Isolation and Characterization of Rat Liver Free and Membrane-Bound Polysomal Messenger Ribonucleoprotein Particles†

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ABSTRACT: Rat liver mRNA-labeled free and membrane-bound polysomes uncontaminated with nuclear or cytoplasmic ribonucleoprotein particles were dissociated with EDTA and the released messenger ribonucleoprotein particles isolated using an oligo(dT)-cellulose column. Seventy percent of the labeled mRNP applied bound to the column. Binding of mRNP was dependent on the presence of a poly(A) segment in the RNA. The nonbound material contained most of the ribosomal subunits and also messenger-sized poly(A^-) RNA molecules associated with protein. The free mRNP fraction bound to the column, washed with 250 mM NaCl, and subsequently eluted contained seven proteins ranging in molecular weight from 52 000 to 138 000 only one of which was found in the fraction not bound to the column. Furthermore, these proteins were shown to have a higher affinity for poly(A^+) RNA as compared with rRNA. The membrane-bound mRNP contained five proteins, four of which were identical with those associated with free mRNP. Membrane-bound mRNP were disrupted at a lower salt concentration than the free. The proteins found associated with free and membrane-bound polysomal mRNP appeared to be clustered in the poly(A) region of the molecule. The implications of these findings are discussed.

Eukaryotic polysomal mRNA appears to be associated with specific proteins. Although it is probable that this interaction is not initiated by cell disruption, the function of these proteins remains unknown (Lewin, 1974). Two proteins are consistently observed to be associated with mRNA in a wide variety of cell types. Usually a 49 000–52 000 and a 72 000–78 000 molecular weight protein are found as major components, although a number of minor high molecular weight proteins have also been observed frequently (Blobel, 1973; Morel et al., 1973; Bryan and Hayashi, 1973). The 78 000 molecular weight protein has been shown to interact with the poly(A)[1] region of mRNA (Blobel, 1973; Kish and Pederson, 1976) and a role in the transport of these RNAs from the nucleus to the cytoplasm has been postulated (Schwartz and Darnell, 1976). The 52 000 molecular weight protein appears to have properties similar to those of the initiation factor IF-MP (Hellerman and Shafritz, 1975).

Recent evidence has suggested the existence of a direct physical interaction between mRNA and intracellular membranes (Cardelli et al., 1976; Milcarek and Penman, 1974; Lande et al., 1975). The nature and role of this interaction remain uncertain but could include stabilization of mRNA templates, segregation of mRNA into free and bound polysome classes and/or provision of a "link" between the nucleus and cytoplasm during mRNA transport from the former (Faiferman et al., 1971). Although a good deal of work has been done with free polysomal mRNP, little data exist concerning membrane-bound polysomal mRNP. Since proteins are likely to be involved in the binding of mRNA to RER (rough endoplasmic reticulum) membranes and most mRNA appears to be in the form of a ribonucleoprotein complex, we undertook the studies described herein to compare membrane-bound polysomal mRNP with free polysomal mRNP.

Materials and Methods

Animals, Chemicals, and Labeling. Male Holtzman rats weighing approximately 300 g were given food and water ad libitum. These animals were used in all experiments. Buffers used were as follows. STKM: sucrose molarity as indicated, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4. HSB (high salt buffer): 10 mM Tris-HCl or triethanolamine, 10 mM EDTA, 250 mM NaCl, pH 7.5. ELI: HSB made in 25% formamide. ELIII: HSB made in 50% formamide containing 500 mM NaCl. Sample buffer: 10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, 62 mM Tris-HCl.

CsCl and sucrose were purchased from Schwarz/Mann Division of Becton; [^{3}H]poly(U) was obtained from Miles Laboratories; [^{3}H]orotate was from Amersham Co.; poly(U)-Sepharose was from P-L Biochemicals; oligo(dT)-cellulose type T2 was from Collaborative Research; gel electrophoresis media were from Bio-Rad. Specific labeling of messenger and ribosomal RNA was accomplished as described previously (Cardelli et al., 1976).

Preparation of Polysomal mRNP. Bound and free polysomes were prepared as follows. An S2 (supernatant from rat liver homogenate centrifuged at 12 000 rpm for 10 min) preparation was fractionated on a discontinuous sucrose gradient as described previously (Cardelli et al., 1976) into a free polysome pellet, RER (rough endoplasmic reticulum) membrane band, and SER (smooth endoplasmic reticulum) membrane band. The free polysome pellet was rinsed with H_2O and frozen at −70 °C. RER membranes were collected and

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[1] Abbreviations used: STKM, sucrose molarity as indicated, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4; EDTA, ethylenediaminetetraacetic acid; HSB, 10 mM Tris-HCl or triethanolamine, 10 mM EDTA, 250 mM NaCl, pH 7.5; Tris, tris(hydroxymethyl)aminomethane; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; RNP, ribonucleoprotein; ELI, HSB made in 25% formamide; ELIII, HSB made in 50% formamide containing 500 mM NaCl; NETS buffer, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.2% sodium dodecyl sulfate; sample buffer, 10% glycerol, 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, 62 mM Tris-HCl; DOC, deoxycholic acid.

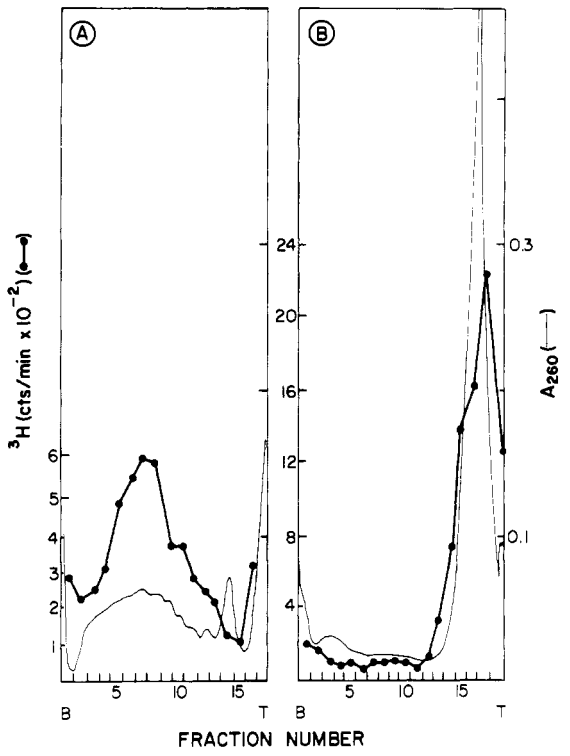

FIGURE 1: Sucrose density gradient centrifugation of mRNA-labeled free polysomes. Free polysomes labeled and isolated as described in Materials and Methods were resuspended in TKM to a concentration of 27.6 A_{260}/mL. An aliquot was adjusted to 15 mM EDTA and 11 A_{260} units fractionated on 10–40% STKM gradients (SW41, 40 000 rpm, 40 min, 4 °C). (A) Polysomes minus EDTA or (B) plus EDTA.

diluted once with TKM (50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4). Heparin was added giving a final concentration of 100 μg/mL followed by 0.1 volume of either 15% DOC (deoxycholic acid) (w/v) or 10% Triton X-100 (w/v). After incubation at 4 °C for 30 min the detergent-treated preparation was layered onto 4 mL of 2.0 M STKM (TKM in 2.0 M sucrose) containing 100 μg/mL heparin. The tubes were capped and centrifuged for 20 h at 45 000 rpm in a 50 Ti rotor at 4 °C. Prior to use the resuspended membrane-bound polysomes were centrifuged at 10 000 rpm for 10 min in a SS-34 rotor in order to remove particulate matter. Control experiments revealed that our bound polysomes were essentially uncontaminated with free polysomes.

For the preparation of mRNP, polysome pellets were resuspended at 4 °C in HSB to a concentration of approximately 30 A_{260}/mL, 0.7 mL of this suspension was applied to a 50 mm × 9 mm oligo(dT)-cellulose type T2 column equilibrated with HSB, the columns were washed with HSB, and bound mRNP eluted at 25 °C with ELI buffer followed by ELIII buffer.

Analysis of Polysomes and mRNP. Aliquots of mRNP in ELI buffer and nonbound mRNP in HSB were analyzed on 15–30% linear sucrose gradients containing 50 mM Tris-HCl (pH 7.5), 70 mM KCl, 10 mM EDTA. Samples were centrifuged at 40 000 rpm in an SW41 rotor for 5 h at 4 °C. Polysomes suspended in TKM buffer were fractionated on 10–40% sucrose gradients made up in TKM. Centrifugation was for 60 min at 4 °C in an SW41 rotor at 40 000 rpm. For analysis using CsCl gradients, unfractionated polysomes (+ or − EDTA) and isolated bound and nonbound column fractions were fixed in 4% formaldehyde (pH 7.4) for the indicated times, layered onto pre-formed CsCl gradients (1.30 to 1.74 g/cm^3), and centrifuged at 50 000 rpm in an SW56 rotor for 20 h at 20 °C. Occasionally the column bound ELI buffer-eluted mRNP were dialyzed against 10 mM triethanolamine (pH 7.4), 100 mM NaCl, 5 mM EDTA for 5 h to remove formamide prior to fixation and subsequent examination on gradients. Gradients were fractionated and analyzed for A_{260} absorbing material and radioactivity as previously described (Cardelli et al., 1976). Densities were determined by weighing 20-μL samples obtained with micropipets.

Poly(U)-Sepharose Fractionation of Isolated Polysomal RNA. Polysomes were phenol-$CHCl_3$ extracted as previously described (Cardelli et al., 1976). The RNA was precipitated with ethanol after which the pellet was resuspended in NETS buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.2% sodium dodecyl sulfate) and applied to a poly(U)-Sepharose column equilibrated with the same buffer. After washing with NETS buffer, the column was washed with a solution of 100 mM NaCl, 10 mM Tris-HCl until the A_{260} of the wash was zero and the poly(A^+) RNA eluted in a small volume of double-distilled H_2O.

Polyacrylamide Gel Electrophoresis. Pooled fractions from the oligo(dT)-cellulose column were precipitated overnight with 2 vol of 95% ethanol, pelleted, and resuspended in a small volume of sample buffer. After heating to 70 °C for 15 min, 1 drop of bromophenol blue was added and various volumes, 25–200 μL, were gently applied to the gels cast in silane-treated glass tubes (110 mm long, 6 mm i.d.). The lower gel consisted of 0.1% sodium dodecyl sulfate, 8.75% acrylamide, 0.23% methylenebisacrylamide in 0.375 M Tris (pH 8.8). The upper stacking gel consisted of 3% acrylamide, 0.08% methylene bisacrylamide.

The reservoir buffer contained per liter: 3 g of Tris-HCl, 14.4 g of glycine, and 1 g of sodium dodecyl sulfate. Samples were electrophoresed at 1 mA/gel until the tracking dye reached the lower gel and then 2 mA until the dye band had migrated to 1 cm from the bottom of the tube. After electrophoresis, gels were removed from the tubes, stained for 4 h in 0.05% Coomassie brilliant blue in methanol-acetic acid-H_2O (5:1:5) and destained in 7.5% acetic acid. Gels were scanned at 550 nm using a Gilford linear transport and recording spectrophotometer.

Calculation of the Number of P73 Molecules. A density value of 1.61 g/cm^3 represents an RNA:protein ratio of about 2:1. Assuming an average molecular weight of 500 000 for liver free polysomal mRNA, this gives a value of about 250 000 daltons of protein or about three P73 protein molecules per mRNA.

Results

Lack of Nuclear and Cytoplasmic RNP Contamination in the Polysome Preparation. Since the polysomes used in the isolation of mRNP were recovered as pellets after sedimentation through 2.0 M sucrose, it was necessary to eliminate the possibility of significant nuclear or cytoplasmic RNP contamination. Functional mRNA sediments with polysomes and can be released as a slower sedimenting mRNP in the presence of EDTA or KCl and puromycin. Contaminating cytoplasmic mRNP would be found present in the 20–80S region of a sucrose gradient in the absence of EDTA. Free polysomes were isolated as described in Materials and Methods from livers of rats given a combination of [^{3}H]OA and FOA (mRNA label), resuspended, and fractionated on 10–40% sucrose gradients. Figure 1A reveals that greater than 90% of the label is found in the polysome region (greater than 80 S) indicating the absence from this preparation of a significant amount of cytoplasmic mRNP. To rule out contamination by nuclear RNP, resuspended polysomes were disrupted with EDTA prior to sucrose gradient centrifugation. Nuclear RNP are insensitive

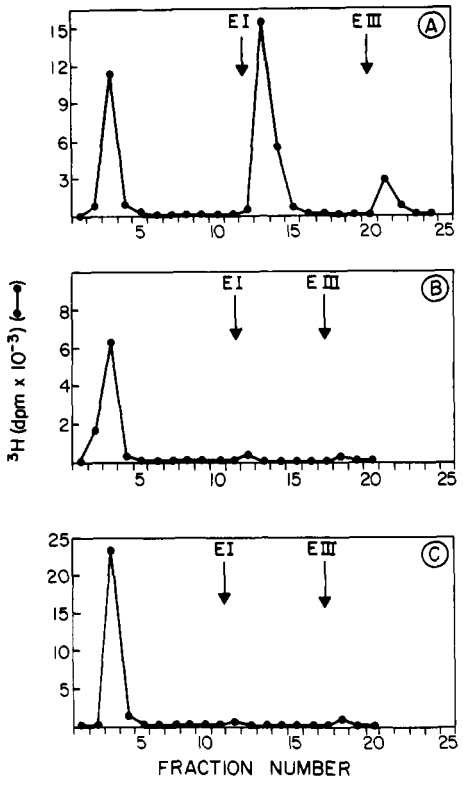

FIGURE 2: Oligo(dT)-cellulose chromatography of EDTA-dissociated free polysomes. Messenger RNA labeled free polysomes resuspended in HSB were either directly applied to an oligo(dT)-cellulose column [(A) 18 A_{260} units] or hybridized with 51 μg of poly(U) for 3.5 h prior to application [(C) 12 A_{260} units]. Columns were washed with 10 mL of HSB, followed by 5 mL of ELI then 5 mL of ELIII buffer. The peak A_{260} fraction in the flow-through of the sample untreated with poly(U) was reapplied to another equilibrated column (B) and processed similar to A and C. Fractions collected from the column were counted in 10 mL of Scintisol.

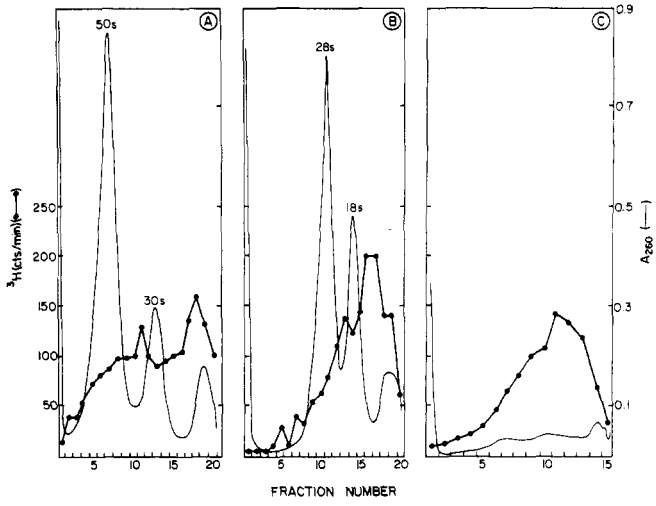

FIGURE 3: Sucrose density gradient centrifugation of mRNP fractions collected from an oligo(dT)-cellulose column. Twenty-two A_{260} units of mRNA-labeled free polysomes in HSB was fractionated on an oligo(dT)-cellulose column into a nonbound and ELI buffer eluted fraction as described in Figure 2. Aliquots of each fraction were either layered directly onto 15–30% sucrose linear gradients or first treated with 1% sodium dodecyl sulfate (final concentration) in the presence of 500 μg/mL heparin and 1 mg/mL bentonite before layering. Samples were centrifuged at 40 000 rpm in an SW41 rotor for 5 h at 4 °C. (A) Nonbound fraction; (B) nonbound fraction, sodium dodecyl sulfate treated; (C) column-bound fraction.

to EDTA (Penman, et al., 1968) and any contaminating nuclear particles would continue to sediment to the region vacated by the polysomes. Most of the label appears to shift to the subunit region after EDTA treatment (Figure 1B) suggesting that nuclear RNP contaminants are absent. mRNA-labeled membrane-bound polysomes also appeared uncontaminated with nuclear and cytoplasmic mRNP when subjected to the above experiments (results not shown). CsCl gradients of formaldehyde-fixed mRNA-labeled polysomes also showed the absence of the characteristic nuclear RNP peak of density 1.40 g/cm^3 and the coincidence of the labeled mRNA with the ribosomal A_{260} peak (results not shown).

Fractionation of mRNA-Labeled, EDTA-Disrupted Free and Bound Polysomes on Oligo(dT)-Cellulose Columns. Messenger RNA-labeled free and bound polysomes were resuspended in HSB and applied to an oligo(dT)-cellulose column equilibrated with the same buffer. After washing the column extensively with HSB, the bound labeled mRNP were eluted in two peaks first with ELI buffer followed by ELIII buffer. As shown in Figure 2A approximately 70% of the applied counts are found in the two eluted peaks (67.4 ± 2.1 for six trials). Although the percentage of applied counts that bound to the column in HSB remained constant, the relative distribution between the ELI and ELIII peaks varied from 2:1 to 4:1. A similar percentage of labeled membrane bound polysomal mRNP bound to these columns (results not shown).

Little of the labeled nonbound material rebinds on a second passage through the column (Figure 2B). If EDTA-dissociated polysomes are incubated with poly(U) prior to column fractionation little labeled mRNP binds suggesting that poly(A) is necessary for binding (Figure 2C). Furthermore, formamide in low salt buffer which releases column bound poly(A$^+$) RNA releases only a small amount of the oligo(dT)-cellulose bound labeled mRNP (data not shown) suggesting that binding is not just mediated by a poly(A):oligo(dT) hybrid. As reported by others (Lindberg and Sundquist, 1974), it appears that the mRNP proteins might be playing a role in binding in addition to that of the poly(A) segment.

Physical Nature of the Oligo(dT)-Cellulose Nonbound and Bound Labeled Fractions. The size distributions of the labeled RNA in the nonbound and bound fractions were determined by centrifugation of aliquots on 15–30% sucrose gradients. It appears that greater than 95% of the applied ribosomal subunits are found in the fraction not bound to the column (compare Figure 3A with 3C). The nonbound fraction consists of labeled RNA which sediments heterogeneously with a peak value in the region of 35 S (Figure 3A). Sodium dodecyl sulfate treatment prior to centrifugation of the nonbound fraction shifts the labeled material to a lower S region, near 14 S (Figure 3B) suggesting that the labeled material not binding to the column consists of mRNA in association with proteins. This possibility is further supported by CsCl gradient analysis of the nonbound fraction after formaldehyde fixation. A labeled peak with a density of 1.55 g/cm^3 was found after fractionation of the gradients. This density coincides with an RNA:protein ratio of about 1 (Figure 4).

We next examined whether the labeled messenger RNA fraction that did not bind to the column lacked poly(A) to rule out the possibility of this RNA segment being "buried" in the mRNP complex and thus inaccessible for binding. Unlabeled free polysomes were dissociated with EDTA and fractionated on an oligo(dT)-cellulose column as described above. Fractions not binding to the column as well as those eluted by ELI and ELIII buffers were ethanol precipitated, deproteinized, and analyzed for poly(A) sequences. Only 10% of the poly(A) sequences are found in the nonbound fraction (Figure 5A), while up to 35% of the labeled mRNP are found there (Figure 5B).

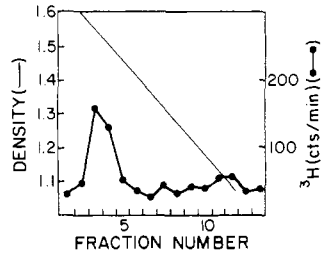

FIGURE 4: CsCl gradient of mRNA-labeled oligo(dT)-cellulose nonbound fractions. Approximately 4 A_{260} units from the fraction of mRNA-labeled EDTA-dissociated polysomes that did not bind to an oligo(dT)-cellulose column was fixed for 20 h in 4% formaldehyde and then centrifuged on linear CsCl gradients (SW56, 50 000 rpm, 20 °C, 20 h). Fractions were collected, weighed to determine density, and counted after the addition of Scintisol.

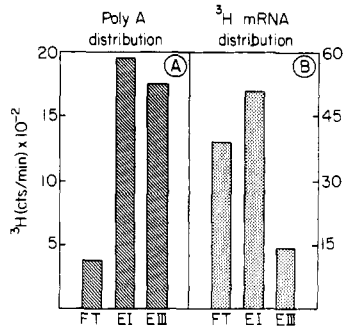

FIGURE 5: The retention of poly(A)-containing mRNP by oligo(dT)-cellulose columns. Unlabeled polysomes resuspended in HSB were fractionated on an oligo(dT)-cellulose column into nonbound and ELI, ELIII eluent fractions as described in Figure 3. After the addition of 500 μg of yeast RNA, samples were ethanol precipitated, washed, and resuspended in 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA with 1 mg/mL bentonite. Samples were analyzed for poly(A) sequences as described previously (Cardelli et al., 1976) after the removal of bentonite. (A) Distribution of poly(A) sequences; (B) distribution of labeled mRNP (see Figure 2).

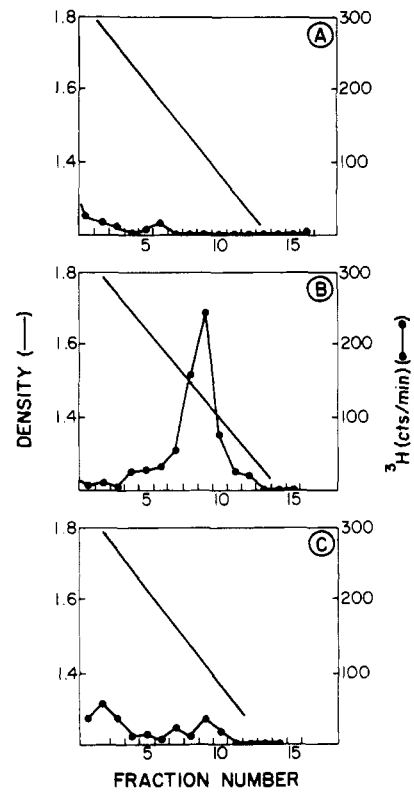

FIGURE 6: CsCl gradient centrifugation of the ELI buffer eluted labeled mRNP fraction. The labeled mRNP fraction recovered from the oligo(dT)-cellulose column with ELI buffer was divided into three equal volumes. One volume was made up to 4% formaldehyde, and the other two dialyzed against 1 L of 10 mM triethanolamine (pH 7.4), 5 mM EDTA, 100 mM NaCl for 3 h. To one of the tubes prior to dialysis was added 5 A_{260} units of phenol-$CHCl_3$ extracted unlabeled polysomal RNA. After dialysis samples were fixed with formaldehyde and fractionated on CsCl gradients as described in Figure 5. (A) Undialyzed mRNP; (B) dialyzed mRNP; (C) dialyzed mRNP in presence of extracted unlabeled polysomal RNA.

Since 10% of the nonbound fraction can rebind (presumably due to poly(A) tails) this suggests that a large percentage of the nonbound labeled mRNP either lack poly(A) segments or their poly(A) tails are too short to form a stable hybrid with column. We favor the former possiblity as described in the Discussion.

The distribution on sucrose gradients of the peak of labeled free mRNP eluted by ELI buffer is shown in Figure 3C. The counts are distributed heterogeneously throughout the gradient with little evidence of ribosomal contamination. Membrane-bound polysomal mRNP showed a similar distribution (results not shown). Sodium dodecyl sulfate treatment prior to centrifugation did not alter the distribution of label suggesting that little protein was associated with this mRNP fraction. Sodium dodecyl sulfate did have an effect on the sedimentation of these particles if they were dialyzed prior to fractionation on gradients (data not shown). This suggested that, when the formamide concentration was lowered by dialysis, the proteins originally bound to the mRNP reattached. If the proteins rebind to the labeled mRNP during dialysis, one might be able to follow this process using CsCl gradients. Figure 6A reveals that labeled mRNP eluted by ELI buffer appear to pellet after formaldehyde fixation and centrifugation in CsCl gradients suggesting the absence of any mRNA-protein interaction. If the particles are dialyzed prior to the above treatment, then a prominent peak of label appears around 1.45 g/cm^3 (Figure 6B) in agreement with values for the density of mRNP determined by others (Kumar and Pederson, 1975; Irwin et al., 1975). The following experiment was performed in order to rule out the unlikely possibility that labeled mRNP fractionated on CsCl gradients lack associated proteins because formamide interferes with the cross-linking by formaldehyde of the protein and mRNA in the mRNP, the non-cross-linked complexes then being dissociated by the high concentration of CsCl. If formamide actually disrupts the mRNP, then an excess of unlabeled phenol-$CHCl_3$ extracted RNA added prior to dialysis should compete with the labeled mRNA for these rebinding proteins and prevent the appearance of the 1.45 g/cm^3 peak. This appears to be the case as shown by Figure 6C. This experiment does not reveal which RNA species is competing, i.e., mRNA, rRNA, etc. If the proteins, which interact with mRNA, function in some aspect of its metabolism and do not simply have an affinity for RNA in general, then the affinity of the proteins for mRNA should be greater than for rRNA. In order to investigate this question, unlabeled extracted free polysomal RNA was fractionated into poly(A^+) and poly(A^-) class on a poly(U)-Sepharose column. Equal amounts of the unlabeled poly(A^+) RNA (mRNA) and poly(A^-) RNA (rRNA) were added separately to aliquots of labeled ELI buffer eluted mRNP and samples were processed as described in Figure 6. It appears that little protein rebinds to the labeled mRNA in the presence of unlabeled poly(A^+) RNA (Figure 7C), while poly(A^-) RNA does not effectively compete for this protein (compare Figure 7D with 7B). Doubling the amount of unlabeled poly(A^-) RNA added still does not inhibit rebinding of the proteins to the labeled mRNA

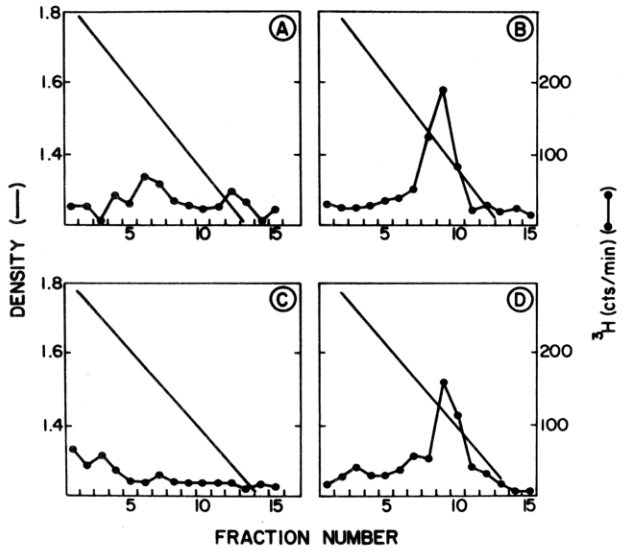

FIGURE 7: CsCl gradients of mRNA-labeled ELI buffer eluted fractions dialyzed in the presence of unlabeled poly(A^+) and poly(A^-) RNA. A mRNA-labeled ELI buffer eluted fraction (see Figure 7) was divided into four aliquots. One sample was fixed with formaldehyde without dialysis (A), while the other three were dialyzed prior to fixation. One microgram of unlabeled poly(A^+) and poly(A^-) extracted RNA was added to two of the later three samples prior to dialysis. Following formaldehyde fixation samples were centrifuged on CsCl gradients as described in Figure 7. (A) Undialyzed; (B) dialyzed; (C) dialyzed in the presence of poly(A^+) RNA; (D) dialyzed in the presence of poly(A^-) RNA.

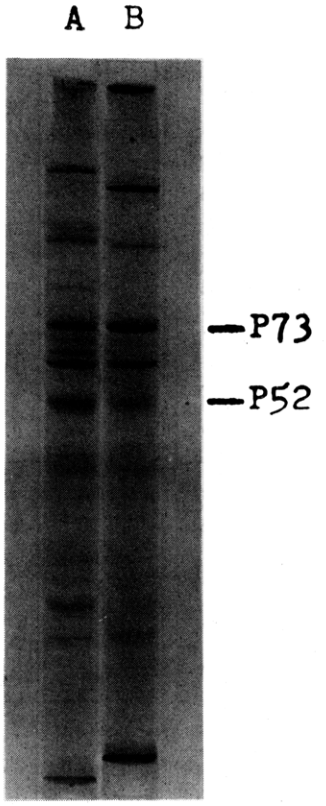

FIGURE 8: Gel electrophoresis of free and membrane-bound oligo(dT)-cellulose isolated polysomal mRNP. Free and membrane-bound polysomal mRNP eluted from columns with ELI buffer were precipitated overnight with ethanol. Pellets were dissolved in sample buffer and 50-μL aliquots subjected to polyacrylamide gel electrophoresis as described under Materials and Methods. (A) Free polysomal mRNP proteins; (B) membrane-bound polysomal mRNP proteins.

TABLE I: Molecular Weights of mRNP Proteins.[a]

Free mRNP		Bound mRNP	
Protein	Mol wt	Protein	Mol wt
*P138	138 000	*P134	134 000
P109	109 000		
P104	103 700	P104	103 700
*P73	73 000	*P73	73 000
P66	65 700		
*P62	62 000	P62	62 000
*P52	52 300	P52	52 300

[a] Proteins preceded by asterisks are major bands on gels.

(data not shown). These data are in agreement with other recent reports (Barrieux et al., 1976) and are consistent with the idea that these proteins function in mRNA metabolism and recognize sequences found in mRNA but not in rRNA.

Protein Components of Oligo(dT)-Cellulose Isolated Free and Membrane-Bound Ribonucleoprotein Particles. Free and membrane-bound polysomes were dissociated with EDTA and fractionated on columns as described in Figure 1. ELI fractions 12–15 were pooled, ethanol-precipitated, dissolved in sample buffer, and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels. Figure 8 presents a photograph of the resultant gels after staining with Coomassie brilliant blue. The proteins in the nonbound fraction consist predominantly of lower molecular weight ribosomal proteins (data not shown). Four major and three minor proteins are found in the free mRNP fraction bound to oligo(dT)-cellulose having molecular weights ranging from 52 000–138 000 (Table I). Only the 52 000 molecular weight protein band is seen in the nonbound fraction (data not shown). Five proteins are present in the column-isolated membrane-bound polysomal mRNP, four of which have molecular weights identical with four proteins in free mRNP. Two minor proteins, P66 and P109, present in free mRNP were absent from membrane-bound mRNP. The relative intensities of the membrane-bound mRNP protein bands on the gel differed from that of the free (Figures 8, 10A, 10E). The 73 000 and 134 000 molecular weight proteins were much more prominent relative to the 52 000 molecular weight species. The differences reported above are not due to degradation of the membrane-bound mRNP prior to column fractionation or to the use of Triton X-100 in preparing membrane-bound polysomes. The messenger-labeled RNA extracted from membrane-bound polysomes had a peak *s* value on sucrose gradients (18 S) similar to that of free mRNA. Washing of free polysomes with Triton X-100 did not remove any of the seven mRNA-associated proteins (unpublished results). The following experiment was done to rule out the possibility that these proteins were merely copurifying as opposed to interacting with the mRNA. EDTA-dissociated polysomes were fractionated on oligo(dT)-cellulose columns in the presence of excess poly(U). Under these conditions little labeled poly(A)-containing mRNP binds (Figure 1C). Electrophoresis of the fraction bound under these conditions revealed an almost complete absence of the seven proteins described above for free mRNP supporting the idea that these proteins observed in Figure 8 are actually bound to mRNA (data not shown).

Salt Sensitivity of Free and Membrane-Bound Polysomal mRNP. We decided to examine the effect of an increase in salt concentration on protein–mRNA interactions in both free and membrane-bound polysomal mRNP. Isolated free and membrane-bound polysomes (mRNA labeled) were resuspended in 50 mM triethanolamine, 10 mM EDTA, divided into aliquots, and adjusted to various NaCl concentrations. After

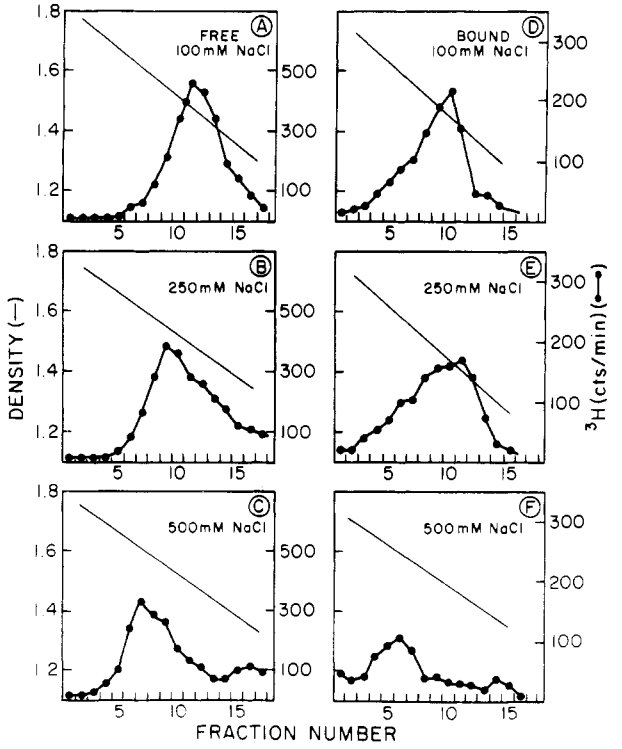

FIGURE 9: CsCl gradients of polysomal mRNP exposed to various ionic strength buffers. Free and membrane-bound mRNA-labeled polysomes were resuspended in 50 mM triethanolamine, 100 mM NaCl, 10 mM EDTA and each divided into 3 equal volumes. NaCl was added bringing the final salt concentration to either 250 or 500 mM. After incubating for 45 min at 4 °C, samples were fixed with formaldehyde and centrifuged on CsCl gradients (SW56, 50 000 rpm, 20 h, 20 °C). Fractionation and scintillation counting was accomplished as described in Figure 4.

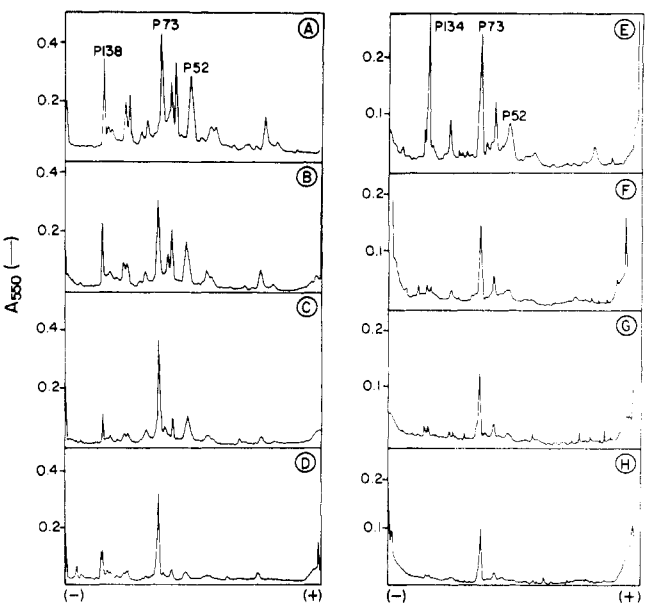

FIGURE 10: Gel electrophoresis of oligo(dT)-cellulose isolated membrane-bound and free polysomal mRNP prepared in the presence of various salt concentrations. Free and membrane-bound polysomes were resuspended in HSB and 15 A_{260} units was applied to two oligo(dT)-cellulose columns. Columns were washed with HSB or with HSB containing 400, 500, or 600 mM NaCl. After washing, mRNP fractions were eluted, ethanol precipitated, and subjected to electrophoresis. Gels were scanned at 550 nm after destaining. (A) Free polysomal mRNP washed with 250 mM, (B) 400 mM, (C) 500 mM, or (D) 600 mM NaCl; (E) membrane-bound polysomal mRNP washed with 250 mM, (F) 400 mM, (G) 500 mM, or (H) 600 mM NaCl.

incubation the samples were prepared and examined on CsCl gradients. The free and membrane-bound mRNP incubated in 100 mM NaCl exhibited similar densities on CsCl gradients (Figures 9A and 9D). Incubation of the polysomes in 250 mM NaCl prior to centrifugation shifted the peak of the free mRNP from 1.47 to 1.55 g/cm^3 while having little effect on the density of the membrane-bound mRNP. The densities of both free and membrane-bound mRNP again were similar after incubation in 500 mM NaCl (Figures 9C and 9F). Note that membrane-bound mRNP appear to lose more protein (greater density shift) in the salt concentration range of 0.25–0.5 M NaCl. These results which were highly reproducible suggest that increasing salt does not have an equivalent effect on free and membrane-bound mRNP. This finding was also supported by following the loss of specific proteins from column-bound mRNP when the salt concentration in the wash buffer was increased. The normal protein components present in the isolated free and membrane-bound mRNP after washing with 250 mM NaCl are revealed in the gel scans shown in Figures 10A and 10E. When mRNP proteins were examined on gels after washing with 400 mM NaCl, a marked change had occurred. While the column-fractionated free mRNP still contained seven proteins with only a slight loss of some of the minor bands (Figure 10B), the membrane-bound mRNP retained mainly the 73 000 molecular weight protein (Figure 10F). These data are consistent with that of the CsCl gradient experiments and suggest that the membrane-bound polysomal mRNA–protein interaction is more sensitive to an increase in salt concentration above 250 mM than that of the free. The 73 000 molecular weight protein remains associated with mRNA even when all the other proteins are released (Figures 10C, 10D, 10G, 10H).

Binding Sites for the mRNP Proteins. Although a 78 000 molecular weight protein (presumably equivalent to P73 described here) has been shown to interact with the poly(A) region of mRNA (Kish and Pederson, 1976; Schwartz and Darnell, 1976; Blobel, 1973) and has an affinity for synthetic poly(A) (Fukami and Itano, 1976; Schweiger and Mazur, 1975), little is known about the binding sites for the other mRNP proteins. A poly(A)-rich ribonucleoprotein particle has been isolated from RNase-treated EDTA-disrupted HeLa cell polysomes using an affinity column (Kish and Pederson, 1976). If most of the proteins described herein are attached along the length of mRNA or are clustered at the 5′ end, then the poly(A)-rich complex isolated after RNase treatment should lack a number of proteins compared with the control. To examine this possibility, free and membrane-bound polysomes were isolated as described in Materials and Methods, EDTA-disrupted and RNase-treated under conditions similar to those employed by Kish and Pederson (1976) in the isolation of a poly(A)-rich RNP particle. After RNase treatment only 5% of the labeled nonpoly(A) portion of the mRNP is bound to the column compared with the control sample. ELI eluted fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis after ethanol precipitation. It is evident from the scans of the gels shown in Figure 11 that the RNase prepared poly(A)-rich RNP retains all of the same proteins present in the intact mRNP.

Discussion

We describe here the isolation of rat liver free and membrane-bound polysomal mRNP using oligo(dT)-cellulose columns. Contamination of the polysomes with nuclear or cytoplasmic RNP particles was ruled out using a combination of CsCl and sucrose density gradient approaches. Conditions thought to minimize nonspecific adsorption of cytoplasmic

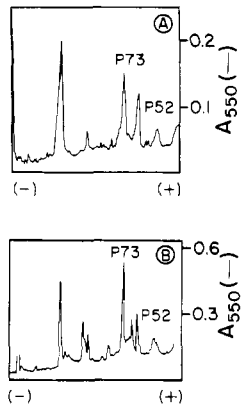

FIGURE 11: Gel electrophoresis of ELI-eluted fractions recovered after oligo(dT)-cellulose column fractionation of RNase-treated free and membrane-bound polysomal mRNP. Free and membrane-bound polysomes resuspended in HSB to a final concentration of 30 A_{260}/mL were digested with pancreatic RNase (5 μg/mL, 30 min, 25 °C) and 20 A_{260} units fractionated on two oligo(dT)-cellulose columns as described in Figure 1. Ethanol-precipitated ELI eluted mRNP fractions were electrophoresed as described in Materials and Methods. After staining with Coomassie brilliant blue and destaining in acetic acid, methanol, gels were scanned at 550 nM. (A) Free polysomal mRNP proteins; (b) membrane-bound polysomal mRNP proteins.

proteins to RNA such as pelleting polysomes through high molarity sucrose and washing the mRNP with 0.25 M NaCl were used (Olsnes, 1971; Baltimore and Huang, 1970). Seventy percent of the applied mRNA-labeled EDTA-dissociated free and membrane bound polysomal mRNP bound to oligo(dT)-cellulose columns.

The poly(A) segment and some other component(s), possibly proteins, were shown to be involved in the binding of the mRNP to the column. Because we employed the FOA procedure which allows only mRNA to incorporate radioactive precursors (Cardelli et al., 1976; Wilkinson et al., 1971), we were able to examine the physical nature of the labeled nonbound as well as bound fractions. A large percentage of the deproteinized nonbound fraction of labeled RNA appeared to consist of intact mRNA molecules. Furthermore using CsCl gradients it was also shown that these messenger-like RNAs were associated with proteins. The lack of binding to the column of these RNAs is probably due to the absence of poly(A) segments or segments too short to allow binding. We favor the former interpretation to explain the lack of binding for the following reasons.

(1) Conditions used during column fractionation, i.e., low temperature and high salt, should favor the binding of mRNP with short poly(A) tails.

(2) No band corresponding to the P73 protein was seen on gels of the nonbound fraction and, since this protein appears to interact with the poly(A) region of the mRNA, this infers the lack of this region on the nonbound mRNP (Blobel, 1973; Kish and Pederson, 1976).

(3) A significant percent of nonhistone mRNA in eukaryotes appears to lack poly(A) (Milcarek et al., 1974; Nemer et al., 1974).

The majority of column bound mRNP were eluted with ELI buffer. Gel electrophoresis revealed the presence of seven proteins associated with free mRNP, and five proteins with membrane-bound mRNP ranging from 52 000 to 138 000 in molecular weight. Four of the membrane-bound mRNP proteins described here, P52, P62, P73, and P104, appear identical with the ones found in free mRNP. The fifth protein associated with bound mRNP, P134, may be similar to the P138 protein found in free mRNP, although the difference in molecular weight between the two is consistently observed. The significance of the absence of proteins P66 and P109 from membrane-bound mRNP will remain uncertain until a function for the mRNP proteins can be determined.

The P52 and P73 proteins seen as major bands on our sodium dodecyl sulfate gels have been found in most mRNP isolated from a variety of cells studied to date (Blobel, 1973; Morel et al., 1973; Bryan and Hayashi, 1973; Kish and Pederson, 1976). The other major protein component described here, P138, may be analogous to the large molecular weight (120 000–130 000) protein found in mRNP isolated by others using techniques similar to ours (Kish and Pederson, 1976; Lindberg and Sundquist, 1974). The variability in the numbers of minor mRNP proteins reported by various investigators is probably due in part to the different methods and ionic conditions used to prepare the complexes. A high salt (0.5 M NaCl) wash is frequently used in the preparation of mRNP and has been suggested as necessary to remove nonspecifically bound proteins (Bryan and Hayashi, 1973; Blobel, 1973). Most mRNP when prepared this way contain only the 52 000 and 78 000 molecular weight proteins. We also found that washing the column-bound mRNP with 0.5 M NaCl leaves only three proteins still as part of the mRNP, two of these being equivalent to those found by others (Figure 10C). A 0.4 M NaCl wash though removes very little protein from the free mRNP (Figure 10B) which suggests the possibility that the minor proteins may not be nonspecifically interacting with mRNA. Furthermore, the seemingly arbitrary choice of 0.5 M NaCl to remove "extraneous" proteins may in fact remove actual mRNP proteins. Salt sensitivity may be a poor criteria to use in judging whether the interaction of a protein with a nucleic acid sequence is specific.

An interesting result concerning salt sensitivity was that bound mRNP appeared to be disrupted at a lower salt concentration then free mRNP. This result may be related to different affinities of these proteins for specific sequences and/or secondary regions in free and membrane-bound mRNA.

We report here that the proteins associated with the oligo(dT)-cellulose bound mRNP appear to have a higher affinity for mRNA sequences as compared with rRNA, a finding consistent with an actual role for these proteins in some aspect of mRNA metabolism.

The type of detergent used in preparation of the bound polysomes determined the amount of mRNA-associated protein isolated. When DOC was used in the preparation, little protein was found bound to the mRNA as revealed by CsCl gradient analysis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (unpublished results). Others have reported similar effects due to DOC (Faiferman et al., 1971; Olsnes, 1970). Consistent with this is the finding that the yield of protein synthesis initiation factors from DOC-prepared membrane-bound polysomes is lower than that of a comparable Triton X-100 preparation (Murty et al., 1975). Because of the DOC effect described above and the sensitivity of the mRNP to formamide (Figure 6), hydrophobic as well as ionic interactions may be important in determining the interaction of proteins with mRNA.

Little evidence exists describing mRNA binding sites for proteins other than the 78 000 molecular weight species. We report that most of the mRNA-associated proteins described here may be clustered in the poly(A) region of the molecule. This was not totally unexpected since others using different preparatory techniques have also found that a number of proteins besides the 78 000 molecular weight species appear to be found associated with poly(A)-enriched mRNA frag-

ments (Blobel, 1973; Schwartz and Darnell, 1976; Kish and Pederson, 1976). If only the P73 protein has an affinity for synthetic poly(A), this implies that the other mRNP proteins either interact with nonpoly(A) sequences proximal to the poly(A) tail or alternately bind to some secondary region involving poly(A). In any case the fact that the poly(A) region is available to interact with oligo(dT)-cellulose suggests that this polynucleotide segment is not completely covered with protein.

Recent evidence has suggested that a variable length of a nonpoly(A) segment rich in uridine and possibly containing double-stranded regions remains covalently attached to a poly(A) segment isolated from the mild RNase treatment of mRNA (Jeffery and Brawerman, 1975; Proudfoot and Brownlee, 1974). Consistent with this is our finding that 5% of the uridine-labeled RNase-treated mRNP binds to affinity columns relative to control samples. Such labeled RNA segments could represent nonpoly(A) sequences up to 75 bases long (assuming an average mRNA length of 1500 bases). Is a 150–250 base poly(A)-rich RNA region sufficiently long to accommodate the number of proteins described? Since the density of free polysomal mRNP exposed to 0.6 M NaCl is approximately 1.61 g/cm^3 and, as shown by gel scans (Figure 10D), most of the protein present is represented by P73 one can calculate that on the average there are 2–3 molecules of this protein per mRNA (see Materials and Methods). By determining the areas under the 7 protein peaks (Figure 10A), correcting for differences in molecular weight and using the number of mRNA-bound P73 proteins as a standard, one can calculate that there are 7–12 proteins present per mRNA molecule. We realize that the Coomassie brilliant blue stain deviates from Beers law and present this range only as an approximation. However, an RNA region approximately 250 bases in length can easily accommodate this number of proteins especially since some of these polypeptide chains may represent subunits of a smaller number of large molecular weight proteins. The finding that these proteins may bind to a specific 3′ mRNA region adds further support for their specificity. Since this laboratory as well as others have shown that mRNA may interact with RER membranes through this region (Cardelli et al., 1976; Milcarek and Penman, 1974; Lande et al., 1975), our data suggest that some of these proteins may play a role in the binding of mRNA to the surface of the endoplasmic reticulum. A simple model for membrane binding of mRNP involving some of these proteins may thus be formulated. Certain mRNAs destined to be translated in a free class of polysomes are inhibited from attaching to RER membranes by the interaction of the P66 and/or P109 proteins with a membrane-recognized mRNA sequence near the 3′ end. Protein dissociation factors (which can cause the release of bound polysomes from membranes) have already been described though the nature of their action remains unknown (Blobel, 1976). Of course there may be membrane proteins which "anchor" mRNA by binding to other regions but which are lost upon Triton X-100 treatment and not observed in the isolated mRNP. Since it now appears established that certain proteins have a high affinity for mRNA future work will have to determine what role if any these proteins play in the various aspects of mRNA metabolism.

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