Eur. J. Biochem. *122*, 453–460 (1982)

The 38000-M_r Poly(A)-Binding Protein of Non-Polysomal Messenger Ribonucleoproteins of Cryptobiotic Gastrulae of *Artemia salina*

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(Received August 10/November 14, 1981)

The 38000-M_r poly(A)-binding protein has been purified to near homogeneity from non-polysomal messenger ribonucleoprotein of *Artemia salina* [Slegers, H., De Herdt, E., and Kondo, M. (1981) *Eur. J. Biochem. 117*, 111–120]. The protein consists of approximately 357 amino acids and is characterized by a high glycine content of 22.5% and the presence of dimethylarginine. From polynucleotide-protein binding experiments a stoichiometry of 9–11 adenylate and 10–12 uridylate residues per protein molecule is calculated. The polypeptide is devoid of poly(A) polymerase and RNase activities. The poly(A)-binding protein and the helix-destabilizing protein HD40 [Marvil, D. K., Nowak, L., and Szer, W. (1980) *J. Biol. Chem. 255*, 6466–6472] have the same mobility in polyacrylamide/dodecylsulphate gel electrophoresis and exhibit a comparable amino acid composition and protein-polynucleotide stoichiometry. Based on the length of poly(A) sequences of mRNA and from protein-poly(A) binding experiments, a repetitive binding of the 38000-M_r protein on the poly(A) sequence is demonstrated. The 38000-M_r protein of cytoplasmic and membrane-bound non-polysomal messenger ribonucleoproteins is also compared.

Messenger RNA of eukaryotic cells interacts with proteins to form ribonucleoproteins [1–3]. In poly(A)-containing mRNP the poly(A) sequence at the 3′ end of its mRNA is one of the main protein-binding sites [4–6]. A 73000–78000-M_r polypeptide is reported to be associated with the poly(A) sequence of mRNP isolated from polysomes of rabbit reticulocytes [7,8], rat liver [4,9], KB cells [10], Ehrlich ascites tumor cells [11,12], HeLa cells [13,14] and duck erythroblasts [15]. The latter protein is not detected in cytoplasmic non-polysomal mRNP of Ehrlich ascites tumor cells and rabbit reticulocytes [12], sea urchin eggs [16], duck erythroblasts [15] or brine shrimp cryptobiotic embryos [6].

Beside the poly(A)-binding proteins of mRNP the existence of a cytoplasmic pool of proteins with affinity for poly(A) has been demonstrated [6,17,18]. The functional significance of poly(A)-associated proteins remains to be established although different possible functions have been proposed, i.e. it has been suggested that the 73000–78000-M_r poly(A)-binding protein is functional in the transport of mRNA from the nucleus to the cytoplasm [13,14,19], the poly(A)-binding proteins may play a role in the protection of mRNA against ribonuclease degradation [3,20,21], an antigenic relationship between the 75000-M_r poly(A)-bound protein and poly(A) polymerase has been demonstrated [22]. In general mRNP proteins may play a role in the storage [23–25] and translation of specific mRNA [15,26,27].

Information is available about the structural organization of the poly(A)-protein complex. Different models have been proposed from the results of RNase degradation experiments, i.e. a repeating structure in which protein-binding sites alternate along the poly(A) sequence with a period of 27 residues [28] and a complex in which the protein-binding site is located near the 5′ end of the poly(A) sequence and extends for about 45 nucleotides toward the 3′ end [29]. More complicated models have been reported containing different size poly(A)-binding proteins [9]. Evidence is also provided that the poly-(A)-binding proteins interact with RNA sequences other than poly(A) [5,30].

To elucidate the function of the poly(A)-binding proteins and the structural organization of the poly(A)-protein complex, a detailed characterization of the proteins with affinity for the poly(A) sequence is necessary. The brine shrimp *Artemia salina* is very suitable for the study of mRNP proteins due to the presence of stored mRNP and the absence of polysomes in cryptobiotic gastrulae [31–33]. The protein composition of poly(A)-containing non-polysomal mRNP has been described previously and 38000-M_r and 23500-M_r polypeptides [6] were identified as the poly(A)-binding proteins. The 38000-M_r protein represents 60–70% of the total amount of mRNP protein and is the poly(A)-binding protein of the main 17-S ribonucleoprotein particle [6].

In this communication we describe the isolation and characterization of the 38000-M_r poly(A)-binding protein. The protein seems to be identical to the helix-destabilizing protein HD40 described by Szer and collaborators [34,35]. Our results support a repeating structural model for the poly-(A)-protein complex.

Abbreviations. HD40, helix-destabilizing protein with a molecular weight of 40000 [34,35]; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; poly(A), poly(riboadenylic acid); poly(U), poly(ribouridylic acid); oligo(dT), oligo(thymidylic acid); mRNA, messenger ribonucleic acid; mRNP, messenger ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; $(A)_4$, adenylyl(3′-5′)adenylyl(3′-5′)-adenylyl(3′-5′)adenosine.

EXPERIMENTAL PROCEDURE

Materials

Artemia salina cryptobiotic embryos were obtained from Metaframe (San Francisco Bay Brand, Newark, CA, USA); oligo(dT)-cellulose from Collaborative Research (Waltham,

MA, USA); poly(A)-Sepharose 4B, poly(U)-Sepharose 4B and low-molecular-weight marker proteins from Pharmacia Fine Chemicals (Uppsala, Sweden); DEAE-cellulose from Whatman Biochem. Ltd (Maidstone, Kent, UK); [^{3}H]-poly(U) (specific activity 39 Ci/mol P), [^{3}H]poly(A) (specific activity 50.9 Ci/mol P) from Miles Laboratories Inc. (Kankakee, IL, USA); $(A)_4$ and T_4 ligase from P-L Biochemicals GmbH (St Goar, FRG), tRNA from Boehringer (Mannheim, FRG), dimethylarginine, Chemical Dynamics Corp. (South Plainfield, NJ, USA); [5′-^{32}P]pCp from New England Nuclear (Dreieich, FRG); ultrafiltration filters from Amicon (Oosterhout, The Netherlands).

Buffers

Buffer A: 20 mM Hepes pH 7.6, 400 mM KCl, 11 mM magnesium acetate, 1 mM dithiothreitol; buffer B: 20 mM Hepes pH 7.6, 100 mM KCl, 9 mM magnesium acetate; buffer C: 20 mM Tris/HCl pH 7.5, 50 mM KCl, 0.6 mM $MgCl_2$, 10 mM 2-mercaptoethanol; buffer D: 90 mM Tris/HCl pH 8.3, 90 mM boric acid, 7 M ureum; buffer E: 90 mM Tris/HCl pH 8.3, 90 mM boric acid, 4 mM EDTA, 0.1% (w/v) sodium dodecylsulfate; buffer F: 50 mM sodium acetate pH 5.0, 5 mM EDTA; buffer G: 5 mM Tris/HCl pH 7.4, 50 mM KCl; buffer I: 10 mM Hepes pH 7.2, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol; 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 M: 10 mM Hepes pH 7.2, 1 mM EDTA, 500 mM NaCl; buffer R: 20 mM Tris/HCl pH 7.5, 200 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol.

Fractionation

100–200 g (dry weight) of cryptobiotic gastrulae of *A. salina* were washed and ground as described [6]. The homogenate in buffer I or buffer K was centrifuged at $6370 \times g$ for 20 min and at 4 °C. From the supernatant a crude membrane fraction was obtained by centrifugation at $20700 \times g$ for 30 min and at 4 °C. The membrane fraction was further purified by centrifugation on a 0.5–1.8 M sucrose gradient in buffer I. Centrifugation was in the Beckman SW 27 rotor at $116000 \times g$ for 85 min and at 4 °C. Membranes banded at a density of 1.17 g/cm^3 [32] and were dissolved by addition of 1 vol. of 2% (v/v) Triton X-100. Undissolved material was removed by low-speed centrifugation at 4 °C. Poly(A)-containing mRNP was prepared from the supernatant by affinity chromatography on oligo(dT)-cellulose in buffer I. Soluble membrane proteins were prepared from the fraction not bound to oligo(dT)-cellulose by differential centrifugation at $360000 \times g$ in a Beckman R60 rotor. Centrifugation was for 125 min at 4 °C. Cytoplasmic poly(A)-containing mRNP was prepared from the postmitochondrial supernatant by affinity chromatography on oligo(dT)-cellulose in buffer L [6].

Proteins used in affinity chromatography on poly(A)-Sepharose 4B were dialyzed against buffer C supplemented with 10% glycerol. Poly(A)-Sepharose 4B was washed with distilled water, 2 M KCl and equilibrated with buffer C before use. Bound proteins were eluted discontinuously with 500 mM, 750 mM and 1.5 M KCl in buffer C or with a salt gradient of 0.05–1.5 M KCl in buffer C. The helix-destabilizing protein HD40 was prepared exactly as described [34]. The crude ribosomes obtained in step 1 of the procedure were dissolved in buffer B and further purified on a 10–30% (w/v) sucrose gradient centrifuged in the Beckman SW27 rotor at $54800 \times g$ for 16.5 h and at 4 °C. Ribosomal subunits were prepared from ribosomes purified by centrifugation through a 10-ml 50% (w/w) sucrose layer. Centrifugation was in the Beckman R60 rotor at $251800 \times g$ for 17 h and at 4 °C. The pellet was dissolved in buffer A and was layered on a 15–50% (w/w) sucrose gradient. Centrifugation was in the Beckman zonal Ti14 rotor at $91000 \times g$ for 18 h and at 4 °C. Ribosomal RNA was removed from gradient fractions by precipitation with 2 M LiCl. Proteins were precipitated from the supernatant with 10% trichloroacetic acid and analysed by polyacrylamide/dodecylsulfate gel electrophoresis.

RNA Preparation

Poly(A)-containing RNA was isolated from the postmitochondrial supernatant prepared in buffer K and 150 mM sucrose from 200 g (dry weight) of cryptobiotic embryos. RNA was obtained by digestion of the postmitochondrial supernatant with proteinase K (2.5 µg/mg protein) for 5 h at 20 °C and in the presence of 0.5% (w/v) sodium dodecylsulfate. The digest was adjusted to 500 mM NaCl and stirred with oligo(dT)-cellulose (1 g/50 ml) for 15 h. After extensive washing with binding buffer L, bound poly(A)-containing RNA was eluted from oligo(dT)-cellulose in 10 mM Hepes pH 7.5 and at 40 °C. RNA was precipitated twice with 2 vol. ethanol and 0.1 vol. 20% potassium acetate (w/v) pH 5.0 and further purified by precipitation in 2 M LiCl.

RNA 3′-end labelling was as described by England et al. [36]. RNA was precipitated from the reaction mixture with 2 vol. ethanol and dissolved in formamide before analysis by polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis

Analysis of proteins by 10% (w/v) polyacrylamide/dodecylsulfate gel electrophoresis was as described [6]. Electrophoresis on 12.5% (w/v) polyacrylamide gels was performed according to the procedure of Laemmli [37]. Sequences up to ± 150 nucleotides were analyzed by 20% (w/v) polyacrylamide gel electrophoresis (acrylamide/bisacrylamide, 39/1, w/w) in buffer D. Electrophoresis was at 300 V and in buffer E. Gels with a thickness of 0.7 mm and a length of 20 cm were preelectrophorezed for 12 h at 300 V. RNA samples dissolved in formamide were denatured by heating at 70 °C for 5 min. Autoradiograms were scanned with a Zeineh soft laser densitometer (Biomed Instruments Inc., Chicago, IL, USA).

Amino Acid Analysis

Protein samples were hydrolyzed in 6 M HCl at 110 ± 2 °C in evacuated tubes under nitrogen for 24 h. The amino acid composition was determined with a Jeol J.L. C 6 AH automatic amino acid analyzer (Jeol, Tokyo, Japan) according to Spackman et al. [38]. Tyrosine and tryptophan were estimated spectrophotometrically [39]. Half-cystine was determined as cysteic acid [40,41]. Dimethylarginine was purified from conjugated dyes as described by Chang et al. [42]. The amino acid concentration was measured by the Kjeldahl method (see below). The ninhydrin color yield ratio of dimethylarginine/arginine is 1.69.

Determination of Protein Concentration by Absorbance at 280 nm

The relation between protein concentration and absorbance at 280 nm was determined from the nitrogen content

of protein samples measured by the Kjeldahl method [43]. Ammonia resulting from protein destruction in 18 M H_2SO_4 was distilled into 25 ml of 2% H_3BO_3 with a Kjeltic System I 1002 distilling unit (Tecator, Helsingborg, Sweden). The amount of ammonia was determined from potentiometric titration.

Assays

Poly(A) sequences were assayed by hybridization with [^{3}H]poly(U) as described [33].

Poly(A) polymerase activity was measured in 50 mM Tris/HCl pH 8.3, 2 mM $MnCl_2$, 0.12 mM [^{3}H]ATP (41 counts min^{-1} $pmol^{-1}$) and 15–50 µg $(A)_4$ as primer. The reaction mixture of 150 µl was incubated at 37 °C for 30 min. The reaction was stopped by addition of 1 ml 5% (w/v) trichloroacetic acid, 2% (w/v) tetrasodium pyrophosphate. After 5 min the acid-precipitable radioactivity was collected on glass-fiber filters.

The complex formation between proteins and [^{3}H]poly(U) (38000 counts min^{-1} μg^{-1}) or [^{3}H]poly(A) (41000 counts min^{-1} μg^{-1}) was done in buffer G. Polynucleotide (± 0.8 µg) was mixed with HD40 or poly(A)-binding protein. A 50-µl reaction mixture was incubated for 1 min at 0 °C, diluted with 1 ml of ice-cold buffer G and slowly filtered through a Millipore filter (HA 0.45 µm). The filters were soaked in 0.5 M KOH for 30 min, rinsed with distilled water and equilibrated with buffer G before use.

RESULTS

Purification of Poly(A)-Binding Proteins from Non-polysomal mRNP

Previously we have shown that 38000-M_r and 23500-M_r proteins are the main poly(A)-binding proteins of the free cytoplasmic mRNP and the mRNP associated with ribosomes in non-polysomal complexes [6]. The main 38000-M_r protein was purified from cytoplasmic mRNP in three steps.

Step 1. Poly(A)-containing mRNP was separated from the postmitochondrial supernatant by affinity chromatography on oligo(dT)-cellulose in buffer L. After extensive washing of the column with buffer L, mRNP proteins were released from the bound particles by differential salt washing with 500 mM KCl and 1 M KCl in 10 mM Hepes, pH 7.2. The eluted proteins were analyzed by sodium dodecylsulfate gel electrophoresis (Fig. 1). The main proteins are those mRNP proteins previously identified [6].

Step 2. Proteins eluted with 1 M KCl were dialyzed against buffer C and fractionated by affinity chromatography on poly(A)-Sepharose 4B. Bound proteins were eluted in buffer C supplemented with 500 mM, 750 mM and 1.5 M KCl. The 38000-M_r and 23500-M_r poly(A)-binding proteins were completely absent from the 500 mM KCl wash. A small amount of poly(A)-binding proteins is enriched in the 750 mM KCl wash. The majority of the 38000-M_r protein eluted in the 1.5 M KCl fraction (Fig. 1).

Step 3. The 1.5 M KCl eluate was concentrated by ultrafiltration on an Amicon PM10 filter. A fraction of the 23500-M_r poly(A)-binding protein was removed in this step. The concentrated 38000-M_r protein sample was dialyzed against buffer R and freed from contaminating RNA by chromatography on DEAE-cellulose. The 38000-M_r protein was not retained in these conditions. Analysis by sodium dodecylsulfate gel electrophoresis showed that the 38000-M_r protein preparation has a purity of more then 95% (Fig. 1).

Characterization of the 38000-M_r Poly(A)-Binding Protein

The molecular weight of the main poly(A)-binding protein was determined by sodium dodecylsulfate gel electrophoresis and varied between 38000 and 38900 with a mean value of 38700.

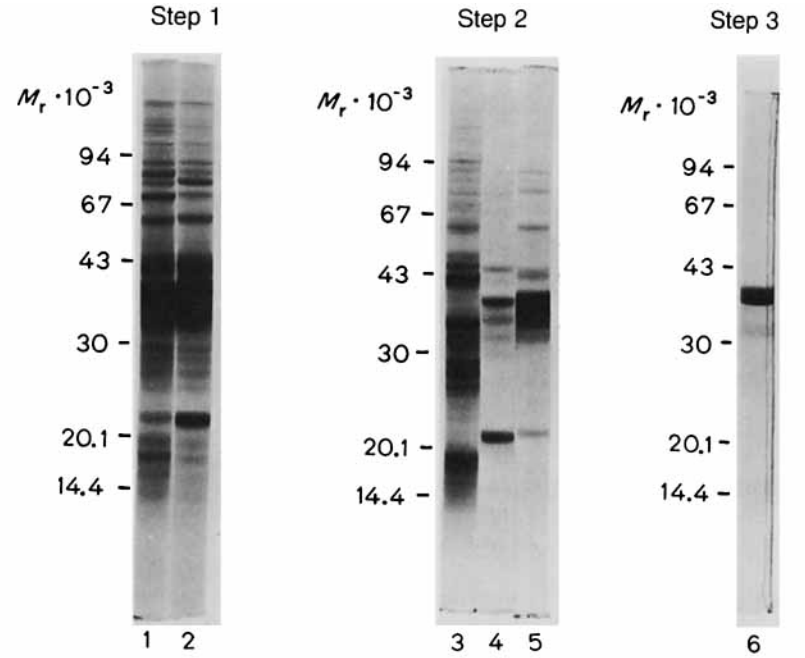

Fig. 1. *Analysis by sodium dodecylsulfate gel electrophoresis of fractions obtained in the purification of poly(A)-binding protein.* Step 1: proteins released from mRNP bound to oligo(dT)-cellulose in 10 mM Hepes pH 7.2 and 500 mM KCl (1), and 1 M KCl (2). Step 2: affinity chromatography on poly(A)-Sepharose 4B of mRNP proteins eluted by 1 M KCl. Bound proteins were eluted in buffer C and 500 mM KCl (3), 750 mM KCl (4) and 1.5 M KCl (5). Step 3: purification of poly(A)-binding proteins by ultrafiltration and DEAE-cellulose chromatography (6)

Table 1. *Amino acid composition of the 38000-*M_r *poly(A)-binding protein and the helix-destabilizing protein HD40*

Amino acid	Amount in		
	poly(A)-binding protein	HD40	
		(ours)[a]	[34][b]
	mol/100 mol protein		
Lysine	8.9	9.2	7.7
Histidine	1.7	2.1	1.8
Arginine	2.4	3.5	2.2
Dimethylarginine	1.5	1.2	1.4
Aspartic acid/asparagine	9.7	9.6	10.5
Threonine	3.6	3.9	3.9
Serine	3.4	5.1	5.1
Glutamic acid/glutamine	11.5	11.9	11.4
Proline	4.4	3.4	4.6
Glycine	22.5	18.2	19.1
Alanine	5.3	6.0	7.3
Cysteine	0	0	0
Valine	4.8	5.4	5.5
Methionine	3.4	2.6	1.5
Isoleucine	3.4	3.7	3.6
Leucine	3.2	4.6	3.8
Tyrosine	5.4	4.8	5.5
Phenylalanine	3.9	4.0	4.1
Tryptophan	0.8	0.7	0.9

[a] Prepared in our laboratory exactly as described by Szer et al. [34,35].

[b] Taken from [34].

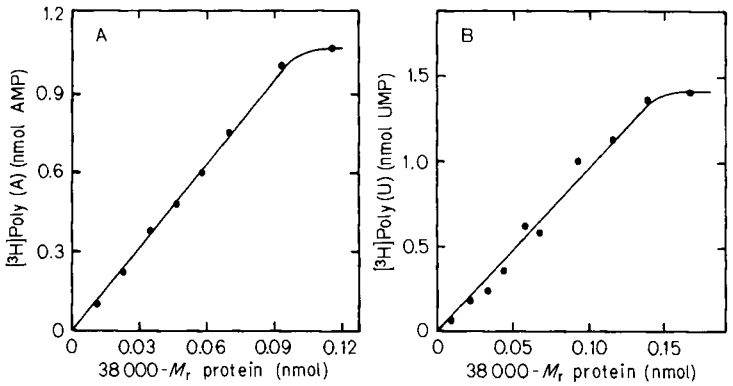

Fig. 2. *Stoichiometry of polynucleotide-protein complexes.* A constant amount of [^{3}H]poly(A) equivalent to 1.59 nmol AMP (A) or [^{3}H]poly(U) equivalent to 2.65 nmol UMP (B) was mixed with different amounts of 38000-M_r protein in buffer G. The amount of polynucleotides retained on nitrocellulose filter is plotted as equivalents of AMP or UMP against protein concentration

The spectrum of the protein has a minimum at 253 nm, a maximum at 276 nm and a shoulder at 285 nm. A purified sample is characterized by an A_{280}/A_{260} ratio of 1.44 and an A_{max}/A_{min} ratio of 1.89.

From a minimum nitrogen content of 16.37% calculated from the amino acid composition (Asx and Glx taken as aspartic acid and glutamic acid), a minimum absorption coefficient of $A^{1\%}_{280\,nm} = 8.2\ cm^{-1}$ was measured from nitrogen determinations of purified protein samples. The maximum value of $A^{1\%}_{280\,nm}$ is $9.8\ cm^{-1}$. The amino acid composition is presented in Table 1. The protein has a very high glycine content of 22.5% but is devoid of cysteine. An amino acid eluted between ammonia and arginine in the position of dimethylarginine; four or five dimethylarginine residues are present in the poly(A)-binding protein. The amino acid composition of the 38000-M_r poly(A)-binding protein is different from the composition of a mixture of poly(A)-binding proteins isolated from rat liver [9]. Although the amount of charged amino acids is similar, a large difference is observed in the amount of hydrophobic and polar uncharged amino acids.

Previously we have demonstrated that the 38000-M_r protein is bound to poly(A)-Sepharose 4B and poly(U)-Sepharose 4B but not to rRNA-Sepharose 4B [6,46]. The stoichiometry of protein-polynucleotide complexes was calculated from nitrocellulose filter-binding experiments. The results are presented in Fig. 2. A stoichiometry of one 38000-M_r protein molecule per 9–11 adenylate residues and 10–12 uridylate residues was calculated.

Identification of 38000-M_r *Poly(A)-Binding Protein*

Assays with purified poly(A)-containing mRNP, salt-washed mRNP proteins or purified 38000-M_r poly(A)-binding protein failed to detect any poly(A) polymerase activity with $(A)_4$ as primer. In the assay conditions used an average length of 36 nucleotides was added to $(A)_4$ by cytoplasmic poly(A) polymerase (E. Roggen and H. Slegers, unpublished results). Nor was it possible to demonstrate any RNase activity in a purified 38000-M_r protein sample. Szer and collaborators [34,35] isolated a helix-destabilizing protein HD40 from a crude ribosomal fraction of *Artemia salina* cryptobiotic embryos. However, ribosomes prepared by differential centrifugation and purified by sucrose gradient centrifugation as well as the derived ribosomal subunits did not contain a 38000–40000-M_r protein (Fig. 3). The presence of the latter protein in the crude ribosomal pellet indicated a possible contamination by soluble proteins or mRNP particles. The resemblance in molecular weight prompted us to compare the HD40 protein and the 38000-M_r poly(A)-binding protein isolated from cytoplasmic non-polysomal mRNP. HD40 prepared exactly as described by Szer and collaborators [34, 35] and the poly(A)-binding protein were coelectrophorezed on sodium dodecylsulfate/polyacrylamide gels (Fig. 4). Both proteins have the same mobility indicating similar molecular weights. In Table 1 the amino acid composition of HD40 prepared in both laboratories is compared with the composition of the poly(A)-binding protein. Apart from a few experimental differences, the similarity in composition is obvious. A possible identity between both proteins is further strengthened by the comparable stoichiometry of protein-polynucleotide complexes of HD40 and 38000-M_r proteins [34,35].

Structure of the Poly(A)-protein Complex

Poly(A)-containing mRNA is very sensitive to nuclease degradation resulting in the removal of the poly(A) sequence. In $MgCl_2$-containing buffers and in the absence of RNase inhibitors, the poly(A) sequence isolated by affinity chromatography on oligo(dT)-cellulose sedimented at a mean value of 4 S (data not shown). The latter value is equivalent to a length of 70–80 nucleotides. This value is lower then the length of 126 nucleotides reported by James and Tata [47] but is in agreement with a length of 65–70 nucleotides measured by Nilsson and Hultin [48]. The length was determined more accurately by polyacrylamide gel electrophoresis of 3'-end-labelled poly(A)-containing RNA. The conditions used re-

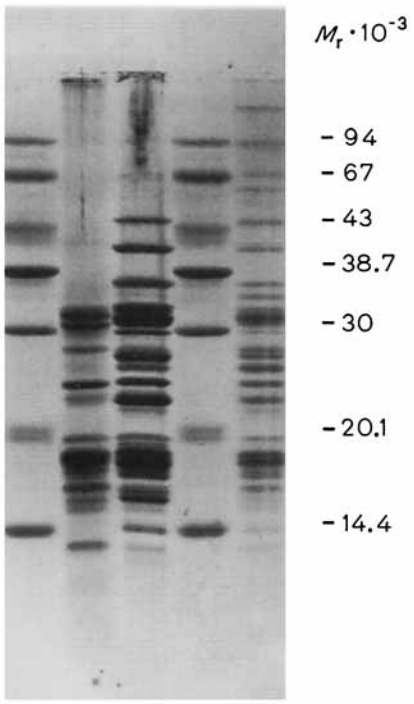

Fig. 3. *Absence of 38000-*M_r *poly(A)-binding protein in purified ribosomes and ribosomal subunits.* A crude ribosomal fraction was obtained by differential centrifugation in buffer B. 200 A_{260} units were purified on a linear 10–30% (w/v) sucrose gradient in buffer B. Centrifugation was in the Beckman SW27 rotor at 54800 × *g* for 16.5 h and at 4 °C. Fractions of the gradient were precipitated with trichloroacetic acid and analyzed on 12.5% polyacrylamide gels as described by Laemmli [37]. Lane 1, marker proteins and 38000-M_r protein; lane 2, 40-S ribosomal protein; lane 3, 60-S ribosomal proteins; lane 4, marker proteins and 38000-M_r protein; lane 5, 80-S ribosomal proteins

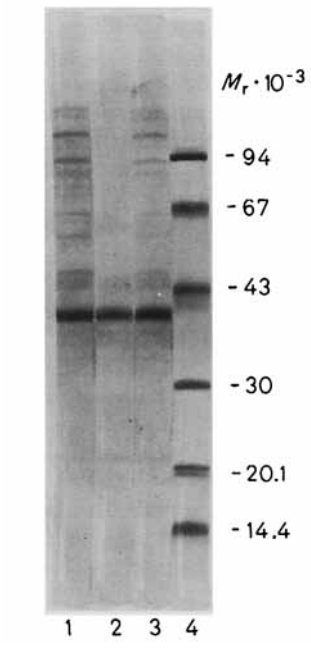

Fig. 4. *Comparison of HD40 helix-destabilizing protein and 38000-*M_r *poly(A)-binding protein by sodium dodecylsulfate/polyacrylamide gel electrophoresis.* Lane 1, HD40 protein isolated as described [34,35]; lane 2, 38000-M_r poly(A)-binding protein; lane 3, mixture of HD40 and poly(A)-binding protein; lane 4, marker proteins. Electrophoresis was described previously [6]

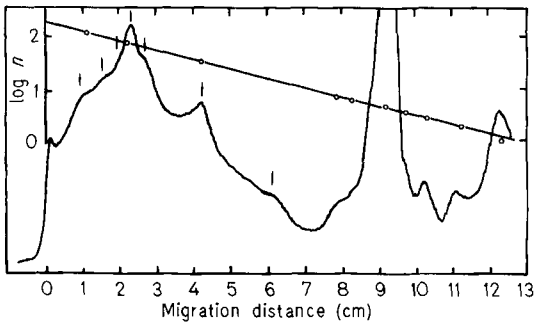

Fig. 5. *Analysis of poly(A) sequences by gel electrophoresis.* Poly(A)-containing RNA was purified from a proteinase-K-digested postmitochondrial supernatant by affinity chromatography on oligo(dT)-cellulose. Labelled poly(A)-containing RNA was analyzed on 20% (w/v) polyacrylamide gels in buffer D. The autoradiogram was scanned with a densitometer. Unlabelled tRNA and 5-S rRNA were run on parallel lanes. The length $n = 31 \pm 1$ was determined separately from a 'ladder' obtained by random degradation and was used as internal marker. The 'ladder' $n = 1$ up to $n = 7$ results from RNA degradation and was used to determine the oligonucleotide length/migration distance relation more accurately. The poly(A) length (Table 2, expt 3) is indicated

Table 2. *Length of poly(A) sequences*

Peak	Length in expt				Length difference between successive oligonucleotides
	1	2	3	mean	
1	126	138	138	134	19–20
2	112	120	112	114–115	17–18
3	97	100	94	97	22
4	76	75	73	75	20
5	57	53	56	55	24
6[a]	31	31	31	31	19–20
7	12	n.d.[b]	13	12–13	

[a] The length of sequence 6 was determined from a 'ladder' obtained by random degradation and was used as internal marker.
[b] Not detected.

sulted in the separation of sequences up to approximately 150 nucleotides. The main sequence had a length of 75 nucleotides. Additional sequences were detected ranging from 12 up to 138 nucleotides. The distribution is heterogeneous, but at least five discrete bands were clearly resolved (Fig. 5, Table 2). A length difference of approximately 20 ± 2 nucleotides is observed between successive oligonucleotides indicating a periodicity in poly(A) length *in vivo*. The periodicity obtained from poly(A) sequence analysis is compared with the stoichiometry measured from protein-binding studies and the stoichiometry estimated from the mean poly(A) length and protein content of mRNP. From the protein content of mRNP, of which 60–70% is 38000-M_r poly(A)-binding protein [6], we can calculate that six or seven proteins are bound to a poly(A) sequence of 75 nucleotides, equivalent to a stoichiometry of 11–13 nucleotides. Approximately the same value is obtained from protein-polynucleotide binding experiments. The stoichiometry of the 38000-M_r protein complex with poly(A) and the periodicity in poly(A) length, which is twice the stoichiometric value, support a pairwise repetition of 38000-M_r proteins on the poly(A) sequence.

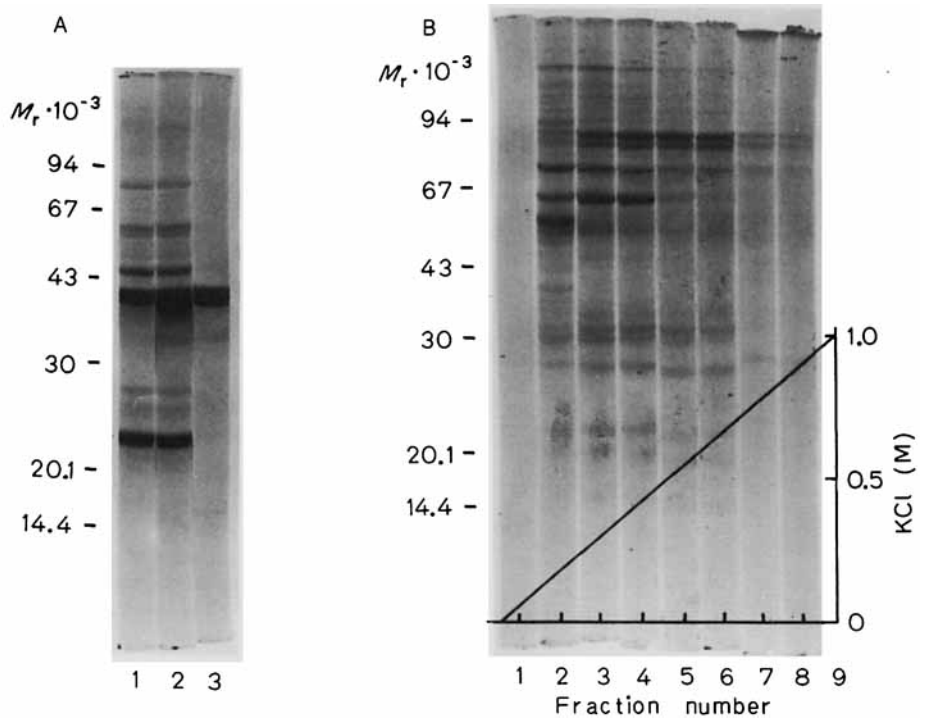

Fig. 6. *Analysis of membrane-associated poly(A)-binding proteins by dodecylsulfate gel electrophoresis.* (A) Comparison of the composition of membrane-bound poly(A)-containing mRNP with the poly(A)-binding protein of cytoplasmic mRNP. Lane 1, proteins of membrane-bound mRNP; lane 2, mixture of proteins of membrane-bound mRNP and the poly(A)-bound protein of cytoplasmic mRNP; lane 3, poly(A)-bound protein of cytoplasmic mRNP. (B) Affinity chromatography on poly(A)-Sepharose 4B of membrane proteins. Bound proteins were eluted in buffer C with a linear salt gradient of 0.05–1.5 M KCl. Lanes 1–8, fractions eluted between 0.05–0.9 M KCl

Membrane-Associated Poly(A)-Binding Proteins

The cytoplasmic poly(A)-binding proteins were compared with those isolated from the membrane fraction. The membrane fraction was purified by differential centrifugation, dissolved in 1% (v/v) Triton X-100 and subjected to affinity chromatography on oligo(dT)-cellulose to isolate poly(A)-containing mRNP. The protein composition of mRNP is presented in Fig. 6A. Proteins with M_r of 76000, 60000, 45000, 38000 and 21500 constitute the membrane-bound mRNP. The composition is comparable to those of poly(A)-containing free mRNP. Coelectrophoresis of the main 38000-M_r poly(A)-binding protein of free mRNP with membrane-bound mRNP proteins indicate that this protein is also the main constituent of membrane-bound mRNP.

From the fraction not bound to oligo(dT)-cellulose membrane proteins were isolated and chromatographed on poly-(A)-Sepharose 4B. The bound proteins were released by a salt gradient and analyzed by polyacrylamide/dodecylsulfate gel electrophoresis (Fig. 6B). A discrete number of proteins were eluted from the poly(A)-Sepharose 4B column. These proteins have M_r of 133500, 90000, 86000, 73000, 60000 and 53000, clearly lacking the poly(A)-binding proteins of free and membrane-bound mRNP.

These observations indicate that the 38000-M_r poly(A)-bound protein is present on non-polysomal mRNP of different cellular compartments and therefore may probably not be involved in the binding of mRNP to membranes. Beside a pool of cytoplasmic poly(A)-binding proteins [6] a class of membrane-bound proteins can be isolated with affinity for the poly(A) sequence. The function of the latter proteins is still unknown.

DISCUSSION

Cytoplasmic non-polysomal poly(A)-containing messenger ribonucleoproteins of cryptobiotic gastrulae of *Artemia salina* are complexed with a discrete number of proteins with M_r of 87000, 76000, 65000, 50000, 45000, 38000 and 23500. The 38000-M_r and 23500-M_r proteins are poly(A)-binding proteins [6]. The latter proteins are present in a free state in the cytoplasm and on mRNP isolated from the cytoplasm and from the membrane fraction. Membranes did not contain these poly(A)-binding proteins, indicating that their localization is restricted to mRNP and to the free cytoplasmic protein pool.

The main 38000-M_r poly(A)-binding protein was isolated and purified from cytoplasmic non-polysomal mRNP. This protein consists of approximately 357 amino acids. It is very rich in glycine but is devoid of cysteine. The high glycine content and the presence of dimethylarginine are also characteristics of hnRNP proteins and indicate a possible relationsship between poly(A)-binding proteins and some hnRNP proteins [44, 45].

The molecular weight, amino acid composition and the protein-poly(A) stoichiometry are comparable to those of HD40, a helix-destabilizing protein isolated by Szer and collaborators [34, 35] from a crude ribosomal fraction of *A. salina*. From preparations of HD40 in our laboratory, we may conclude that it is not a component of 80-S ribosomes. HD40 seems to be identical to the 38000-M_r poly(A)-binding protein and is observed in the ribosomal fraction by association of mRNP with ribosomes or ribosomal subunits [6, 33]. The latter mRNP has exactly the same protein composition as the free mRNP and also contains the 38000-M_r protein as the

main poly(A)-binding protein [6]. The length of the poly(A) sequences of cytoplasmic poly(A)-containing RNA varied from 12 up to 138 nucleotides. The main component has a length of 75 nucleotides in agreement with the value determined by Nilsson and Hultin [48]. A periodicity of 20 ± 2 nucleotides has been observed in poly(A) shortening. A value half that was obtained for the protein-poly(A) stoichiometry. These results support a model where protected and unprotected regions against ribonuclease degradation alternate in the poly(A) segment of mRNP. A pairwise repetition of 38000-M_r protein is consistent with the observed periodicities. The existence of a repeating structure in the poly(A) sequence has been demonstrated in mouse cells [28]. The poly(A) sequence of mRNP is complexed with a 75000-M_r protein and fragments produced by digestion with T_2 RNase showed a periodicity of 27 residues.

The cleavage of the poly(A) tail in a periodical manner has also been reported for duck globin mRNP [49]. The proposed repeating structure is different from the model described by Adams et al. [29]. These authors assume a binding region of 15.3 nm between the 76000-M_r protein and the poly(A) sequence. Our results indicate a much smaller binding region. Szer and collaborators [34,35] reported a recognition site of five nucleotides for the HD40 helix-destabilizing protein. Although the function of the poly(A)-binding proteins is unknown, some of the previously proposed functions can be eliminated from our results. A poly(A) polymerase activity is often proposed for the poly(A)-binding proteins. Several observations demonstrate that the main poly(A)-binding protein is different from poly(A) polymerase: (a) it is unlikely that this enzyme is complexed in a periodical manner with internal regions of the poly(A) sequence; (b) no poly(A) polymerase activity is demonstrated for mRNP proteins; (c) in *A. salina* the molecular weight of 70000 for poly(A) polymerase [50] is different from the molecular weight of the main poly(A)-binding proteins.

The same 38000-M_r poly(A)-binding protein is present on mRNP from different cellular compartments, indicating that it is not involved in membrane binding. However no conclusion about a transport function can be drawn from this result. Recently Moffet and Webb [51] have isolated cytoplasmic transport factors. Some of these factors have comparable molecular weights to poly(A)-binding proteins.

Our observation of the presence of poly(A)-binding proteins in the membrane and free cytoplasmic fractions which are distinct from mRNP-associated poly(A)-binding proteins demonstrate the existence of at least two classes of proteins with affinity for the poly(A) sequence. These classes are probably involved in different mRNP-associated processes.

At neutral pH poly(A) has a single-stranded stacked structure. Binding of the helix-destabilizing protein HD40 to poly(A) alter the circular dichroic spectrum of the polymer indicating an unstacking of the bases [35]. This unstacking may result from the intercalation of aromatic residues between two neighbouring adenine bases [52]. It may be noted that the same perturbation of the circular dichroic spectrum has been observed in the binding of basic oligopeptides to single-stranded poly(A). The configurational change does not result in an unstacking of the polymer but in a change of the winding angle [53,54]. The unstacking or change in winding angle may be involved in the regulation of polyadenylation or may be a mechanism to make stored mRNA available for the translational machinery [55]. It is already proposed that the stored mRNA is not translated due to interaction of the 3′ and 5′ ends [5,30,56,57]. The interactions in which the poly(A) sequence participates may be modified by the unstacking-stacking mechanism triggered by the association or dissociation of the poly(A)-binding protein from the poly(A)-protein complex or by poly(A) shortening.

The authors are grateful to Mr H. Backhovens for his excellent technical assistance. Dr A. Vandenberghe is acknowledged for suggestions on RNA analysis. E.D.H. is a Research Assistant of the Belgian National Fund for Scientific Research. E.P. is a fellow of the Belgian Institute for Scientific Research in Agriculture and Industry. This investigation is supported by grants from *Fonds voor Kollektief Fundamenteel Onderzoek* of the Belgian National Fund for Scientific Research.

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