Messenger RNA Binding Protein Purified from Reticulocyte Polyribosomes†

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ABSTRACT: One of the proteins in the 0.5 M KCl eluate of rabbit reticulocyte polyribosomes which bind poly(A)-rich mRNA has been purified to apparent homogeneity using ammonium sulfate fractionation and phosphocellulose, hydroxylapatite, and diethylaminoethylcellulose column chromatography. The protein appears to contain two subunits of 66 700 and 56 400 apparent molecular weights with a 1:1 stoichiometry, since an apparent molecular weight of 110 000 was determined using Sephadex G-200 chromatography and an $s^0_{20,w}$ of 5.6 was obtained with rate-zonal sedimentation. The mRNA binding activity banded at pH 5.2–5.5 on isoelectric-focusing polyacrylamide gel electrophoresis. Protein-dependent binding appeared to be specific, since other natural or synthetic RNAs, including tRNA, ribosomal RNA, and poly(riboadenylic acid), were 90- to 250-fold less effective than mRNA at competing for binding of [^{3}H]poly(adenylic acid)-rich mRNA. Poly(riboguanylic acid), however, was even more efficiently bound by this protein than mRNA.

While the factors required for initiation of protein synthesis in prokaryotes have been characterized (Caskey et al., 1972), the factors required for initiation of translation of natural mRNAs in eukaryotic systems are incompletely defined. Proteins which function in the binding of Met-tRNA$_f$ to the small ribosomal subunit in a GTP[1]-dependent or GTP-independent manner have been identified, purified, and characterized (Prichard et al., 1970; Leader and Wool, 1972; Gassor and Moldave, 1972; Levin et al., 1973; Dettman and Stanley, 1973; Gupta et al., 1973; Schreier and Staehelin, 1973; Zasloff and Ochoa, 1973; Merrick and Anderson, 1975; Safer et al., 1975a,b; Walton and Gill, 1975; Adams et al., 1975). A number of additional proteins have been suggested to be involved in translation of natural mRNAs, including a protein apparently analogous to prokaryotic IF-3 (Leader and Wool, 1972; Caskey et al., 1972; Prichard et al., 1970; Schreier and Staehelin, 1973; Adams et al., 1975) and additional protein factors (Gassor and Moldave, 1972; Shafritz et al., 1972; Wigle and Smith, 1973; Cashion and Stanley, 1974; Adams et al., 1975; Merrick et al., 1975). Using a protein-synthesizing system derived from *Artemia salina* embryos, the GTP-dependent Met-tRNA$_f$ binding protein, the protein analogous to IF-3 and two other proteins, largely permitted mRNA translation in this system putatively deficient in initiation factors (Filipowicz et al., 1976); in other systems, requirements for four or six factors for initiation of eukaryotic protein synthesis have been reported (Cashion and Stanley, 1974; Staehelin et al., 1976). The importance of factors found in so-called mRNA-protein particles (Perry and Kelly, 1968; Olsnes, 1970; Spohr et al., 1970; Blobel, 1971, 1972, 1973; Bryan and Hayashi, 1973; Lindberg and Sundquist, 1974; Barrieux et al., 1975) in initiation of mRNA synthesis has also been suggested (Ilan and Ilan, 1973; Cashion and Stanley, 1974). Factors which are critical for introduction of mRNA into the initiation complex might be expected to have a high affinity for mRNA binding to specific regions common to mRNAs. The purification and partial characterization of a protein eluted from rabbit reticulocyte polyribosomes which exhibits high affinity for mRNA and appears to be distinct from other proteins implicated in eukaryotic initiation complex formation are described in this report.

Experimental Procedures

Materials

ATP, GTP, GDP, and GMP were purchased from P-L Biochemicals, Incorp. Synthetic homoribopolymers, the random heteropolymer, poly(AUG), and [^{3}H]poly(G) (37.7 μCi/μmol of P) were purchased from Miles Laboratories, Incorp.; they were sized by sedimentation through linear sucrose gradients and ethanol precipitation prior to use. Carrier-free Na^{125}I and [5,6-^{3}H]uridine (37.6 Ci/mmol) were purchased from New England Nuclear Corp. 7-Methylguanosine was purchased from Cyclo Chemicals and 7-methylguanosine 5′-monophosphate from Terra Marine Bioreagents. 4S, 18S, and 28S RNA were prepared by rate-zonal sedimentation from Ehrlich ascites cells.

Methods

Preparation of [^{3}H]mRNA. [^{3}H]Uridylate-labeled poly(A)-rich mRNA (4.16×10^3 cpm/μg) was prepared from Ehrlich ascites tumor cell cytoplasmic RNA as previously described (Barrieux et al., 1975) using oligo(dT) cellulose chromatography (Aviv and Leder, 1972).

Preparation of mRNA Binding Factor. Rabbit reticulocytes were extensively washed to remove the buffy coat, and polyribosomes were prepared as previously described (Walton and Gill, 1975). The 0.5 M KCl eluate of polyribosomes was adjusted to 70% saturation with respect to ammonium sulfate; the precipitate was collected by centrifugation at 15 000g for 15 min and resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 100 mM KCl

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‡ Recipient of PHS Research Career Development Award No. AM00078 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

[1] Abbreviations used are: poly(A), poly(riboadenylic acid); poly(G), poly(riboguanylic acid); tRNA, transfer RNA; rRNA, ribosomal RNA; GTP, GDP, and GMP, guanosine tri-, di-, and monophosphates; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE I: Purification of mRNA Binding Factor.[a]

Step	Total Protein (mg)	Total Act. (μg of mRNA bound)	Sp Act. (μg of mRNA/μg of protein)
0.5 M KCl eluate	380		
70% ammonium sulfate fraction	350		
Phosphocellulose chromatography	7	252	0.036
Hydroxylapatite chromatography	0.24	151	0.630
DEAE-cellulose chromatography	0.18	130	0.720

[a] The 0.5 M KCl eluate of reticulocyte polyribosomes was purified as described under Methods; aliquots of each fraction were used to quantitate mRNA binding. Results are the average of duplicate determinations.

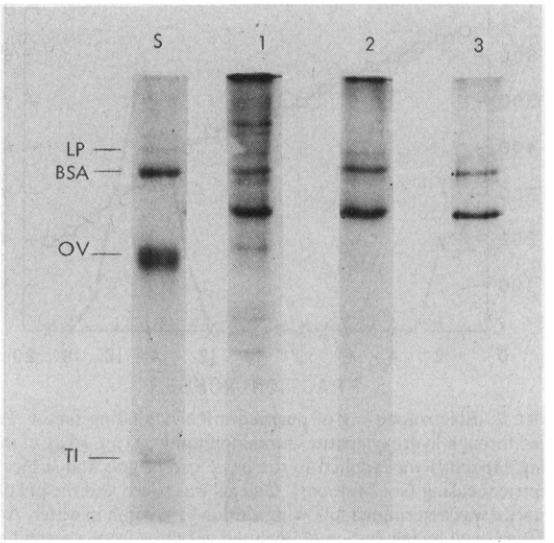

FIGURE 1: Polyacrylamide gel analysis of mRNA-binding protein. Fractions were iodinated, adjusted to 1% sodium dodecyl sulfate reduced, subjected to polyacrylamide gel electrophoresis using a 6 to 12% polyacrylamide gradient, and analyzed via radioautography (see Methods). S = standards: bovine serum albumin (BSA, 66 500); ovalbumin (OV, 45 500); soybean trypsin inhibitor (TI, 21 500); (1) protein purified through the phosphocellulose step; (2) protein purified through DEAE-cellulose and; (3) fractions of pI 5.2–5.5 eluted with 1% sodium dodecyl sulfate following isoelectric focusing (see legend, Figure 3). The position of migration of the traces of immobilized iodinated lactoperoxidase dissociating during the iodination procedure is indicated LP.

(buffer A). It was dialyzed against the same buffer for 18 h and applied to a 1.5 × 5 cm phosphocellulose column previously equilibrated against the same buffer. The column was washed extensively with buffer A until no further material was eluted; batch elution was performed using buffer A containing 0.2 M KCl; the eluted material was dialyzed against 10 mM potassium phosphate (pH 7.5), 10% glycerol, 1 mM dithiothreitol (buffer B) and applied to a 1 × 5 cm hydroxylapatite column previously equilibrated against the same buffer. The column was successively eluted with buffer B containing 0.1, 0.2, 0.4, and 0.6 M potassium phosphate. The eluted material was extensively dialyzed against buffer A and applied to a 0.5 × 10 cm DEAE-cellulose column previously equilibrated with the same buffer. Batch elutions were performed using buffer A containing 0.1, 0.2, 0.4, and 0.6 M KCl. The 0.2 M KCl fractions were concentrated by dialysis against buffer A containing 9% (w/v) polyethylene glycol (molecular weight 15 000–20 000) and stored at −70 °C. Protein was quantitated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Isoelectric focusing was performed in 4% polyacrylamide gel as previously described (Righetti and Drysdale, 1970), except that the NaOH concentration was 0.05 N and the time of focusing was 12–14 h. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and radioautography were performed as previously described (Barrieux et al., 1975).

Assay for mRNA Binding. The assay was performed in a final volume of 50 μl, containing 20 mM Tris-HCl (pH 7.5), 30 mM KCl, 1 mM dithiothreitol (buffer C), 0.5–2 μg of [^{3}H]poly(A)-rich mRNA, the indicated amounts of other RNAs, and the protein fraction to be assayed. Unless otherwise stated, incubations were for 10 min at 30 °C, and reactions were terminated by the addition of 1 ml of ice-cold buffer C, applied to nitrocellulose filters (Millipore, 0.45 μm) and washed with 20 ml of buffer C. The filters were dried and counted in 5 ml of toluene–Liquifluor. The binding of [^{3}H]poly(A)-rich RNA to Millipore filters in the absence of added protein represented <0.3% of added RNA, and was subtracted from each experimental result.

Results

Identification of mRNA Binding Activity in Ribosomal Eluate. Multiple mRNA binding activities appeared to be present in the 0.5 M KCl ribosomal wash, since significant mRNA binding was found in the 0.1, 0.2, 0.4, and 0.6 M KCl fractions following phosphocellulose chromatography (data not shown). Ten to fifteen percent of the total [^{3}H]mRNA binding activity was recovered in the 0.2 M KCl eluate; this activity demonstrated an identical elution profile on rechromatography. Virtually all of the mRNA binding activity in this fraction was retained on hydroxylapatite and >80% of the activity was eluted in the 0.4 M potassium phosphate fraction. The mRNA binding activity in this fraction was recovered in the 0.2 M KCl eluate following DEAE-cellulose chromatography. A summary of the purification scheme is presented in Table I. Since the mRNA binding activity of the 0.5 M KCl eluate was heterogeneous, total recovery could not be accurately determined. Binding of mRNA was dependent upon concentration of protein added, and upon time of incubation. Binding at 30 °C was essentially complete at 15–30 s; even at 4 °C, binding was complete by 30–60 s.

Characterization of the mRNA Binding Protein. Analysis of the DEAE-cellulose fraction by polyacrylamide gel electrophoresis following protein iodination and reduction in sodium dodecyl sulfate revealed two proteins of 56 400 ± 2500 and 66 700 ± 1500 apparent molecular weights by protein staining. Radioautography, which permitted detection of even nanogram quantities of proteins, revealed minimal amounts of other proteins and traces of iodinated lactoperoxidase, a contaminant of the iodination procedure (Figure 1). Isoelectric focusing under nondenaturing conditions revealed one major peak of mRNA binding activity having an isoelectric point of 5.2–5.5 (Figure 2). A minor peak observed at the basic end of the gel where the sample was applied was believed to represent aggregates too large to penetrate the gel (Figure 2). Protein in the mRNA binding area was eluted with 1% sodium dodecyl sulfate, iodinated, reduced, and subjected to sodium dodecyl sulfate, polyacrylamide gel electrophoresis; both the 56 400 and 66 700 molecular weight proteins were presnet (Figure 1). Analysis by rate-zonal sedimentation (Figure 3) revealed an

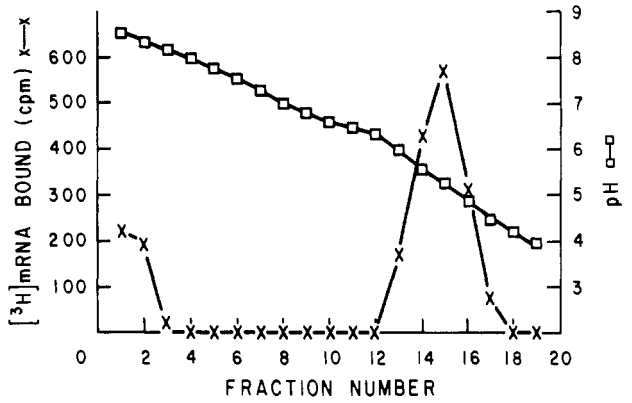

FIGURE 2: Electrofocusing of purified mRNA binding factor. Protein purified through hydroxylapatite chromatography (20 μg, 15 μg of mRNA binding capacity) was applied to the basic end of gels and subjected to isoelectric focusing (see Methods). One gel was sliced and the pH of each 2-mm slice was determined following elution for 30 min in water. Activity was determined by incubation of uneluted gel slices from a second gel for 10 min at 30 °C in a mixture containing 5 μg of [^{3}H]mRNA (12 500 cpm/μg); the gel slices were then extensively washed with buffer C, placed on a nitrocellulose filter, washed with 20 ml of buffer C, dried, and counted in toluene–Liquifluor. The nonspecific binding (40 cpm), determined by incubation of slices of a gel to which no protein had been applied, was subtracted from each result.

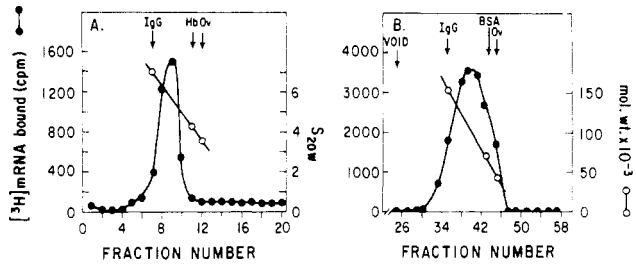

FIGURE 3: Rate-zonal sedimentation and Sephadex G-200 chromatography of mRNA binding protein. Protein purified through hydroxylapatite step (5 μg, 6 μg of mRNA binding capacity) was: (A) applied to a 10–30% linear glycerol density gradient in 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and centrifuged at 4 °C for 19 h at 45 000 rpm using an SW 56 rotor. Fractions containing 140 μl were collected from the bottom, 15-μl aliquots were assayed for mRNA binding activity (see Methods), and the remainder was analyzed by polyacrylamide gel electrophoresis; (B) applied in 0.5 ml to a 0.2 × 100 cm Sephadex G-200 column previously equilibrated with 10 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, and eluted, using the same buffer. Fractions of 2.2 ml were collected and aliquots of 0.4 ml were assayed for mRNA binding activity. In both A and B, the standards used were IgG, bovine serum albumin (BSA), horse hemoglobin (Hb), and ovalbumin (Ov). Migration of standards on Sephadex G-200 chromatography was determined in parallel by measuring A_{280} prior to and following chromatography of the mRNA binding protein; peak fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis to confirm their identity.

$s_{20,w}$ of 5.6–5.8, suggesting a molecular weight of >100 000; analysis of peak fractions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, confirmed the presence of both the 56 400 and 66 700 molecular weight proteins. Similar results were obtained using Sephadex G-200 chromatography (Figure 3). The mRNA binding protein eluted with an apparent molecular weight of 100 000; when subsequently subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, two proteins of 56 000 and 67 000 apparent molecular weights were observed.

Specificity of the mRNA Binding Protein. The relative affinity of the protein for mRNA compared to other natural and

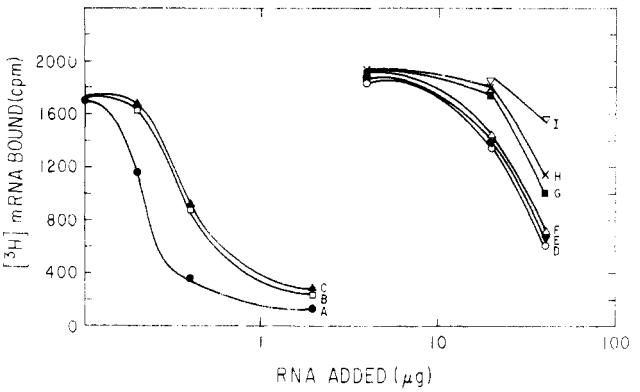

FIGURE 4: Competition of mRNA binding by natural and synthetic RNAs. mRNA binding protein purified through the hydroxylapatite step (0.1 μg) was added to a reaction mixture containing 0.4 μg of [^{3}H]mRNA (16 500 cpm/μg) and the indicated amount of unlabeled RNA. Binding was quantitated following incubation for 10 min at 30 °C; results are the average of duplicate determinations differing by less than 3%; [^{3}H]mRNA retention in the absence of added protein (50 cpm) was subtracted from each value. Unlabeled RNA added was: (A) poly(G), (B) poly(A)-rich mRNA from Ehrlich ascites tumor cells, (C) poly(A)-rich mRNA from rabbit reticulocytes, (D) tRNA, (E) 18S ribosomal RNA, (F) poly(A), (G) poly(A)·poly(U), (H) poly(AUG), (I) poly(U).

TABLE II: Nucleotide Effects on mRNA Binding.[a]

Addition	[^{3}H]mRNA Bound (μg)
None	1.13
GTP (1 mM)	1.08
GDP (1 mM)	0.99
GMP (1 mM)	1.13
7MG (1 mM)	1.13
7MGP (1 mM)	1.08
mRNA (0.1 μM)	<0.01
poly(G) (0.1 μM)	<0.01

[a] mRNA-binding protein (2 μg) purified through the hydroxylapatite step was added to the reaction mixture containing 5 μg of [^{3}H]mRNA (12 500 cpm/μg) and the indicated concentration of GTP, GDP, GMP, 7-methylguanosine (7MG) or 7-methylguanosine 5′-monophosphate (7MGP) and binding was quantitated as described under Methods; the nonspecific retention of [^{3}H]mRNA (45 cpm), determined by omission of binding protein, from the assay was subtracted from each result. Results are the average of duplicate determinations differing by less than 2%.

synthetic RNAs was assessed by quantitating the competition by various species of RNA of radiolabeled poly(A)-rich RNA binding. Messenger RNA from either Ehrlich ascites tumor cell or rabbit reticulocytes were 90- to 250-fold more effective than tRNA, 18S ribosomal RNA, poly(A), poly(U), poly(A)·poly(U), poly(AUG) (Figure 4), or 28S ribosomal RNA or poly(C) (data not shown) at competing for binding, when compared on the basis of micrograms of RNA added. The binding of mRNA was not affected by addition of 1 mM GTP, GDP, GMP, 7-methylguanosine, or 7-methylguanosine 5′-monophosphate (Table II). In contrast, poly(G) was even more effective than mRNA at competing for binding of [^{3}H]mRNA. [^{3}H]poly(G) itself was bound by the mRNA-binding protein, and its binding was competed much more effectively by addition of unlabeled poly(G) than by unlabeled mRNA (Table III). As predicted by the competition studies, the protein exhibited very low binding activity with respect to [^{3}H]poly(A) or [^{3}H]poly(U).

TABLE III: Binding of Poly(G).[a]

Addition	[^{3}H]poly(G) Bound(cpm)
None	84 000
poly(G) (1 μg)	7 000
poly(G) (10 μg)	<1 000
mRNA (1 μg)	82 000
mRNA (10 μg)	43 000
poly(A) (1 μg)	84 000
poly(A) (10 μg)	83 000

[a] mRNA-binding protein (2 μg) purified through the hydroxylapatite step and 0.1 μg of [^{3}H]poly(G) (37.7 μCi/μmol of P) were added to the buffer used for mRNA binding assays prior to incubation at 30 °C for 10 min; where indicated, poly(G)-, poly(A)-, or poly(A)-rich RNA was also added. Retention on nitrocellulose filters was determined as described under Methods; background for assay, determined by omission of the binding protein (2500 cpm), was subtracted from each value.

Discussion

Although many factors required for the translation of eukaryotic mRNAs have been identified (e.g., Prichard et al., 1970; Schreier and Staehelin, 1973; Cashion and Stanley, 1974; Filipowicz et al., 1976), the factors required for insertion of natural mRNAs into the initiation complex remain incompletely defined. Proteins involved in mRNA translation might be expected to have a specific and high affinity for natural mRNAs. Multiple proteins with mRNA binding activity were found to be present in a 0.5 M KCl eluate of reticulocyte polyribosomes. One of these proteins has been purified and appears to have relative specificity for mRNA binding, since most other species of natural or synthetic RNAs were 90- to 250-fold less effective than mRNA at competing for protein-dependent mRNA binding. This magnitude of relative affinities for various species of RNAs has been observed in the case of several initiation factors; thus, the GTP-dependent Met-tRNA$_f$ binding protein has a high affinity of binding for mRNA (Kaempfer, 1974; Hellerman and Shafritz, 1975; Barrieux and Rosenfeld, 1976), and, although it binds other natural and synthetic RNAs such as poly(A) (Hellerman and Shafritz, 1975), it does so with a very much lower affinity (Majumdar et al., 1975, Barrieux and Rosenfeld, 1976). The protein appears to be an acidic protein with an apparent molecular weight around 110 000, as determined using Sephadex-200 chromatography and rate-zonal sedimentation. After reduction, this protein could be dissociated into two subunits of apparent molecular weight 56 400 and 66 700, suggesting a 1:1 stoichiometry. On the basis of the behavior during the purification procedures and properties of the protein, the mRNA binding protein appears different from characterized translation factors, including elongation factors (Hardesty and McKeehan, 1971; Chuany and Weissbach, 1972; Merrick et al., 1975; Safer et al., 1975b), the Met-tRNA$_f$ binding proteins (Levin et al., 1973; Schreier and Staehelin, 1973; Dettman and Stanley, 1973; Gupta et al., 1973; Walton and Gill, 1975; Adams et al., 1975), and the proteins involved in formation of the puromycin-sensitive initiation complex with the 60S ribosomal subunit (Levin et al., 1973; Schreier and Staehelin, 1973; Cashion and Stanley, 1974; Suzuki and Goldberg, 1974; Nombela et al., 1975). The binding of mRNA was so rapid that the K_M for poly(A)-rich mRNA or the V_{max} could not be accurately determined. The very low affinity of this protein for poly(A) and its high affinity for poly(G), exceeding the affinity for even natural mRNA, suggested that a region other than the poly(A) tract was involved in binding. The observation that reticulocyte mRNA, where globin mRNAs represent >90% of the total mRNA, and Ehrlich ascites tumor mRNA had similar affinities for the protein suggested that the region involved in binding was common to most, if not all, mRNAs. The region appears, therefore, to be different from the loops identified at the 3′ terminus proximal to the poly(A) tract (Proudfoot and Brownlee, 1974) or a region at the 5′ portion of mRNA complementary to a region of 18S ribosomal RNA which was reported to involve (A–U) sequences (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). Although no oligo(G) or poly(G) tract has been identified in eukarotic mRNAs, such a region has been proposed in certain viral mRNAs (Pang and Phillips, 1975). This protein is distinct from the translation factor initially reported to be competed by 1000-fold excess of 7-methylguanosine 5′-monophosphate (Hickey et al., 1976) and subsequently suggested to be the initiation factor IF-M$_3$ (Shafritz et al., 1976), since addition of even 10^7 molar excess of that compound failed to inhibit mRNA binding. However, a role for the unique sequence of methylated bases at the 5′ termini of mRNA (Muthukrishnan et al., 1975; Both et al., 1975) in binding of this protein is not excluded. The relationship of this protein to minor protein bands observed in mRNPs remains speculative; the subunits had apparent molecular weights similar to several of the minor polyribosomal mRNP proteins, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Barrieux et al., 1975). Any putative role in the process of mRNA translation remains to be defined for this mRNA binding protein.

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