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Non-polysomal Poly(A)-Containing Messenger Ribonucleoproteins of Cryptobiotic Gastrulae of *Artemia salina*

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Non-polysomal poly(A)-containing messenger ribonucleoprotein (mRNP) of *Artemia salina* has been isolated by thermal chromatography on oligo(dT)-cellulose in moderate (250 mM) and low (50 mM NaCl and 5 mM $MgCl_2$) ionic strength. The purified particles sedimented between 5 S and 30 S and banded at a density of 1.38–1.40 g/cm^3 and 1.26–1.27 g/cm^3 in CsCl and sucrose isopycnic centrifugation, respectively. The translatability of the mRNP in a cell-free system depended on the conditions of isolation. The protein composition of the free mRNP is independent of the conditions used in oligo(dT)-cellulose chromatography. The proteins have M_r of 87000, 76000, 65000, 50000, 45000, 38000 and 23500. A specific set of proteins is associated with different ribonucleoproteins, although some proteins are present on multiple particles. The main 17 ± 2-S particle is composed of proteins with M_r of 87000, 76000, 45000 and 38000. Approximately the same proteins were present on free mRNP and mRNP isolated from non-polysomal mRNP-ribosome complexes. Poly(A)-binding proteins have M_r of 38000 and 23500. The 38000-M_r protein comprised at least 60% of the total mRNP protein. Poly(A)-binding proteins with M_r of 38000 and 76000 are also present in a free state in the cytoplasm. A relation between the main poly(A)-binding mRNP protein and the helix-destabilizing protein HD40 [Marvil, D. K., Nowak, L., and Szer, W. (1980) *J. Biol. Chem. 255*, 6466–6472] is discussed.

Free ribonucleoprotein particles were first detected in the cytoplasm of fish embryos in 1964 [1]. Since then, the existence of mRNP has been reported in a great number of eukaryotic organisms and in different cellular locations indicating their universal nature. Several review articles on mRNP have been published [2–4]. The protein moiety of mRNP depends on the cellular location. An exchange of proteins on mRNP has been observed after its transport from the nucleus to the cytoplasm and after its entry into polysomes [4–11]. The organization and functional significance of mRNP has still to be resolved. Although qualitative similarities in protein compositions of mRNP have been demonstrated in several eukaryotic cell types by affinity chromatography on oligo(dT)-cellulose [11], the composition of non-polysomal mRNP has led to conflicting results [2–4]. The differences may be explained in part by the various techniques used in the isolation of mRNP [12–16] but also the studied systems may contain different ratios of distinct functional forms of free cytoplasmic mRNP differing in protein moiety, i.e. mRNP in transit and masked stored mRNP [17–19]. The masked mRNP is most abundantly present in less active cells and becomes translatable after activation by alterations in the mRNP structure. The masking of mRNP activity may be due to translational control RNA [20–22], to inhibition by mRNP proteins [23], to an inhibitor ribonucleoprotein particle [24] or to interaction of the 3′-poly(A)-protein complex with mRNA sequences near the 5′ end [25]. The different inhibitory phenomena could be related to each other.

Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; poly(A), poly(U), oligo(dT), poly(riboadenylic acid), poly(ribouridylic acid), oligo(thymidylic acid); mRNA, mRNP, messenger ribonucleic acid, messenger ribonucleoprotein; TMV, tobacco mosaic virus.

Artemia salina is very suitable for the study of non-polysomal mRNP. Under certain conditions the embryos of the brine shrimp enter a cryptobiotic phase after gastrulation. No polysomes have been observed in the dormant embryos and their rapid formation after redevelopment is due to the presence of stored mRNP [26–28]. The cytoplasmic poly(A)-containing mRNP exhibits a heterogeneous sedimentation distribution, consisting of several discrete peaks ranging from 10 S to 120 S. This poly(A)-containing mRNP exists in a repressed form due to the binding of a translational control RNA [21]. The latter binding does not prevent the association of ribosomes and ribosomal subunits to mRNP. Approximately 50% of the poly(A)-containing mRNP is located in non-polysomal mRNP-ribosome complexes [29]. In contrast, the poly(A)-lacking mRNP is not repressed and has been characterized as a 22-S particle containing 9-S mRNA coding for a 26000-M_r basic protein [30].

In this communication we describe the isolation and characterization of the non-polysomal poly(A)-containing mRNP. The protein composition is determined and the poly(A)-binding proteins are identified. The isolation of intact mRNP and the identification of the mRNA-associated proteins are a prerequisite for the study of the functional properties of mRNP proteins.

A preliminary report has been presented at a symposium on the biochemistry of *Artemia* development [31].

MATERIALS AND METHODS

Buffers

Buffer J: 10 mM Hepes pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl; buffer K: 10 mM Hepes pH 7.2, 1 mM EDTA, 50 mM

NaCl; buffer L: 10 mM Hepes pH 7.2, 1 mM EDTA, 250 mM NaCl; buffer N: 10 mM Hepes pH 7.2, 10 mM KCl, 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol; buffer S: 10 mM Tris/HCl pH 8.3, 2% sodium dodecylsulfate (w/v), 2% 2-mercaptoethanol (v/v), 20% glycerol (v/v); buffer T: 10 mM Hepes pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 0.01% (w/v) heparin.

Fractionation

100–200 g (dry weight) of cryptobiotic gastrulae of *Artemia salina* (Metaframe, San Francisco Bay Brand, Newark, CA, USA) were separated from sand and metal impurities by flotation in saturated NaCl, washed extensively with distilled water and homogenization buffer (buffer J or K). The washed gastrulae were ground at 4 °C in a motor-driven mortar (Retsch, Düsseldorf, FRG) in the presence of a small amount of buffer (50 µl/g gastrulae) containing 0.5 mg/ml heparin and 150 mM sucrose. The homogenate diluted to 3 ml/g gastrulae was filtered through cheese cloth. The postmitochondrial supernatant was prepared by two successive centrifugations of the filtrate at $20700 \times g$ for 30 min and at 4 °C in a Beckman JA 20 rotor. The postmitochondrial supernatant was analyzed on a linear 10–30% (w/v) sucrose gradient in buffer J centrifuged in a Beckman SW27 rotor. Conditions of centrifugation are described in the figure legend. The postribosomal supernatant was obtained by differential centrifugation of the postmitochondrial supernatant at $360000 \times g$ in a Beckman R60 rotor for 50 min and at 4 °C.

Buoyant Density Centrifugation

For isopycnic centrifugation in sucrose, samples were placed on three layers (9 ml each) of sucrose solutions (30% 50% and 70% w/w) in buffer J or K supplemented with 0.1 mg/ml of heparin. Centrifugation was in a Beckman R60 rotor at $251800 \times g$ for 90 h and at 4 °C.

Buoyant density of mRNP was also determined by CsCl isopycnic centrifugation. Poly(A)-containing mRNP was fixed by dialysis against 5% neutralized formaldehyde in buffer K for 12 h and at 4 °C and then another 24 h against buffer K. The fixed ribonucleoprotein was placed on three layers (1.3 ml each) of CsCl (20%, 35% and 50% w/w). Centrifugation was in a Beckman SW65 rotor at $301000 \times g$ for 25 h and at 4 °C.

The fractions of sucrose and CsCl isopycnic gradients were collected by sucking through a stainless-steel needle from the bottom of the centrifuge tubes. Densities were determined by weighing samples in a 100-µl constriction pipette. The protein content was determined by the method of Ovchinnikov et al. [32]. Poly(A) sequences were assayed by hybridization with [^{3}H]poly(U) (Miles Laboratories Inc., Kankakee, IL, USA) [29].

Affinity Chromatography on Oligo(dT)-cellulose and Poly(A)-Sepharose 4B

Olido(dT)-cellulose type T2 (Collaborative Research, Waltham, Ma, USA) was treated with 0.1 M NaOH for 10 min, extensively washed with distilled water and equilibrated with the appropriate binding buffer. Postribosomal supernatant prepared in buffer J was immediately chromatographed on oligo(dT)-cellulose. Postribosomal supernatant prepared in buffer K was brought to 250 mM NaCl before chromatography. Batch binding was used in analytical experiments. 0.1 g oligo(dT)-cellulose was washed successively by adding 1-ml fractions of binding and elution buffer. The melting temperature of mRNP-oligo(dT) complexes was measured by raising the temperature discontinuously. Added 1-ml fractions were removed after 10 min of equilibration at the indicated temperature and assayed for poly(A) sequences [29]. Column procedures were used in the preparative isolation of poly(A)-containing mRNP. After chromatography of postribosomal supernatant the oligo(dT)-cellulose column (1.2×5 cm) was washed with the binding buffer to bring the A_{260} of the effluent to zero. Subsequently the bound material was eluted with 10 mM Hepes pH 7.2 at 36 °C and concentrated by ultrafiltration (Amicon, Oosterhout, The Netherlands) using a PM 30 membrane pretreated with 5% formaldehyde.

Poly(A)-containing mRNP was analyzed on 10–30% sucrose gradients in buffer T. Conditions of centrifugation are described in the figure legends.

1 ml of poly(A)-containing mRNP (5–10 A_{260} units) was digested in 10 mM Hepes pH 7.2 with 50 units of T_1 RNase (Sankyo Chemical Co., Tokyo, Japan) and 2 µg pancreatic RNase (Sigma Chemical Co., St Louis, MO, USA) for 30 min at 30 °C. The digest was rechromatographed on oligo(dT)-cellulose after addition of 1 vol. of twice-concentrated binding buffer.

Poly(A)-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was washed with distilled water, 2 M KCl and equilibrated with buffer L or buffer N. Proteins of mRNP were extracted with LiCl [33], dialyzed against buffer N and chromatographed on poly(A)-Sepharose. Bound proteins were eluted in buffer N with a potassium chloride gradient from 10 mM to 2 M. Postribosomal supernatant prepared in buffer L was chromatographed on poly(A)-Sepharose. The RNA-binding proteins were eluted with a sodium chloride gradient from 250 mM to 1.5 M.

Protein Synthesis in vitro *in Rabbit Reticulocyte Lysate*

Nuclease-treated reticulocyte lysate was prepared exactly as described by Pelham and Jackson [34]. The reaction mixture (25 µl) consisting of 5 µl template sample and 20 µl lysate is described [30]. [^{35}S]Methionine (Radiochemical Centre, Amersham, UK) had a specific activity of 1290 Ci/mmol.

Polyacrylamide/Dodecylsulfate Gel Electrophoresis

Proteins were precipitated overnight with 10% trichloroacetic acid. The pellet collected by centrifugation was washed with ethanol and diethylether and dissolved in buffer S. Samples were denatured by heating at 95 °C for 3 min and analyzed on 10% polyacrylamide gels (0.6×10 cm) containing 0.21% *N,N'*-methylenebisacrylamide in the presence of 0.1% (w/v) sodium dodecylsulfate.

Electrophoresis was as described by Studier [35]. The following proteins were used as standards for molecular weight estimation: phosphorylase *b* (94000), serum albumin (68000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (21500) and α-lactalbumin (14000).

RESULTS

Heterogeneity of the Cytoplasmic Non-polysomal Poly(A)-Containing mRNP

Previously we have shown that approximately 50% of the cytoplasmic poly(A)-containing mRNP is complexed with ribosomes and ribosomal subunits. These complexes sedi-

mented faster than 50 S and were dissociated in the presence of EDTA [29]. The postribosomal ribonucleoprotein present in the postmitochondrial supernatant were further characterized by sucrose gradient centrifugation. These particles were only separated if centrifugation was prolonged until the 40-S ribosomal subunit was sedimented to the bottom of the tube (Fig. 1). The poly(A)-containing mRNP was localized by hybridization with [^{3}H]poly(U). The distribution of poly(A) sequences revealed the presence of several discretely sedimenting peaks with sedimentation coefficients of (34 ± 2) S, 28–20 S and 17–13 S.

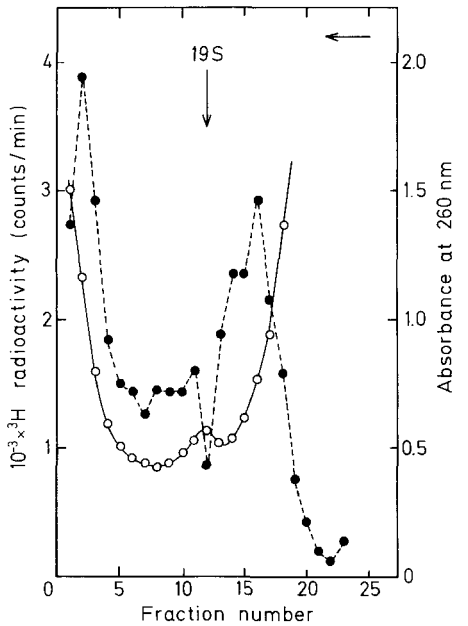


Fig. 1. *Sucrose gradient analysis of postribosomal poly(A)-containing mRNP.* The postmitochondrial supernatant was prepared in buffer J and analyzed on a 10–30% (w/v) sucrose gradient in the same buffer. Centrifugation was in the Beckman SW27 rotor for 17 h at 131 000 × *g* and at 4 °C. The direction of centrifugation is indicated. RNA was extracted from each fraction of the gradient and its poly(A) content determined by hybridization with [^{3}H]poly(U). (●——●) Radioactivity; (○——○) absorbance at 260 nm. The 19-S protein complex [36] was used as internal marker

The cytoplasmic ribonucleoprotein was separated into a ribosomal pellet and a postribosomal supernatant by differential centrifugation. Both fractions were subjected to isopycnic centrifugation in sucrose. The density distribution of the poly(A)-containing mRNP was obtained by a 90-h centrifugation at 251 800 × *g* in a three-layer system of sucrose (Fig. 2). In the latter conditions ribosomes are at a density of 1.35–1.36 g/cm^3 and poly(A)-containing mRNP is at or close to its equilibrium position [15, 29]. Only 18% and 15% of the poly(A) sequences of the postribosomal supernatant and of the ribosomal fraction are located at a density below 1.2 g/cm^3 and represent degraded mRNP or small particles unable to attain equilibrium. The mRNP complexed with ribosomes and the free mRNP exhibited a different density distribution. The free poly(A)-containing mRNP banded at a density of 1.26–1.27 g/cm^3 and that of the ribosomal fraction at a density of 1.29–1.30 g/cm^3.

Isolation of Free Poly(A)-Containing mRNP on Oligo(dT)-cellulose

The high salt concentrations (400–500 mM) normally used in affinity chromatography on oligo(dT)-cellulose probably results in the removal of some functional proteins from mRNP. Therefore the isolation of poly(A)-containing mRNP has been compared in low and moderate ionic strength. The stability of the interaction of postribosomal poly(A)-containing mRNP with oligo(dT)-cellulose was determined in buffer J and buffer L (Fig. 3). After extensive washing the bound material was eluted by stepwise increase of the temperature. A melting temperature of 51 °C was measured in buffer L and 43 °C in buffer J. In 10 mM Hepes pH 7.2 a melting temperature of 30 °C was observed independent of the ionic conditions used in the mRNP binding. Instead of reducing the melting temperature of poly(A) · oligo(dT) by

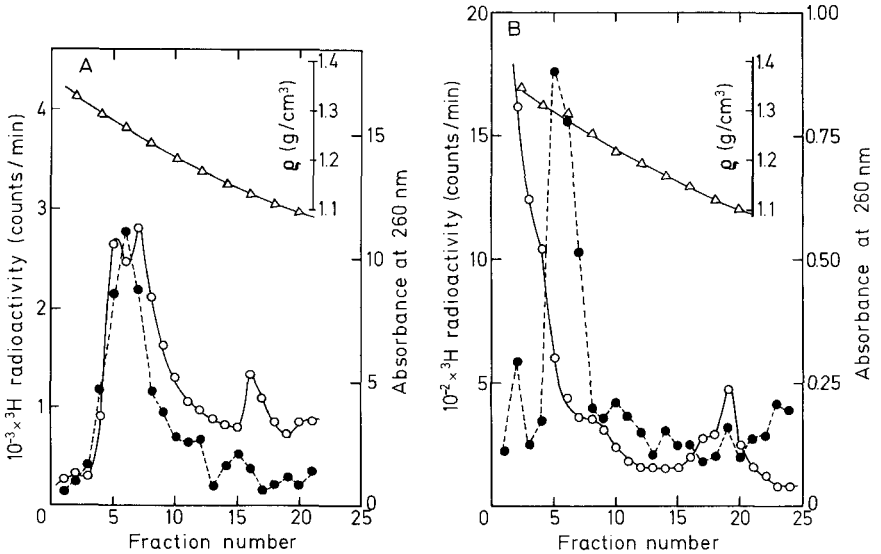


Fig. 2. *Analysis of the ribosomal fraction (B) and the postribosomal supernatant (A) by isopycnic centrifugation in sucrose.* Both fractions were obtained from the postmitochondrial supernatant prepared in buffer J by differential centrifugation. The postribosomal supernatant (A) and the ribosomal fraction (B) were centrifuged on a three-layer sucrose system (30%, 50%, 70% w/w; 9 ml each). Centrifugation was in the Beckman R60 rotor for 90 h at 251 800 × *g* and at 4 °C. Poly(A) sequences were determined by hybridization with [^{3}H]poly(U). (●——●) *Radioactivity*; (○——○) absorbance at 260 nm; (△——△) density

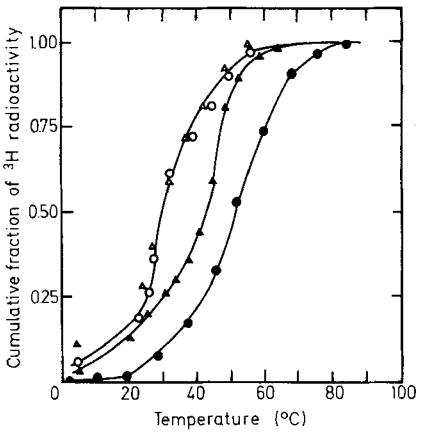


Fig. 3. *Interaction of free poly(A)-containing mRNP with oligo(dT)-cellulose.* Poly(A)-containing mRNP present in the postribosomal supernatant was bound to oligo(dT)-cellulose in buffers J and L. The stability of the binding was determined by differential thermal elution in buffer J (▲——▲), buffer L (●——●) and in 10 mM Hepes pH 7.2 after binding in buffer J (△——△) and after binding in buffer L (○ ——○). The amount of poly(A) sequences present in each fraction was determined by hybridization with [^{3}H]poly(U). The results are expressed as a cumulative fraction of [^{3}H]poly(U) radioactivity

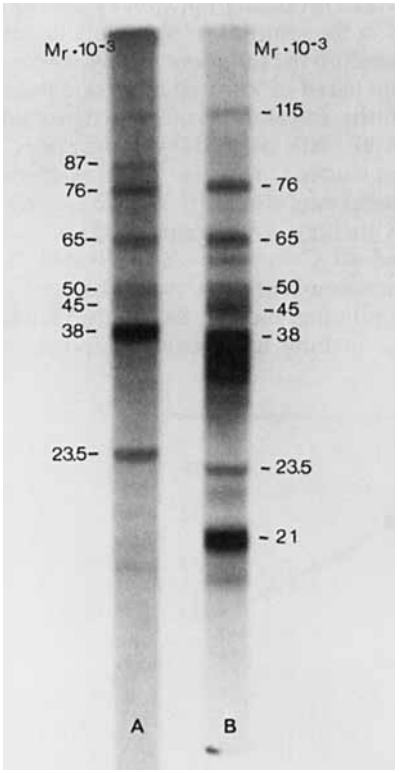


Fig. 4. *Protein composition of free poly(A)-containing mRNP.* Free mRNP was bound to oligo(dT)-cellulose in buffer J and L. The bound mRNP was eluted with 10 mM Hepes pH 7.2 at 36 °C, concentrated by Amicon ultrafiltration and analyzed by dodecylsulfate/polyacrylamide gel electrophoresis. Proteins of mRNP prepared in buffer J (A) and in buffer L (B)

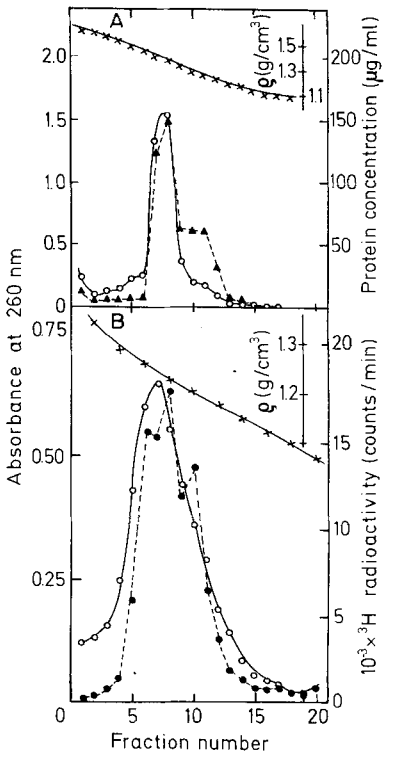


Fig. 5. *Analysis of free poly(A)-containing mRNP by isopycnic centrifugation.* Poly(A)-containing mRNP was prepared by affinity chromatography in buffer L. The mRNP was eluted with 10 mM Hepes, pH 7.2 at 36 °C and concentrated by Amicon ultrafiltration. (A) Isopycnic centrifugation in CsCl. mRNP was fixed with formaldehyde and placed on three layers of CsCl (20%, 35%, 50% w/w). Centrifugation was in the Beckman SW65 rotor at 301000 × *g* for 25 h and at 4 °C. (▲---▲) Protein content; (○——○) absorbance at 260 nm; (×——×) density. (B) Isopycnic centrifugation in sucrose. mRNP was placed on three layers of sucrose (30%, 50%, 70% w/w). Centrifugation was in the Beckman R60 rotor for 90 h at 251800 × *g* at 4 °C. Poly(A) sequences were determined by hybridization with [^{3}H]poly(U). (●---●) Radioactivity; (○——○) absorbance at 260 nm; (×——×) density

the addition of formamide an increase in temperature is sufficient to elute the bound poly(A)-containing mRNP. 75% of the poly(A) sequences can be eluted by a temperature increase to 36 °C and more than 95% are eluted at 53 °C. In all subsequent experiments poly(A)-containing mRNP was prepared by thermal elution at 36 °C.

The proteins eluted from oligo(dT)-cellulose were analyzed by dodecylsulfate gel electrophoresis (Fig. 4). The protein composition is practically independent of the binding conditions used. Proteins with M_r of 76000, 65000, 50000, 45000, 38000 and 23500 are common for both preparations. The 38000-M_r protein is the main component and represents 60–70% of the total amount of protein present in free poly(A)-containing mRNP.

Additional proteins with M_r 87000, 115000 and 21000, were present in mRNP prepared in buffer J and buffer L. The similarity of the protein composition demonstrated the absence of extensive adventitious binding. Control experiments were performed to examine if binding in low ionic strength was mediated by interaction between poly(A) and oligo(dT) and to determine the amount of unspecific binding.

Isolated poly(A)-containing mRNP was hybridized with an excess of poly(U) in buffer J and rechromatographed on oligo(dT)-cellulose. The protein content of each fraction was measured by the method of Ovchinnikov et al. [32]. Only 5–6% of the proteins eluted with 10 mM Hepes pH 7.2 at 36 °C. The same result was obtained with oligo(dT)-cellulose

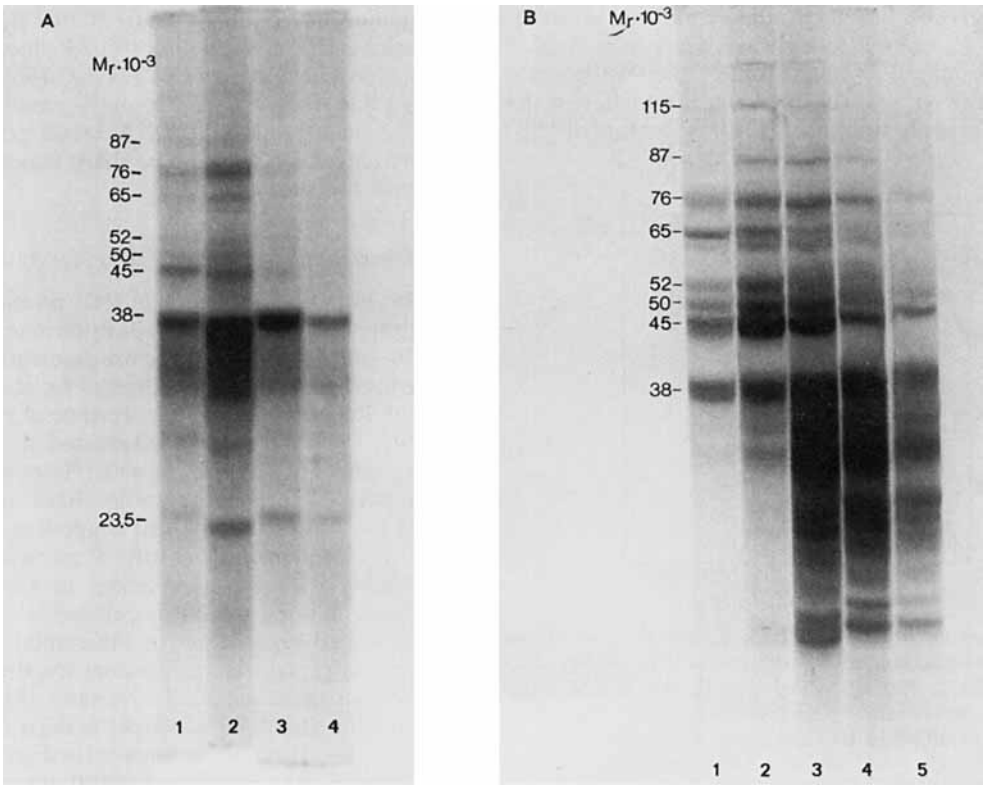


Fig. 6. *Protein composition of free poly(A)-containing mRNP after isopycnic centrifugation.* Poly(A)-containing mRNP was prepared by affinity chromatography in buffer L. The mRNP was eluted with 10 mM Hepes pH 7.2 at 36 °C and concentrated by Amicon ultrafiltration. (A) Isopycnic centrifugation in CsCl. Unfixed mRNP was placed on three layers of CsCl (20%, 35%, 50% w/w. Density fractions were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis: lane 1, 1.33 g/cm^3; lane 2, 1.28 g/cm^3; lane 3, 1.24 g/cm^3; lane 4, 1.21 g/cm^3. (B) Isopycnic centrifugation in sucrose. Fractions from Fig. 5B were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis: lane 1, 1.28 g/cm^3; lane 2, 1.24 g/cm^3; lane 3, 1.215 g/cm^3; lane 4, 1.19 g/cm^3; lane 5, 1.16 g/cm^3

saturated with poly(A). Only 4–5% of the total protein was rebound to oligo(dT)-cellulose.

Buoyant Density of Isolated Free poly(A)-Containing mRNP

The integrity of free poly(A)-containing mRNP isolated in buffer L was demonstrated by estimation of the buoyant density in CsCl and in sucrose. mRNP was fixed with formaldehyde, placed on three layers of CsCl and centrifuged to equilibrium (Fig. 5A). The fixed mRNP was located by measurement of the absorbance at 260 nm and the protein content. The particles banded at a density of 1.38–1.40 g/cm^3. The protein content coincided with the absorbance at 260 nm. 17% of the proteins were tailing and banded at a density of 1.22–1.26 g/cm^3. A protein content of 71% was calculated from the measured RNA and protein concentration compared to 78% estimated from the buoyant density.

Unfixed mRNP subjected to isopycnic centrifugation in CsCl was only partially disassembled and banded at a density of 1.28 g/cm^3. Fractions of the latter centrifugation were analyzed by dodecylsulfate gel electrophoresis (Fig. 6A). Proteins with M_r of 87000, 76000, 65000, 52000, 50000, 45000, 38000 and 23500 constituted the poly(A)-containing mRNP.

In sucrose isopycnic centrifugation free poly(A)-containing mRNP was located by measurement of the absorbance at 260 nm and by hybridization of RNA with [^{3}H]poly(U) (Fig. 5B). The mRNP is distributed over a broad density range with a mean density of 1.25–1.26 g/cm^3 compared to a value of 1.26–1.27 g/cm^3 measured before affinity chromatography on oligo(dT)-cellulose. The absorbance at 260 nm and the [^{3}H]poly(U) radioactivity coincided, indicating that the majority of the poly(A) sequences are not cleaved from the mRNP. 32% of the poly(A) sequences are located at a density below 1.2 g/cm^3. The latter fraction represents degraded ribonucleoprotein or small particles unable to attain equilibrium in these conditions. Fractions of the sucrose isopycnic centrifugation were also analyzed by dodecylsulfate gel electrophoresis (Fig. 6B). The proteins are distributed over a broad density range. Proteins with M_r of 87000, 76000, 65000, 52000, 50000, 45000 and 38000 are present in the 1.24–1.27-g/cm^3 density region. Additional low-molecular-weight proteins are observed below 1.24 g/cm^3. The majority of these proteins were not present after chromatography on oligo(dT)-cellulose and probably resulted from protein degradation.

Free poly(A)-containing mRNP prepared in buffer J was more susceptible to nuclease degradation [23]. The integrity of the ribonucleoprotein was only demonstrated if heparin (0.1 mg/ml) was used in each purification step.

Translation of Free Poly(A)-Containing mRNP

Previously we have demonstrated that poly(A)-containing mRNA exists in a repressed form. Inhibition is due to the presence of an oligonucleotide associated with mRNA which can be dissociated from the complex by EDTA treatment [21]. The isolated free poly(A)-containing mRNP particles

have similar properties. The latter mRNP prepared in buffer J is not translated in a rabbit reticulocyte lysate in contrast to that prepared in buffer L. Comparison of the translation of template-active mRNP and the extracted RNA showed that RNA is more efficiently translated (Fig. 7). The rate of [^{35}S]-methionine incorporation mediated by 1 A_{260} unit of poly(A)-containing RNA is 1.9 times faster than for mRNP. The translation characteristics of poly(A)-containing mRNP are comparable with those previously measured for the 22-S poly(A)-lacking mRNP [30]. A broad potassium optimum centered around 85 mM and a sharp magnesium optimum at 1.75 mM has been observed.

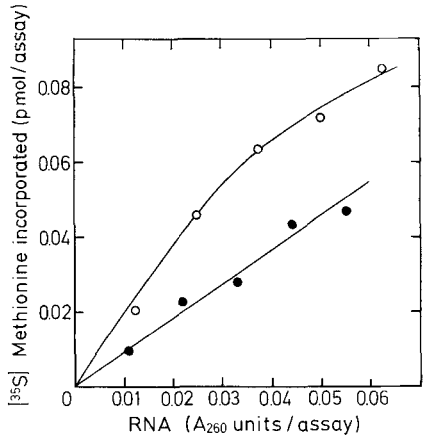
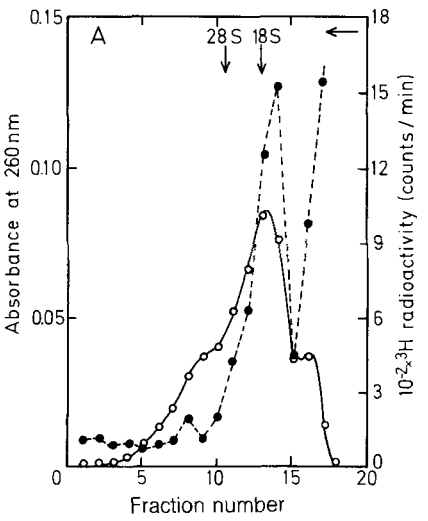


Fig. 7. *Comparison of the translation of poly(A)-containing mRNP and its mRNA.* Poly(A)-containing mRNP was prepared by affinity chromatography in buffer L. The mRNP was eluted with 10 mM Hepes pH 7.2 at 36 °C and concentrated by Amicon ultrafiltration. The mRNA was isolated from the mRNP by LiCl extraction. Translation of mRNP (●——●) and mRNA (○——○) was in a rabbit reticulocyte cell-free system. TMV RNA has an initial [^{35}S]methionine incorporation of 0.078 pmol/μg RNA in the 25-μl system, the system is saturated with 8 μg TMV RNA

Size Distribution of Isolated Free Poly(A)-Containing mRNP

The size distribution of mRNP purified by chromatography on oligo(dT)-cellulose was determined by centrifugation on a 10–30% (w/v) linear sucrose gradient (Fig. 8A). A heterogeneous distribution was observed by absorbance measurement at 260 nm revealing the presence of a few discrete components. The main particle sedimented at 17 S. Each fraction of the gradient was hybridized with [^{3}H]poly(U). The majority of the poly(A) sequences were localized in the 17-S fraction and at the top of the gradient suggesting a partial cleavage of poly(A) sequences from mRNP particles. The results are comparable with those presented in Fig. 1. The 34 ± 2-S poly(A)-containing mRNP is pelleted in the preparation of postribosomal supernatant by differential centrifugation.

Proteins present in the gradient fractions were identified by dodecylsulfate gel electrophoresis (Fig. 8B). The main protein with M_r 38000 is present in each of the sedimenting regions. Several proteins sedimented in discrete zones. Proteins with M_r of 87000, 76000 and 45000 are associated with the main 17-S mRNP, proteins with M_r of 50000 and 45000 sedimented in the 20–25-S zone and proteins with M_r of



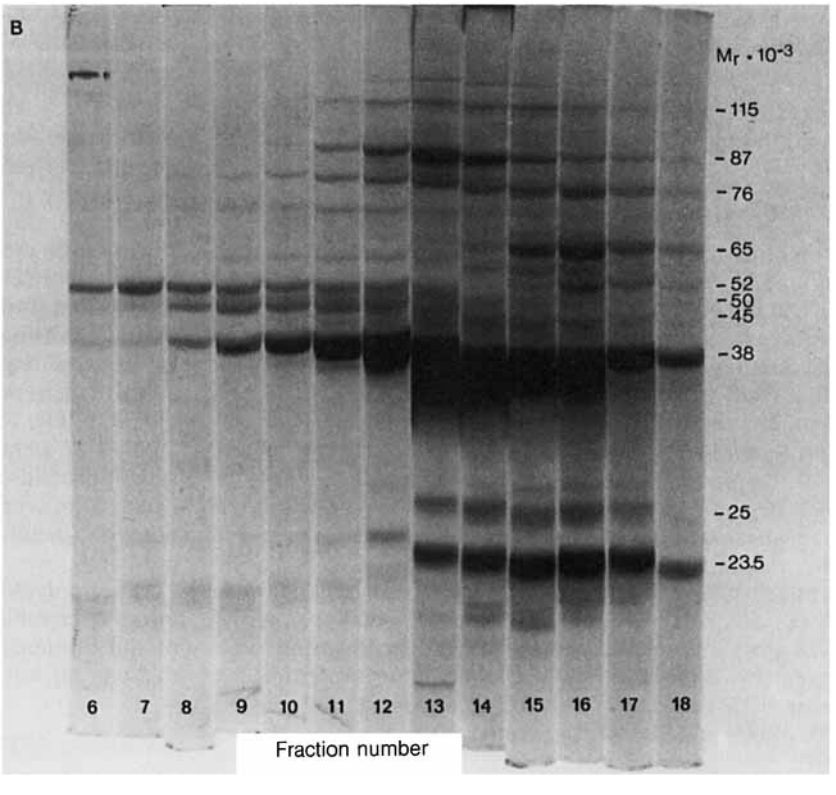


Fig. 8. *(A) Analysis of isolated free poly(A)-containing mRNP by sucrose gradient centrifugation and (B) analysis of gradient fractions 6–18 by dodecylsulfate/polyacrylamide gel electrophoresis.* Poly(A)-containing mRNP was prepared by affinity chromatography in buffer L. The mRNP was eluted with 10 mM Hepes pH 7.2 at 36 °C, concentrated by Amicon ultrafiltration and then analyzed by 10–30% (w/v) sucrose gradients in buffer T. Centrifugation was in a Beckman SW40 rotor at 161000 × *g* for 10 h and at 4 °C. Poly(A) sequences were located by hybridization with [^{3}H]poly(U). rRNA was used as marker. The direction of centrifugation is indicated. (●——●) Radioactivity; (○——○) absorbance at 260 nm

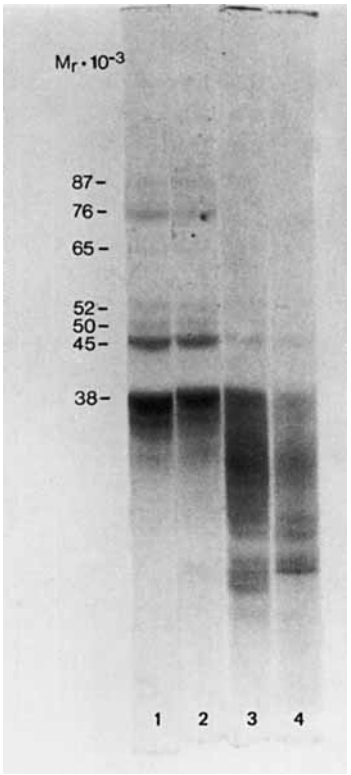


Fig. 9. *Protein composition of poly(A)-containing mRNP isolated from the ribosomal fraction.* A ribosomal pellet was prepared from the post-mitochondrial supernatant by differential centrifugation. The pellet was dissolved in buffer L and chromatographed on oligo(dT)-cellulose. Poly(A)-containing mRNP was eluted with 10 mM Hepes pH 7.2 at 36 °C, concentrated by Amicon ultrafiltration and placed on three layers of sucrose (30%, 50%, 70% w/w). Isopycnic centrifugation was carried out in the Beckman R60 rotor for 90 h at 251800 × *g* and at 4 °C. Density fractions were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis: lane 1, 1.28 g/cm^3; lane 2, 1.25 g/cm^3; lane 3, 1.22 g/cm^3; lane 4, 1.185 g/cm^3

52000 and 23500 in the 5–10-S zone. These results suggest a specific set of proteins associated with each mRNP size class.

Comparison of Protein Moiety of Free and Ribosomal Poly(A)-Containing mRNP

Non-polysomal poly(A)-containing mRNP associated with ribosomes and ribosomal subunits was isolated from the ribosomal pellet. The pellet was dissolved in buffer L and chromatographed on oligo(dT)-cellulose. The ribosomal poly-(A)-containing mRNP was further purified by isopycnic centrifugation in sucrose; it banded at the same mean density of 1.25–1.26 g/cm^3 as measured for free poly(A)-containing mRNP. Fractions of the sucrose isopycnic centrifugation were analyzed by dodecylsulfate gel electrophoresis (Fig. 9). Proteins with M_r of 87000, 76000, 50000, 45000 and 38000 were identified. The same proteins were also present in free poly(A)-containing mRNP, indicating no extensive exchange of proteins during preinitiation complex formation by ribosome binding.

Identification of Poly(A)-Binding Proteins

Proteins were extracted from free poly(A)-containing mRNP with 2 M LiCl. The extraction was nearly complete and only a small amount of a 126000-M_r protein was present in the RNA pellet. The extracted proteins were dialyzed against buffer N and chromatographed on poly(A)-Sepharose. After extensive washing of the column with buffer N the bound proteins were released by a salt gradient (Fig. 10A). Proteins with M_r of 76000, 38000 and 23500 were bound to poly(A)-Sepharose and eluted between 100 mM and 500 mM KCl. The 76000-M_r protein is only present in a small amount. To exclude the possibility that minor poly(A)-binding proteins were displaced by competition with the 38000-M_r protein for poly(A), they were also identified after degradation of mRNP with ribonucleases. Displacement of proteins from rRNA-Sepharose has been observed in the isolation of RNA-binding proteins [32]. Poly(A)-containing mRNP was incubated with T_1 RNase and pancreatic RNase and the poly(A)-protein complex isolated by chromatography on oligo(dT)-cellulose. The bound complex was eluted with 10 mM Hepes pH 7.2 at 36 °C. Besides proteins with M_r of 38000 and 23500 an additional protein with M_r of 45000 was observed (Fig. 10B). The presence of this latter protein may be due to displacement or to binding to an adjacent non-poly(A) region protected from RNase degradation by this protein. The 76000-M_r protein was not present in the poly(A)-protein complex.

Relation between mRNP Proteins and RNA-Binding Proteins

RNA-binding proteins prepared on *Escherichia coli* rRNA and *Artemia salina* rRNA linked to Sepharose 4B have been identified previously [36]. The main proteins have M_r of 112000, 90000, 68000, 55000, 43000 and 27000. Among the proteins of poly(A)-containing mRNP only the 87000-M_r and 65000-M_r proteins coelectrophorized with the 90000-M_r and 68000-M_r RNA-binding proteins [36, 37].

Different results were obtained with poly(A)-Sepharose. The main poly(A)-binding proteins have M_r of 76000 and 38000 (Fig. 11). Both proteins eluted from poly(A)-Sepharose in a specific salt range. The 38000-M_r protein, not observed among the rRNA-binding proteins, has the highest affinity for poly(A) and is a component of poly(A)-containing mRNP.

Our observations indicate that only a discrete number of the main RNA-binding proteins can be involved in the formation of stored mRNP. Some of the minor RNA-binding proteins are also present in mRNP. eIF-2 has been identified recently as a component of stored mRNP (our unpublished results).

DISCUSSION

Non-polysomal poly(A)-containing mRNP was isolated and characterized using thermal chromatography on oligo-(dT)-cellulose combined with isopycnic centrifugation. Chromatography on oligo(dT)-cellulose in high ionic strength (500 mM KCl) and thermal elution has been used in the isolation of free and polysomal mRNP [11, 16, 38]. Although high salt concentration prevents non-specific association of cytoplasmic proteins to mRNA [39, 40], it probably removes proteins from mRNA particles [13, 41]. We did not observe extensive differences in the protein composition of mRNP prepared in moderate (250 mM NaCl) and low salt concentration (50 mM NaCl and 5 mM $MgCl_2$). Although chromatography in low ionic conditions resulted in the recovery of more than 35 proteins in sea urchin egg mRNP [16], mRNP isolated from *Artemia salina* contained only eight or nine proteins.

In spite of the reproducibility in protein composition, it is not known if the same protein composition exists *in vivo*. It is not unlikely that proteins with a low affinity for mRNA

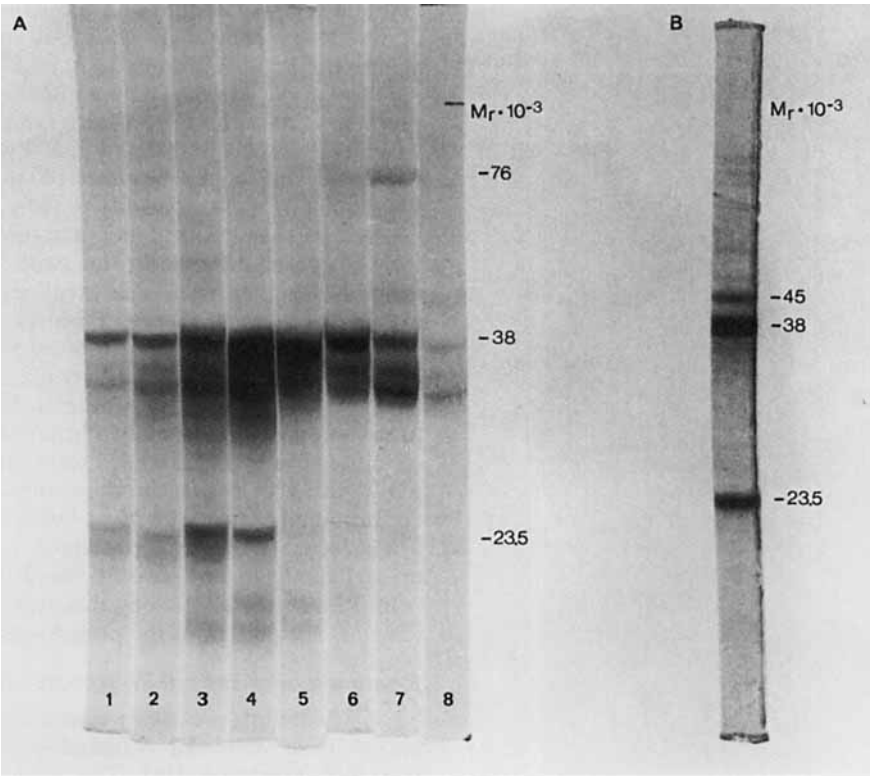


Fig. 10. *Identification of poly(A)-binding proteins.* (A) Affinity chromatography on poly(A)-Sepharose. Proteins were extracted from the free poly(A)-containing mRNP with LiCl, dialyzed against buffer N and chromatographed on poly(A)-Sepharose. Bound proteins were eluted from the column with a linear potassium chloride gradient from 10 mM up to 2 M. Proteins eluted in different fractions were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis: lane 1–8, fractions eluted with 10–650 mM KCl. (B) Identification from the poly(A)-protein complex. Free poly(A)-containing mRNP was incubated with T_1 RNase and pancreatic RNase. The digest was chromatographed on oligo(dT)-cellulose in buffer L and the bound complex eluted with 10 mM Hepes pH 7.2 at 36 °C. The eluted proteins were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis

are replaced by other stronger binding proteins during the fractionation and purification of mRNP. Displacement of proteins from rRNA-Sepharose has been observed in the isolation of RNA-binding proteins from rabbit reticulocytes [32]. However in high ionic strength, protein displacement may be enhanced by reducing the stability of protein–nucleic-acid interaction [42]. Different criteria show that the isolated material represents the native mRNP. (a) The isolated ribonucleoprotein has a poly(A) sequence as shown by hybridization with [^{3}H]poly(U) and binding to oligo(dT)-cellulose. (b) It has a characteristic density and sedimentation distribution comparable before and after oligo(dT)-cellulose chromatography. (c) It has a characteristic protein pattern; ionic conditions altering the RNA-protein interaction did not significantly influence the protein composition. (d) Ribosomal proteins were absent when the ribonucleoprotein was prepared from the ribosomal fraction. (e) The ribonucleoprotein can be isolated in a repressed or translatable state depending on the conditions of isolation. (f) Identification of rRNA-binding proteins shows that only two proteins have similar molecular weights as compared with mRNP proteins, indicating a high specificity of protein-mRNA interaction.

The free poly(A)-containing mRNP is characterized by a high protein content of 71% in agreement with the results of others [3,43–45]. The proteins are identified and have M_r of 87000, 76000, 65000, 50000, 45000, 38000 and 23500. Although Jain and Sarkar [46] concluded that differences in the protein composition of ribonucleoproteins from different species are negligible, significant differences have been reported for free mRNP [12–16]. From sucrose gradient centrifugation we may conclude that a specific set of proteins is associated with different mRNP classes. Some of these proteins, e.g. poly(A)-binding proteins, are present on multiple particles.

The mRNP present in the ribosomal fraction showed the same protein composition as free mRNP, indicating no extensive protein exchange on formation of non-polysomal mRNP-ribosome complexes. The 38000-*Mr* and 23500-M_r proteins have been identified as poly(A)-binding proteins. An additional protein with M_r of 45000 was present on the poly(A)-protein complex after ribonuclease degradation of mRNP. The latter protein is probably bound to an mRNA sequence adjacent to the 3′ poly(A) stretch. The 38000-M_r protein represents 60–70% of the total amount of mRNP protein and is present on the majority of the mRNP isolated from the postmitochondrial supernatant. The location of a large amount of protein on the poly(A) sequence of non-polysomal mRNP is in agreement with the results obtained with *Physarum polycephalum* by Jeffery and coworkers [47, 48]. They concluded that the existence of mRNA complexed with protein was correlated with the length of the poly(A) sequence and that the disruption of the poly(A)-protein complex was triggered by poly(A) shortening.

Preliminary experiments from our laboratory have shown that the 38000-M_r protein comigrated in dodecylsulfate/polyacrylamide gel electrophoresis with the protein HD40 (helix-destabilizing protein) isolated from a crude ribosomal fraction of *A. salina* by Szer and collaborators [49]. The latter

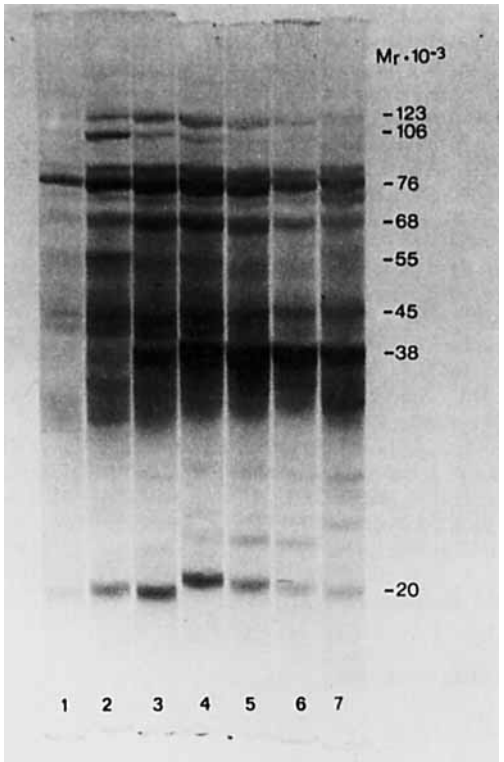


Fig. 11. *Cytoplasmic poly(A)-binding proteins.* The postribosomal supernatant prepared in buffer L was chromatographed on poly(A)-Sepharose. Bound proteins were eluted with a linear sodium chloride gradient from 250 mM to 1.5 M. Different fractions were analyzed by dodecylsulfate/polyacrylamide gel electrophoresis: lane 1–7, fractions eluted from 0.25 M to 1.5 M NaCl

protein has no tendency for self-aggregation and sediments at 2.4 S. The protein has a recognition site of five nucleotides and a stoichiometry of one HD40 molecule per 12–15 nucleotides [50]. Based on these findings, an average poly(A) stretch of 126 nucleotides [51] can accomodate eight poly(A)-binding protein molecules of M_r 38000. From the estimated molecular weight of 500000 of 17-S poly(A)-containing mRNP and a saturation of the poly(A) sequence, a protein content of 60% is calculated. So the high protein content of free mRNP is consistent with a discrete number of additional proteins. The HD40 protein is not specific for an RNA sequence [49]. However in mRNP this protein is not observed in the fraction of a ribonuclease digest not bound to oligo-(dT)-cellulose and it is also not bound to rRNA-Sepharose, indicating a specific location on the poly(A) sequence. The HD40 protein binds strongly to single-stranded RNA but has no effect on the conformation of double-stranded RNA. RNA degradation is also significantly reduced in the presence of this protein [49, 50]. These properties suggest an important role in mRNP translation and stability.

It is established that polysomal mRNP contains a common 76000-M_r protein attached to the poly(A)segment [9–11, 52, 53]. In contrast the poly(A) sequence of non-polysomal mRNP is complexed with different proteins [54, 55] (and our unpublished results). Although 38000-M_r and 76000-M_r poly(A)-binding proteins exist in a free state in the cytoplasm of *A. salina*, only the former is the main protein present on mRNP. It is not known if the 76000-M_r protein is related to the polysomal poly(A)-binding protein found in other systems. The repression of mRNA is due to the presence of translational control RNA [21]. The high uridylic acid content of this RNA [22] indicates a possible interaction with the poly(A) sequence of mRNA. The existence of an analogous translational control RNA complexed in a stoichiometric ratio with poly(A) has been demonstrated in myosin mRNA [56]. The poly(A)-binding proteins may play a role in the storage and unmasking phenomena of repressed poly(A)-containing mRNP of *A. salina*. We propose that all important events related to mRNP stability, translatability and storage are correlated with the structure and/or composition of the poly(A)-protein complex.

Further studies are in progress in this laboratory to elucidate the function of mRNP proteins. The absence of poly(A) polymerase, the presence of initiation factor eIF-2 and the relation of the 38000-M_r poly(A)-binding protein with HD40 will be reported in subsequent communications.

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