 °C) prior to centrifugation in a Beckman SW50.1 rotor at 142000g_{av} for 18 h (4 °C). The absorbance at 260 nm of the gradients was monitored with a 5-mm path length flow cell attached to a recording spectrophotometer. The density of every fourth 0.24-mL fraction was determined by weighing 100 μL in a precalibrated micropipet.

Metrizamide gradients were prepared by mixing pooled fractions of the preparative sucrose gradients with an equal volume of 80% (w/v) metrizamide, made to a final concentration of PIB-20. The resulting solution had a density of 1.221 g/cm^3 and was centrifuged in a Beckman SW50.1 rotor at 187000g_{av} for 24 h at 5 °C (Buckingham & Gros, 1975), forming a sigmoidal gradient. The densities of the 0.24-mL fractions were determined as described above and from their refractive indexes (Rickwood & Birnie, 1975). After the addition of 20 μg of a carrier [yeast tRNA purified of any poly(A) species on oligo(dT)–cellulose], fractions were precipitated according to Dessev & Grancharov (1973).

[^{3}H]Poly(U) Hybridization. All RNPs were ethanol-precipitated, digested with $NaDodSO_4$ and proteinase K, and hybridized to [^{3}H]poly(U) as described by Kaumeyer et al. (1978). After RNase A digestion of unhybridized [^{3}H]poly(U), samples were precipitated for only 10 min after the addition of an equal volume of 5% (w/v) Cl_3AcOH (Williams & Klett, 1978). The precipitates were collected on glass-fiber filters (Gelman), washed with 10 mL of 2.5% Cl_3AcOH, dried, digested for 1 h (22 °C) with 0.6 mL of NCS (Amersham/Searle) and 0.1 mL of water, and then counted in a toluene-based scintillation fluid. The number of hybridized counts was linear with respect to sample concentration, independent of the concentration of nonadenylated RNA, and competitively inhibited by the addition of unlabeled poly(U). Quenching did not vary between samples.

Cell-Free Translation. The methods of Roberts & Paterson (1973) were used to prepare a wheat germ S30 cell-free system. The 50-μL reaction mixtures were incubated at 25 °C for 2 h and then stopped by the addition of 1 mL of 7.5% (w/w) Cl_3AcOH containing 0.1 mg/mL unlabeled leucine. Precipitates were heated at 95 °C for 10 min, chilled for 30 min at 0 °C, collected on glass-fiber filters, and washed with 10 mL of 7.5% Cl_3AcOH. The filters were dried, digested with NCS, and counted in scintillation fluid to determine the incorporation of [^{3}H]leucine, as described above.

Isolation of Protein from RNPs. Total egg RNPs were dissociated by 2 M LiCl (Chao, 1961; Curry & Hersh, 1961/1962; Huynh-Van-Tan & Schapira, 1978), 2 M LiCl and 4 M ultrapure urea (Traub & Nomura, 1969), or 66% acetic acid with 0.1 M Mg^{2+} (Hardy et al., 1969; Sherton & Wool, 1974). The proteins obtained by these three methods were found to be identical on sodium dodecyl sulfate and isoelectric focusing gels (Laemmli, 1970; O'Farrell, 1975). Proteins from purified mRNPs were prepared by using 2 M LiCl and 4 M ultrapure urea. Dissociated proteins were concentrated by negative-pressure dialysis against 0.2% acetic acid for 24 h (4 °C) and then lyophilized.

Gel Electrophoresis. Sodium dodecyl sulfate electrophoresis was performed with 17.5 cm × 12 cm × 1.5 mm vertical slab gels according to Laemmli (1970) and O'Farrell et al. (1973). The separating gels either contained 10% (w/v) acrylamide or 10–15% exponential polyacrylamide gradients were made by using 20% (v/v) of the higher concentration gel solution. Gels were stained in 50% methanol, 10% acetic acid, and 0.25% Coomassie brilliant blue R for 2 h (22 °C), destained in 7.5% acetic acid, and scanned at 560 nm by using a Gilford linear transport and a recording spectrophotometer. Molecular weights were estimated from a semilog plot using molecular weight standards.

Results

Isolation and Characterization of Total RNPs from Sea Urchin Eggs. The amount of poly(A)-containing material from sea urchin eggs which sediments in 10–30% sucrose gradients has been increased several-fold over that of Kaumeyer et al. (1978) by using isolation buffers which have concentrations of K^+, Mg^{2+}, Na^+, PO_4^{2-}, Cl^-, and H^+ similar to those present in the cytosol (Rothschild & Barnes, 1953; Steinhardt et al., 1971; Shen & Steinhardt, 1978). The utility of inorganic phosphate and heparin as ribonuclease inhibitors and the addition of poly(A)-deficient yeast RNA to compete for ribonucleases were tested to maximize the amount of poly(A)-containing mRNPs which sediments in 10–30% sucrose gradients. The peak of poly(A)-containing RNA hybridizable to [^{3}H]poly(U) in the preparative sucrose gradients was 60–70 S for all isolation conditions (Figure 1), in agreement with previous reports (Kaumeyer et al., 1978). Isolation with PIB-67 resulted in a higher yield of poly(A)-containing mRNPs than isolation in IB or PIB-20 (Figure 1). Furthermore, higher yields of poly(A)-containing mRNPs were obtained by using PIB-20 rather than IB, demonstrating that

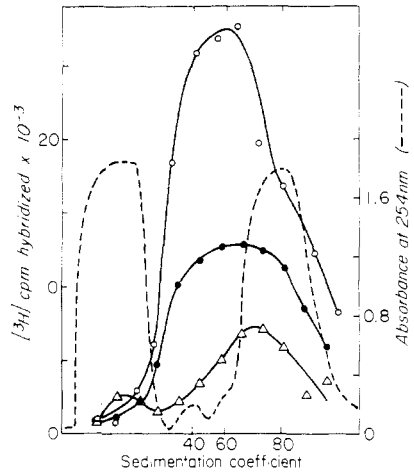

FIGURE 1: Effect of phosphate concentration on the recovery of poly(A)-containing mRNP particles from *S. purpuratus* eggs. Homogenization buffers and sucrose gradients were made to the concentration of IB (△), PIB-20 (●), or PIB-67 (○). Eggs were homogenized and centrifuged on 10–30% sucrose gradients as described under Materials and Methods. Centrifugation was from left to right. After ethanol precipitation and digestion by $NaDodSO_4$ and proteinase K, 50 μL of each 2-mL fraction was hybridized to 88 000 dpm of [^{3}H]poly(U) as described under Materials and Methods. The absorbance profiles of all three gradients at 254 nm were as indicated.

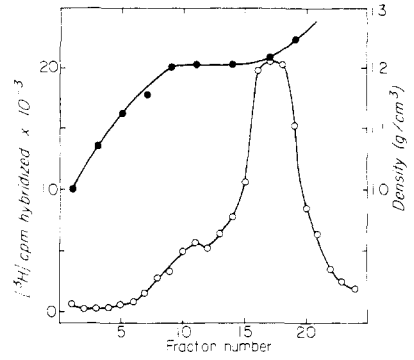

FIGURE 2: Metrizamide gradient analysis of total RNPs from *S. purpuratus* eggs isolated in PIB-20. Centrifugation was from left to right. For details see Materials and Methods. For localization of the poly(A)-containing mRNP particles, 0.24-mL fractions were hybridized to 88 000 dpm of [^{3}H]poly(U) (○).

the yield of poly(A)-containing mRNPs increased with increasing concentrations of PO_4^{2-}. The addition of heparin or yeast RNA to buffers containing PO_4^{2-} did not improve the recovery of poly(A)-containing mRNPs and they were not used in subsequent isolations (data not shown). At present, we do not know whether the recovery of poly(A)-containing mRNPs which pellet in the 10–30% sucrose gradients is enhanced by isolation in phosphate buffers or whether some pelleting mRNPs contribute to the detectable 60S mRNPs following isolation in phosphate buffers.

The poly(A)-containing mRNPs present in the pooled 40–90S fractions of preparative sucrose gradients of total RNPs prepared in PIB-20 were shown to have a peak buoyant density of 1.22 g/cm^3 in metrizamide (Figure 2). This buoyant density is similar to the 1.205–1.220 g/cm^3 density of free mRNPs from muscle cells (Buckingham & Gros, 1975). In contrast, most heterogeneous nuclear RNP particles have higher peak densities in metrizamide, in the range of 1.27–1.29 g/cm^3 (Blanchard et al., 1977; Karn et al., 1977; Gattoni et al., 1977). The presence of a small peak of [^{3}H]poly(U)-hybridizable RNA with a corresponding density of 1.21 g/cm^3 suggested that the free mRNPs were heterogeneous in their protein content.

Table I: Recovery of Poly(A)-Containing mRNPs from Oligo(dT)-Cellulose[a]

sample	cpm of [^{3}H]-poly(U) hybridized per $A_{260\,nm}$ RNA unit	% of hybridizable poly(A) added to column
unbound material	2 056	3
45 °C low salt eluted	100 825	83
formamide eluted	70 466	14

[a] Unbound material of the *L. pictus* crude mRNP fraction was removed with PIB-67. mRNPs were eluted at 45 °C with EB and at 6 °C with FEB, as described under Materials and Methods. No more than 0.1 A_{260nm} RNA unit was hybridized with 88 000 dpm of [^{3}H]poly(U). The RNA concentration was determined after $NaDodSO_4$–proteinase K digestion of the RNP fractions and precipitation of the free RNA. The percent hybridized for each fraction was calculated from the amount eluted in each fraction and the information in column two.

The total egg RNPs, isolated in the K^+ and Na^+ buffers of Jenkins et al. (1978) and pelleted from the 40–90S region of preparative sucrose gradients, were translated in a wheat germ cell-free system and found to be three- to fivefold less active in directing protein synthesis than either total egg RNA or RNA purified from the RNPs. Na^+-isolated mRNPs were twice as active as K^+-isolated RNPs in directing protein synthesis as found by Jenkins et al. (1978). Although Jenkins et al. (1978) found that mRNPs and purified mRNA required different Mg^{2+} concentrations for optimal translation (3.0 and 3.5 mM, respectively), in our hands both mRNPs and purified mRNA usually translated best at 3.5 mM Mg^{2+}.

Purification and Characterization of Poly(A)-Containing mRNPs. Free mRNPs from *S. purpuratus* and *L. pictus* eggs and polyribosomal mRNPs from *L. pictus* blastula embryos were purified substantially free of ribosomal material by specific binding of poly(A)-containing mRNPs to oligo(dT)–cellulose. Low-salt buffers at elevated temperatures and formamide were required for 100% elution of the poly(A)-containing material from the oligo(dT)–cellulose, as similarly found by Jain et al. (1979) for chick embryo muscle mRNPs. For example, as shown in Table I, 97% of the poly(A)-containing material bound to oligo(dT)–cellulose in the high-salt buffer PIB-67 and 83% of the [^{3}H]poly(U)-hybridizable RNPs eluted from oligo(dT)–cellulose with the low-salt buffer EB, at 45 °C. Fourteen percent of the hybridizable material applied to the column was resistant to elution at 45 °C but was eluted with a 50% formamide buffer, FEB, at 6 °C. In our initial experiments, we found that only 6% of the hybridizable material was eluted when EB was at 4 °C (data not shown). In all our investigations we continued to use the two-step elution procedure for purifying poly(A)-containing mRNPs since the populations of complexes eluted by each step contained two distinct patterns of proteins as shown below.

The poly(A)-containing complexes isolated with PIB-67 and eluted at 45 °C from the oligo(dT)–cellulose column were characterized by density and sedimentation as largely intact mRNP particles. This fraction had a peak buoyant density of 1.43 g/cm^3 (range of 1.38–1.47 g/cm^3) in Cs_2SO_4 (Figure 3C), similar to that previously found for unpurified poly(A)-containing mRNPs from sea urchin eggs (peak 1.46 g/cm^3, range 1.35–1.57 g/cm^3; Kaumeyer et al., 1978) and for purified poly(A)-containing mRNPs from KB cells (1.38–1.52 g/cm^3; Lindberg & Sundquist, 1974) and significantly higher than that of *L. pictus* hnRNP (ρ = 1.28 g/cm^3; Wilt et al., 1973). This fraction also contained little material which bands like deproteinized RNA at 1.66 g/cm^3 (Figure 3C) or like

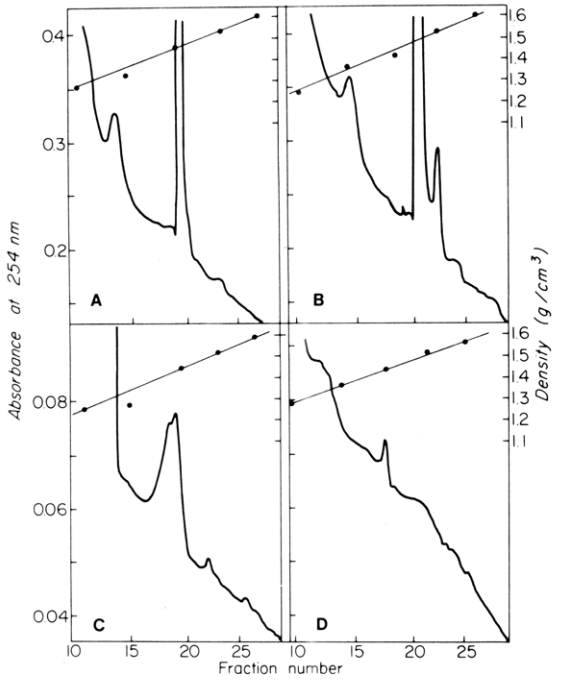

FIGURE 3: Buoyant density in Cs_2SO_4 of material from *S. purpuratus* egg RNPs isolated in PIB-67 and eluted from oligo(dT)–cellulose. (A) 1.7 A_{260nm} units of nonchromatographed total mRNP fraction; (B) 1.7 A_{260nm} units of material not binding to oligo(dT)–cellulose; (C) 0.4 A_{260nm} unit of 45 °C EB-eluted material; (D) 1.1 A_{260nm} units of FEB-eluted material. Centrifugation was from left to right. Absorbance at the top of the gradients was due to gluteraldehyde.

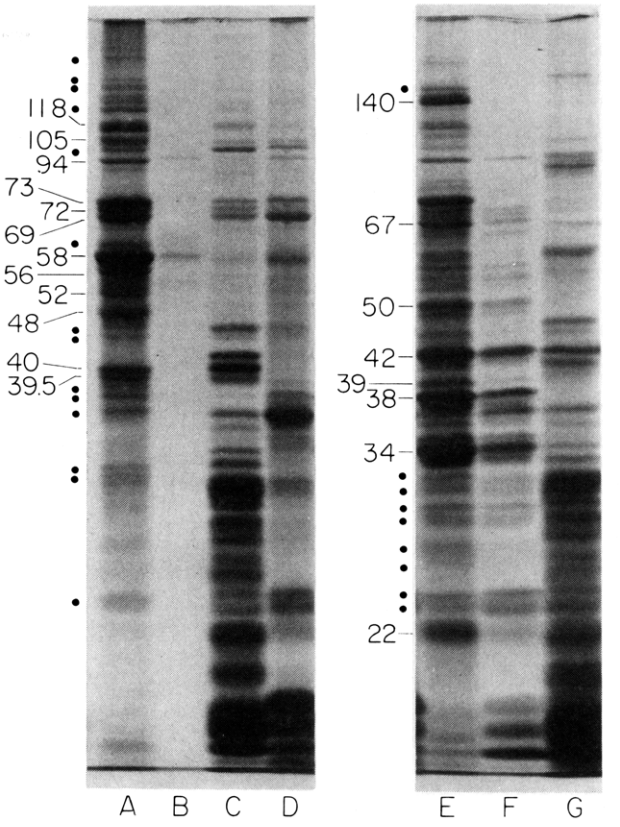

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of *L. pictus* poly(A)-containing mRNP proteins. RNPs were isolated from eggs and blastula and mixed with oligo(dT)–cellulose in a phosphate buffer (PIB-20) and finally with CBB containing EDTA to remove unbound material before elution of the purified mRNPs as described under Materials and Methods. (A) 45 °C EB-eluted fraction from egg RNPs; (B) 45 °C EB-eluted fraction from egg RNPs pretreated with poly(U); the amount of pretreated RNPs was equal to that applied to (A); (C) nonchromatographed intact polyribosomes from the hatched blastula stage; (D) FEB-eluted fraction of hatched blastula polyribosomes; (E) FEB-eluted fraction of egg mRNPs; (F) FEB-eluted fraction from egg RNPs pretreated with poly(U); the amount pretreated was equal to that applied to (E); (G) unbound fraction from egg RNPs. 50–100 μg of protein was applied to lanes A, C–E, and G. Electrophoresis was on 10% acrylamide gels. The molecular weights of major proteins are given ($\times 10^{-3}$), and those of the minor bands, designated by (●), are in (A) 24 000, 32 000, 32 500, 36 500, 38 000, 39 000, 44 000, 46 000, 62 000, 98 000, 135 000, 155 000, 162 000 and 190 000 and in (E) 23 500, 24 000, 26 000, 27 500, 28 500, 30 000, 31 000, and 32 000.

ribosomal material at 1.47–1.52 g/cm³. In the fraction eluted with formamide (Figure 3D), however, small amounts of protein and ribosomal material coeluted with free mRNPs from the oligo(dT)–cellulose column. The retention of mRNPs and free protein on oligo(dT)–cellulose after elution of most of the poly(A)-containing RNA has been observed by previous investigators (Lindberg & Sundquist, 1974; Sundquist et al., 1977). Sucrose gradient centrifugation and hybridization to [³H]poly(U) showed that the size of the mRNP complexes eluted at 45 °C was similar to those in the crude fraction (data not shown). The presence of some slower sedimenting material at the top of the sucrose gradient indicated some degradation of the particles had occurred during purification with oligo(dT)–cellulose or upon recentrifugation in sucrose. However, no 260-nm absorption peaks corresponding to ribosomal material were present in this sucrose gradient.

Proteins of the Purified Poly(A)-Containing mRNPs. Poly(A)-containing complexes isolated from *L. pictus* eggs with PIB-67, washed with CBB containing EDTA after binding to oligo(dT)–cellulose to remove nonspecifically bound material and eluted in two steps as described above, were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as shown in Figure 4. This gel analysis revealed about 35 distinct protein bands in the purified complexes ranging in molecular weight from 22 000 to 190 000 (Figure 4, lanes A and E). Of the major bands, 12 occurred predominantly in the fraction eluted at 45 °C (lane A) and 8 occurred predominantly in the fraction eluted with formamide (lane E) as indicated numerically by their apparent molecular weight ($\times 10^{-3}$) in the figure. These major protein bands of both eluted fractions were also present in the same proportion after isolation of mRNPs with IB or PIB-20 (data not shown). Less prevalent but distinct protein bands present in the fractions eluted at 45 °C and by formamide are indicated by dots on Figure 4. In contrast to the polypeptides associated with the material bound to oligo(dT)–cellulose, most of the polypeptides in the unbound fraction (Figure 4, lane G) had molecular weights of less than 45 000, suggestive of a ribosomal nature (Cox, 1977). Only four of the major bands from the material eluted at 45 °C and with formamide were similar in molecular weight to protein bands present in the unbound fraction. Those were the 34 000-, 42 000-, 67 000-, and 94 000-dalton polypeptides. The identity, however, of these major mRNP polypeptides with those in the unbound fraction has yet to be established by two-dimensional gel electrophoresis.

To assess how many of the polypeptides were specifically bound to oligo(dT)–cellulose as complexes with poly(A)-containing mRNA, total RNPs were hybridized to saturating amounts of nonradioactive poly(U) prior to chromatography with oligo(dT)–cellulose. Only two polypeptides, with molecular weights of 58 000 and 95 000, eluted at 45 °C in low-salt buffer after treatment with poly(U) (Figure 4, lane B). Reduced quantities of many proteins were eluted with formamide from the oligo(dT)–cellulose column after preincubation of the crude mRNP fraction with poly(U) (Figure 4, lane F).

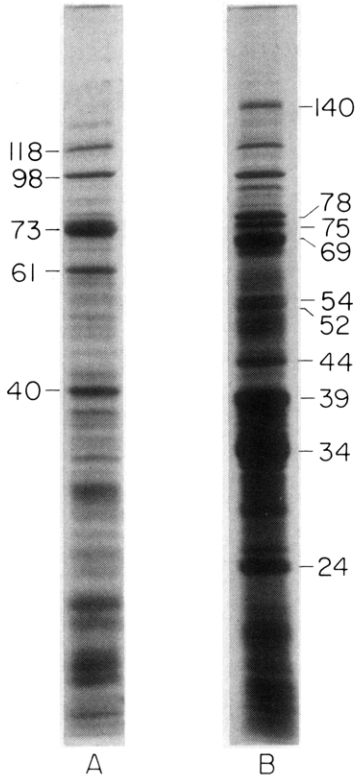

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of *S. purpuratus* poly(A)-containing mRNP proteins. mRNPs bound to oligo(dT)–cellulose in PIB-67 were washed with the same buffer to remove unbound material and then eluted in two steps with 45 °C EB (A) and FEB (B). The higher molecular weight proteins of (A) were present but not visible due to underloading of the lane. Electrophoresis was on a 10–15% exponential polyacrylamide gradient gel. Major proteins are designated as molecular weights ($\times 10^{-3}$).

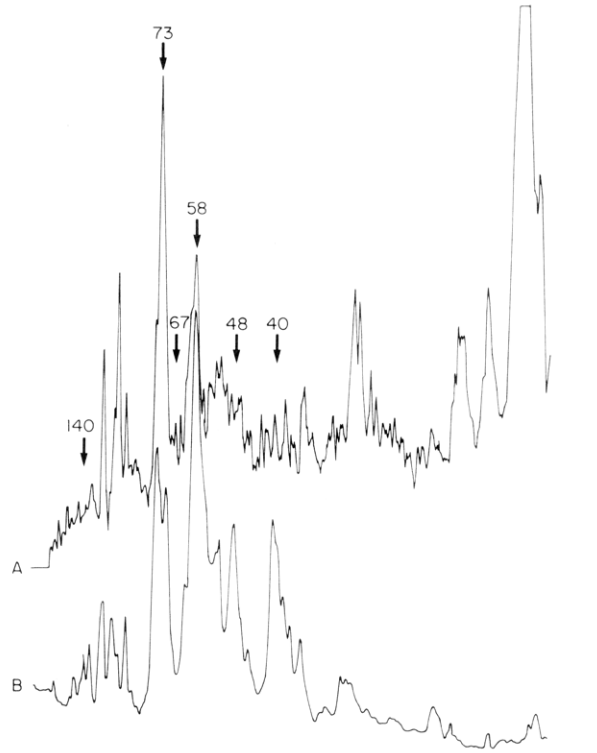

FIGURE 6: Densitometric tracings of $NaDodSO_4$–polyacrylamide gels of proteins from *L. pictus* poly(A)-containing mRNPs. (A) Embryo polyribosomal 45 °C EB-eluted fraction of the oligo(dT)–cellulose column and (B) egg 45 °C EB-eluted fraction of Figure 4, lane A. mRNPs were prepared and electrophoresed as in Figure 4. The origin was at the left. Proteins discussed in the text are designated as molecular weights ($\times 10^{-3}$).

Control experiments showed that no proteins were retained on unmodified cellulose (data not shown), and incomplete hybridization of the poly(A)-containing mRNPs to poly(U) does not explain the retention of polypeptides on oligo(dT)–cellulose since the concentration of poly(U) used was calculated to be in excess. Some polypeptides may, however, bind directly to oligo(dT) and not be part of the mRNP complexes or may bind to oligo(dT) in preference to mRNPs. Similar binding of proteins to poly(U)–Sepharose has been observed by Ovchinnikov et al. (1978), who showed that these proteins normally have a high affinity for RNA. Finally, binding mRNPs to oligo(dT)–cellulose for 1.5 h without physical mixing did not alter the pattern of mRNP proteins (data not shown), suggesting that there were no changes in the proteins binding to the mRNA during isolation on oligo(dT)–cellulose.

The major mRNP proteins of *S. purpuratus* eggs were similar in number and molecular weight to those of *L. pictus* (Figure 5). When a buffer without EDTA and containing Mg^{2+} was used to remove unbound material from the oligo(dT)–cellulose column, *S. purpuratus* mRNP fractions contained many low molecular weight proteins (Figure 5). Similar results were found for *L. pictus* mRNP fractions chromatographed under these conditions. These presumably ribosomal proteins were not removed from the oligo(dT)–cellulose column by washing with PIB-67 containing 500 mM K^+ with 360 mM Cl^-, nor were any mRNP proteins removed (data not shown).

When the polypeptides of oligo(dT)–cellulose purified mRNP fractions from eggs were compared with similarly purified mRNP fractions from blastula polyribosomes by polyacrylamide gel electrophoresis (Figures 4 and 6), we found that about half of the polypeptides were in common and half of the polypeptides were missing in the polyribosome-derived mRNPs. The purified poly(A)-containing polyribosomal mRNPs, in addition, had many proteins with molecular weights of less than 20 000, suggesting that ribosomal proteins were not completely dissociated from polyribosomal mRNPs.

Of the 20 major protein bands from the purified poly(A)-containing mRNP complexes of the *L. pictus* eggs, those with molecular weights of 34 000, 40 000, 42 000, 48 000, 67 000, and 140 000 were not detected or were present in only low concentrations in the purified mRNP fractions from blastula-stage polyribosomes [Figure 6A and Figure 4 (lane D)]. Polypeptides with molecular weights of 34 000, ~40 000, and 42 000 were, however, present in intact polyribosomes (Figure 4, lane C). Whether these are the same polypeptides as those in egg mRNPs, and the significance of their appearance in intact polyribosomes but not in purified mRNPs of polyribosomes, has not yet been established. Three major egg mRNP polypeptides (48 000, 67 000 and 140 000 daltons), however, were absent from both purified polyribosomal poly(A)-containing mRNPs and intact polyribosomes and shown to be associated with poly(A)-containing egg mRNPs by competition with poly(U). In addition, stoichiometric differences exist between the polypeptides associated with free egg mRNPs and polyribosomal mRNPs. For instance, both poly(A)-containing polyribosomal mRNPs [Figure 6A and Figure 4 (lane D)] and intact polyribosomes of *L. pictus* (Figure 4, lane C) have fewer polypeptides banding at 58 000 daltons relative to the 69 000–73 000-dalton bands than do poly(A)-containing egg mRNPs [Figure 4 (lanes A and E) and Figure 6B]. A detailed comparison of the proteins associated with free and polyribosomal poly(A)-containing mRNPs of *S. purpuratus* eggs and embryos, currently in progress, confirms that fewer proteins are associated with the

polyribosomal mRNPs.

Discussion

As a first step toward analyzing how sea urchin egg mRNP proteins regulate the translation of mRNA, we have demonstrated that the recovery of free poly(A)-containing mRNPs from sea urchin eggs can be increased several-fold by the inclusion of PO_4^{2-} in the isolation buffers. Additionally, we have shown that almost all of the poly(A)-containing mRNPs present in an unpurified mRNP fraction isolated under physiological conditions from eggs can be purified from free monoribosomes and subunits by chromatography on oligo(dT)–cellulose. Finally, we have shown that three proteins associated with free poly(A)-containing mRNPs from eggs are different from those of polyribosomal poly(A)-containing mRNPs from embryos.

This is the first analysis of the proteins associated with free poly(A)-containing mRNPs in which physiological ionic conditions were used throughout isolation. Caution regarding the use of nonphysiological ionic conditions in the analysis of mRNPs is justified for the following reasons: (1) the derepression of sea urchin egg mRNPs is temporarily linked to the subtle ionic changes accompanying fertilization (Epel, 1978) and (2) we would not expect potential regulatory proteins of mRNPs to be as tightly associated with mRNPs as core proteins. Therefore, we have not used high salt concentrations as a criterion for identifying mRNP proteins.

The association of the described mRNP proteins with poly(A)-containing mRNA is suggested by (1) the coelution from oligo(dT)–cellulose of the majority of these proteins with most of the poly(A)-containing RNA, (2) the differences in molecular weight between most of the mRNP proteins and the proteins remaining in the unbound fraction of the oligo(dT)–cellulose column, (3) the retention of the mRNP proteins on oligo(dT)–cellulose when either Mg^{2+} or EDTA was used in the buffer to remove unbound material, and (4) the greatly reduced retention of these mRNP proteins on oligo(dT)–cellulose after preincubation of the mRNPs with saturating levels of poly(U). The mRNP nature of the poly(A)-containing mRNA is further suggested by the mRNP-characteristic buoyant densities of these particles in metrizamide and Cs_2SO_4.

At least 16 major and 15 minor proteins were associated with sea urchin egg poly(A)-containing mRNPs, by using the criterion of blocked retention on oligo(dT)–cellulose after pretreatment of the mRNPs with poly(U). Huynh-Van-Tan & Schapira (1978) have also reported up to 30 proteins ranging in molecular weight from 22 000 to greater than 100 000, and the absence of the 78 000-dalton poly(A)-binding polypeptide, in free mRNPs from rabbit reticulocytes purfied by zonal centrifugation using physiological ionic conditions. The mRNP proteins in their study have been grouped roughly into three molecular weight classes of 22 000–40 000, 40 000–70 000, and greater than 70 000. Similar groupings are seen in our gel patterns from an evolutionary divergent species.

All major sea urchin egg mRNP proteins had molecular weights in the 16 000–150 000 range, typical of proteins from other mRNPs [reviewed by Jain & Sarkar (1979)]. Nineteen of the major proteins from *L. pictus* purified mRNPs which were isolated with EDTA-containing buffers had molecular weights greater than 34 000 and, hence, conform to the stringent conditions set by Jain & Sarkar (1979) for purified mRNP particle proteins. The protein patterns from *L. pictus* and *S. purpuratus* egg mRNPs which were isolated in the presence of Mg^{2+} included many proteins in the 15 000–30 000-dalton range. In our hands, Mg^{2+} depletion of the preparations was, therefore, essential for complete removal of contaminating ribosomal proteins from mRNPs.

A 52 000-dalton protein, common to mRNPs from different sources [reviewed by Greenberg (1975)], was present in both *L. pictus* and *S. purpuratus* poly(A)-containing egg mRNPs. However, a 78 000-dalton protein, bound specifically to the 3′-poly(A) region of mRNAs in many other species [reviewed by Jain & Sarkar (1979)], was only observed in gels of *S. purpuratus* material. This poly(A)-binding protein may be present, however, as a 73 000-dalton protein in *L. pictus* material since the 78 000-dalton protein is also absent in *L. pictus* polyribosome-derived mRNPs, where it is typically found.

Although the localization of the egg mRNP proteins on the mRNA has yet to be established, Peters & Jeffery (1978) previously analyzed the poly(A) region of *L. pictus* egg mRNPs by oligo(dT)–cellulose chromatography of a RNase A digested postmitochondrial supernatant. Proteins with molecular weights of 67 000, 76 000, 87 000, and 130 000 were retained on the oligo(dT)–cellulose. These proteins roughly correspond to the *L. pictus* major mRNP proteins of 67 000, 73 000, 94 000, and 140 000 daltons found in our study. Some of the egg mRNP proteins within the molecular weight range of 52 000–58 000 may also be associated with the poly(A) region of the mRNA, since a protein of 56 000 daltons binds the poly(A) region of mRNPs from Ehrlich ascites tumor cells (Jeffery, 1977). One of the three major protein bands which we found to be specific to poly(A)-containing mRNPs of *L. pictus* eggs, the 48 000-dalton band, does not correspond in size to the known poly(A)-binding proteins of Peters & Jeffery (1978), suggesting that it is localized elsewhere on the mRNA.

Comparing mRNPs from chick embryonic muscle, Jain & Sarkar (1979) detected 6 proteins associated with polyribosomal poly(A)-containing mRNPs and ~10 proteins associated with free poly(A)-containing mRNPs. The proteins which were specific to free mRNPs had molecular weights of 44 000, 49 000, 58 000, and 85 000. The 48 000-dalton protein which we found to be specific to free mRNPs of sea urchin eggs may correspond to their 49 000-dalton protein. Unlike Jain and Sarkar, we detect a major 58 000-dalton band in both the free and polyribosome-derived poly(A)-containing mRNPs from *L. pictus* eggs and no 44 000- or 85 000-dalton bands. The 40 000-dalton polypeptide which we found in purified mRNPs from *L. pictus* eggs may, however, be specific to free mRNPs and correspond to Jain and Sarkar's 44 000-dalton polypeptide. Proteins with molecular weights of 67 000 and 140 000 were dissimilar in molecular weight to the free mRNP specific proteins of Jain and Sarkar, yet they were specific to the free mRNPs of *L. pictus* eggs.

Young & Raff (1979) have recently shown that newly synthesized poly(A)-containing mRNAs in early blastula stage embryos of sea urchins, present in either free or polyribosomal mRNPs, form protein-deficient mRNPs consisting of only 10% protein, as determined by their density of about 1.61 g/cm^3 in Cs_2SO_4. In contrast to newly synthesized mRNPs, the oogenetic mRNPs had densities in Cs_2SO_4 of 1.45 g/cm^3 (Young & Raff, 1979) when present either as free mRNPs or as polyribosomal mRNPs and, hence, contained ~50% protein, even when being translated. One of their conclusions is that the postfertilization increase in the template activity of stored mRNPs is not attributable to a significant deproteinization of the stored mRNPs. However, these results have not precluded the possibility that the loss of some protein through limited proteolysis or other means accompanies the activation of stored message. Although we have not yet dis-

tinguished between the proteins present on newly synthesized messages and those present on maternal messages, we have demonstrated that several proteins are present in free poly(A)-containing mRNPs of eggs which are not detected in polyribosomal poly(A)-containing mRNPs of embryos. The next step is to determine if any of these egg mRNP specific proteins are lost from maternal messages following fertilization and whether they suppress the template activity of the mRNP.

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