THERMAL CHROMATOGRAPHY OF EUKARYOTIC MESSENGER RIBONUCLEOPROTEIN PARTICLES ON OLIGO(dT)-CELLULOSE

Evidence for common mRNA-associated proteins in various cell types

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1 Introduction

Eukaryotic mRNAs exist in the cytoplasm as protein-bound complexes (messenger ribonucleoprotein or mRNP) in polysomes (PmRNP) and in several instances as free nonpolysomal (CmRNP) particles [1–3]. The organization and biological function of the proteins in mRNP are not well understood, and the published reports on their variations and diversity remain a matter of considerable dispute [2]. PmRNP derived from a large number of cells contains two major proteins, M_r 50–52 × 10^3 (P52) and 74–78 × 10^3 (P78), and a number of additional polypeptides, usually 2-13, in the 15–150 × 10^3 mol. wt range [2,3,5–16] The P78 protein and a protein of similar size have been reported to be specifically bound to the 3′-poly(A) tracts of PmRNP [5,6,9,12,15] and heterogeneous nuclear RNP (hnRNP) [17]. Several conflicting reports have indicated that the sodium dodecyl sulfate (SDS)–gel electrophoretic patterns of the protein components of PmRNP and CmRNP are either identical [4–6] or different [7,8]. This variability may be partly due to differences in the methods of isolation and also to possible contamination by non-mRNP components [2,3]. Whether or not some common proteins, e.g P78, remain associated with mRNA sequences in CmRNP and PmRNP has not yet been settled [2,3,5–8].

In this report we describe the isolation of poly(A)-containing eukaryotic mRNP by oligo(dT)-cellulose chromatography of subcellular fractions using thermal elution. Using this methodology we have shown that the two classes of mRNP isolated from a wide variety of cells show significant differences in their characteristic protein patterns, although a number of major proteins, including P78, were observed in both classes of mRNP in the cell types examined.

2. Materials and methods

Leg and breast muscles of day 12–14 chick embryos or liver from young adult rats were homogenized in TMKED buffer, 10 mM Tris–HCl, (pH 7.6), 10 mM $MgCl_2$, 0.25 M KCl, 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT) containing 0 25 M sucrose; and postmitochondrial supernatant was prepared as in [18,19]. Ehrlich ascites tumor cells and rabbit reticulocytes were processed to obtain lysates by published procedures [5,20] and fractionated as above. Total polysomes (105 000 × *g*, 1 h) and postpolysomal pellets (255 000 × *g*, 4 h) were used as the source of PmRNP and CmRNP, respectively, and were obtained by further centrifugation of the postmitochondrial supernatant, supplemented with heparin (100 μg/ml) and phenylmethylsulfonyl fluoride (0.25 mM) as in [18,19] Samples of total polysomes, resuspended in TMKED buffer (without

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sucrose) and containing 0.5 M KCl, were centrifuged through a discontinuous sucrose gradient (1.35, 1.6 and 2.0 M sucrose in TMKED buffer at 0.5 M KCl) at 177 000 × *g* for 16 h in a Ti 60 rotor to give KCl-washed free polysomes. After dissociation of the polysomes in 10 mM Tris–HCl, (pH 7.6), 0.25 M KCl and 20 mM EDTA, the samples were used to isolate PmRNP.

About 500 A_{260} units of postpolysomal pellets, suspended in the binding buffer (10 mM Tris–HCl, (pH 7.6), 1 mM EDTA and 0.4 M NaCl) or dissociated polysomes dialyzed against the binding buffer, were stirred with 0.4 g oligo-dT-cellulose (type T_3, Collaborative Res., Inc.) for 6 h at 4°C and packed into a small glass column (5 × 0.6 cm). After washing the column at 4°C stepwise with the binding buffer and a low salt buffer (10 mM NaCl, 10 mM Tris–HCl (pH 7.6)) the bound material was eluted by using a combination of temperature change from 4–45°C and the low salt buffer (fig.1). This was achieved by preheating the buffer and placing the column in a heated water jacket. Finally 50% formamide in low salt buffer was used to remove any residual mRNP not released by elevating the temperature. The fractions containing mRNP were pooled and collected by precipitation with 2 vol. ethanol or centrifugation at 255 000 × *g* for 16 h.

RNA was isolated from the mRNP fractions by extraction with phenol/chloroform/isoamylalcohol [21] and precipitation with ethanol, at pH 5.0, as in [18,19,22]. Radioactive mRNP were isolated by oligo(dT)-cellulose chromatography of the subcellular fractions obtained from chick embryos which were pulse-labeled in ovo by injection with 1 mCi [2,8-^{3}H]-adenosine (spec. act. 45 Ci/mmol, New England Nuclear Corp) and incubation at 37°C for 60 min [22].

Electrophoresis of RNA samples was carried out using 2.5% gels containing 0.5% agarose crosslinked with 0.175% bisacrylamide for 3 h at 4 mA/tube [19]. The gels were scanned at 260 nm, sliced and the radioactivity of each slice was determined [22] The protein components of mRNP were alkylated with iodoacetamide and analyzed by SDS–polyacrylamide gel electrophoresis using 7.5% gels [18,19]. The buoyant density of formaldehyde-fixed mRNP was determined by centrifugation in a preformed CsCl gradient as in [18,19]. The messenger activity of RNA samples was estimated by measuring the amino acid incorporation in a preincubated wheat germ embryo S30 system [23], programmed with the RNA samples as in [18].

3. Results and discussion

The isolation of the two classes of mRNP by oligo(dT)-cellulose chromatography of the subcellular fractions of chick embryonic muscles is shown in fig.1. In both cases about 97% of the applied material was recovered as an early unbound peak. Elution with the low salt buffer at 4°C released about 4–10% of the bound material. The A_{260}/A_{280} ratio of this fraction was about 1.95–2.05, suggesting that it contained mostly protein-free poly(A)$^+$-RNA likely to be present in small amounts in the preparations About 90% of the bound material was subsequently eluted at 45°C with the low salt buffer. The A_{260}/A_{280} ratios of these fractions were 1.40–1.45 (for CmRNP) and 1.55–1.65 (for PmRNP). The remaining 5–10% of the bound material was eluted at 25°C with 50% formamide in low salt buffer, an eluant used for the isolation of eukaryotic hnRNP [12] and PmRNP [6,12]. When subcellular fractions prepared from embryos pulse-labeled with [^{3}H]adenosine were chromatographed, the relative distribution of counts in the 4 fractions gave the same pattern as shown in fig 1 Moreover, rechromatography on oligo(dT)-cellulose of the deproteinized labeled 45°C fractions showed that about 95% of the initial counts could be rebound to and then eluted from the column at 4°C with the low salt buffer, as would be expected of typical poly(A)$^+$RNA.

Electrophoresis of [^{3}H]adenosine labeled RNA obtained by deproteinization of the 45°C fractions (fig 1) shows that both RNA samples gave a similar heterogeneous distribution with about 75% of the counts migrating between 8–25 S (fig.2). This is in agreement with the expected size of most eukaryotic mRNAs [24]. Furthermore, no discrete peaks comigrating with the marker 28, 18 and 4 S RNAs were detected in the samples. Both RNA samples also gave a similar degree of stimulation of amino acid incorporation in a wheat germ embryo S30 system (table 1), thus confirming their mRNA nature. In agreement with this view we have recently observed that total

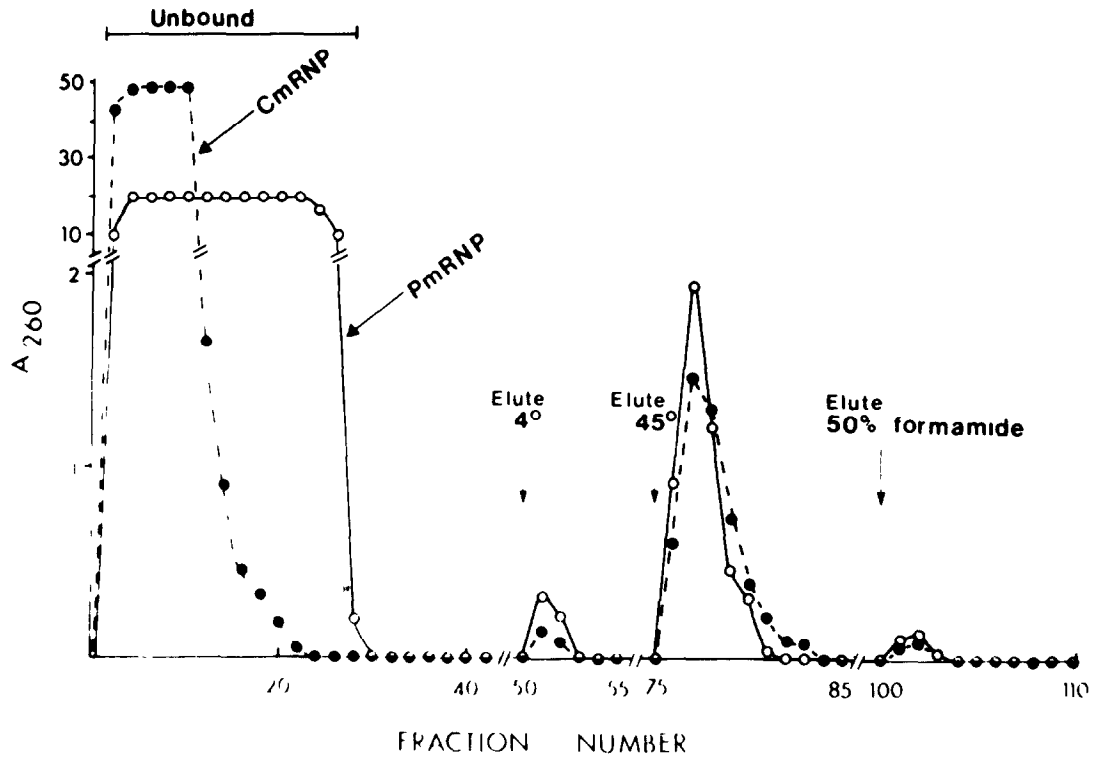

Unbound
CmRNP
PmRNP
50
30
10
2
A 260
Elute 4°
Elute 45°
Elute 50% formamide
20
40
50
55
75
85
100
110
FRACTION NUMBER

Fig 1

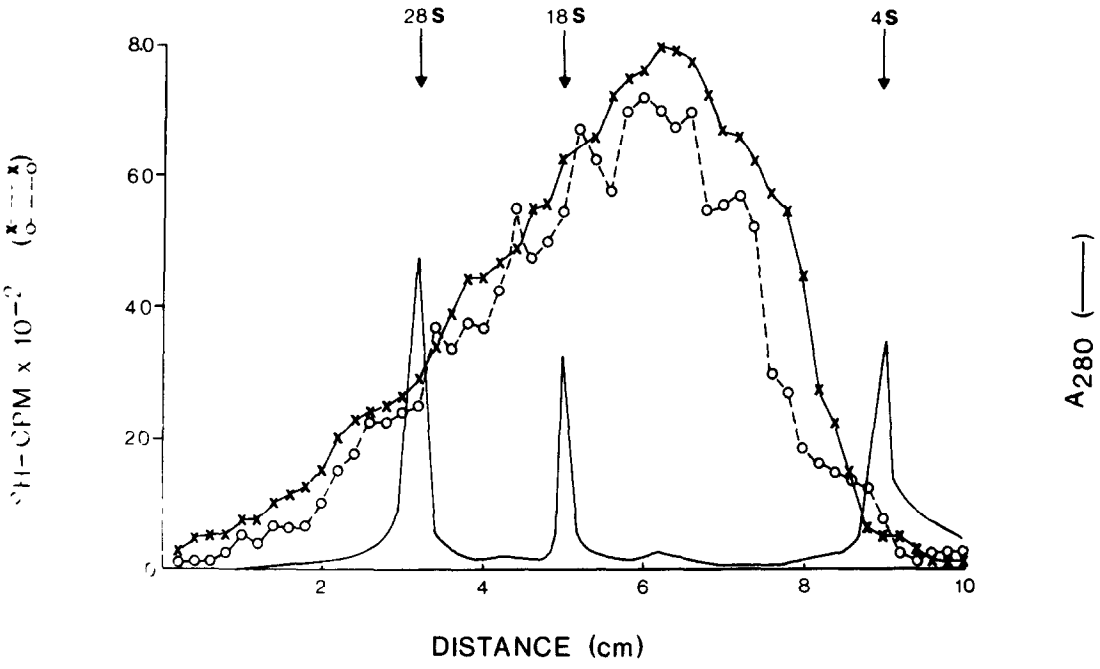

28S
18S
4S
8.0
60
40
20
0
2
4
6
8
10
DISTANCE (cm)
A280

Fig 2

Table 1
Stimulation of amino acid incorporation by RNA samples isolated from embryonic muscle mRNP fractions in wheat germ embryo S30 system

Source of RNA	μg RNA used	Amino [^{14}C] acids incorporated (cpm/assay)	Degree of stimulation
–	–	1100	control
Rabbit globin 9 S mRNA	3	13 100	11.9-fold
RNA from CmRNP	1.5	4300	3.9-fold
RNA from CmRNP	4.5	7500	6.8-fold
RNA from CmRNP	8.5	9400	8.5-fold
RNA from PmRNP	2	4500	4.1-fold
RNA from PmRNP	5	8100	7.4-fold
RNA from PmRNP	9	9800	8.9-fold
Chick muscle 28 S rRNA	12	1000	–
Chick muscle 4 S RNA	20	1250	–

Assays were performed as in [18]. Incubation mixtures contained in total vol. 100 μl 40 μl wheat germ S30 fraction; 20 mM Hepes (pH 7.6); 2 mM DTT; 1 mM ATP; 0.1 mM GTP, 8 mM creatine phosphate; 5 μg creatine phosphokinase, 80 mM KCl; 3 mM magnesium acetate, 2.5 nmol of each of 20 amino acids, indicated amounts of RNA samples, and 3 μCi uniformly-labeled amino [^{14}C] acid mixture (New England Nuclear Corporation)

pulse-labeled poly(A)$^+$RNA and two muscle-specific mRNAs, viz., actin and myosin heavy chain mRNA, are distributed in about a 65·35 ratio in CmRNP and polysomal fractions of chick embryonic muscles (unpublished results).

SDS–polyacrylamide gel electrophoresis of the protein moieties of the 45°C fractions (fig.1) indicate that CmRNP contains 7 major and 4–5 minor bands in the mol. wt 35–110 X 10^3 range (fig.3, gel 2). The major bands of mol. wt 52 000 (P52) and 78 000 (P78) daltons constitute about 40–45% of the total CmRNP proteins (fig.4, scan A in panel A). The unbound fraction from the oligo-dT-cellulose chromatography of post-polysomal material contained the bulk of the cytoplasmic proteins (gel 4). In contrast to CmRNP, the protein pattern for PmRNP was relatively simple (gel 3), containing only 3 major proteins – P52, P58 and P78 – amounting to about 80–85% of the total proteins. Interestingly, P52 and P78 from PmRNP and CmRNP co-electrophorese

Fig.1. Oligo(dT)-cellulose chromatography of subcellular fractions of chick embryonic muscles. For details see section 2. Two separate runs using postpolysomal particles (●- - -●) and EDTA-dissociated polysomes (○——○) for the isolation of CmRNP and PmRNP, respectively, are shown. The arrows indicate the positions where the columns were washed stepwise with different eluants.

Fig.2. Electrophoresis of deproteinized RNA samples obtained from [^{3}H]adenosine-labeled muscle mRNP fractions For details see section 2. Key. A_{280} scan of a gel run of a mixture of marker 28 S, 18 S and 4 S muscle RNAs (——), radioactivities of gel slices of RNA samples, (○- - -○) RNA from PmRNP and (X——X) RNA from CmRNP.

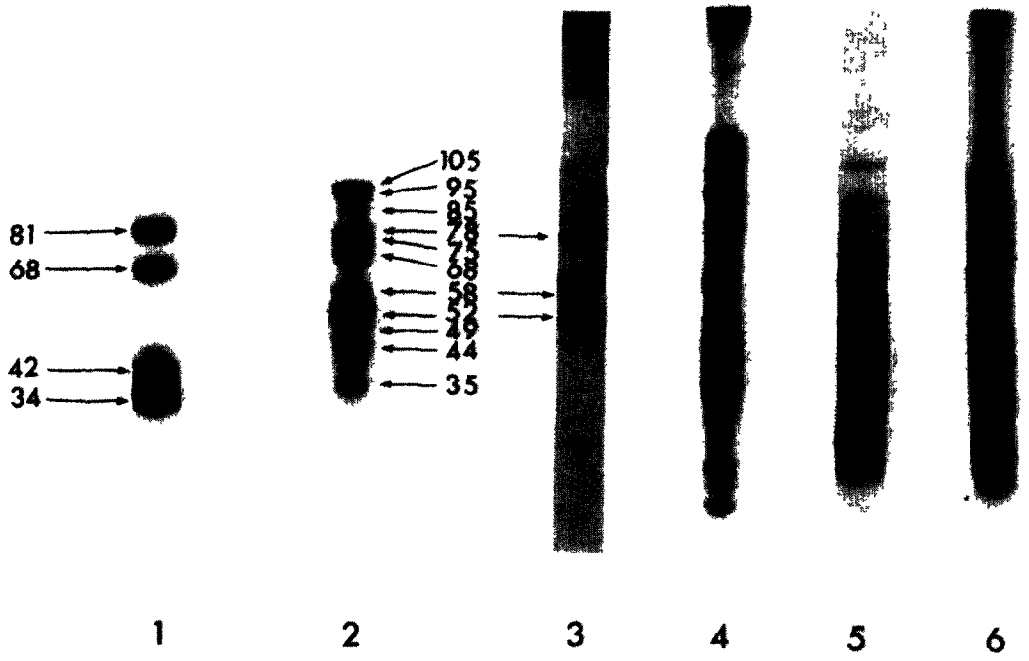
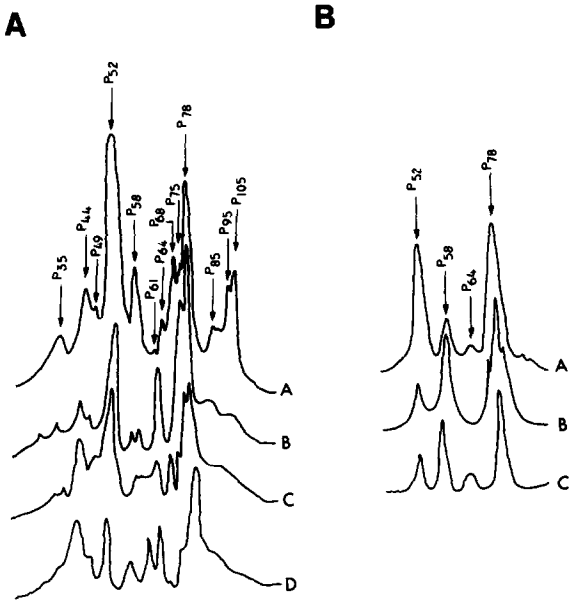


Fig.3 SDS–polyacrylamide gel electrophoresis of protein moieties of 45°C fractions. (see fig.1). For details see section 2. Gel 1 A mixed sample of transferrin (10 μg), bovine serum albumin (8 μg), rabbit muscle actin (8 μg), and tropomyosin (14 μg). Gel 2 0 5 A_{260} unit of CmRNP (50 μg protein). Gel 3: 0.4 A_{260} unit of PmRNP (12 μg). Gel 4 unbound fraction (120 μg) obtained by chromatography of CmRNP (fig.1). Gel 5 unbound fraction obtained by chromatography of PmRNP (80 μg) Gel 6: native 40 S muscle ribosomal subunits (120 μg).



suggesting that the same proteins may be present in both classes of mRNP (data not shown). Typical ribosomal proteins in the mol. wt 15 000–30 000 range which are found in the native 40 S subunit (gel 6) are also found in the unbound fraction from the chromatography of PmRNP (gel 5), indicating that this fraction contains dissociated ribosomal subunits. The high mol. wt proteins in the 50–100 X 10^3 range and banding in gel 6 presumably result from eIF_3 which is known to bind to the native 40 S particle [25] The thermally eluted

Fig.4 SDS–gel electrophoresis of CmRNP and PmRNP proteins isolated from various eukaryotic cells. Amount of samples loaded· CmRNP, 0 4–0 5 A_{260} units (about 45–55 μg proteins), PmRNP, 0.5–0.6 A_{260} units (15–18 μg proteins) Panel A CmRNP particles Panel B. PmRNP particles. Scans A, chick embryonic muscle; B, rat liver, C, rabbit reticulocyte; and D, Ehrlich ascites tumor cells.

mRNP (gels 2, 3) did not contain the typical ribosomal or cellular proteins in the mol. wt 15–30 × 10^3 range which have been previously observed in many mRNP preparations ([7,8], reviewed [2]). The nucleoprotein nature of the 45°C fractions was also indicated by their characteristic buoyant densities (1.40–1.42 g/cm^3 for CmRNP, and 1.50–1.53 g/cm^3 for PmRNP). In sucrose gradients both CmRNP and PmRNP gave a polydisperse and similar size distribution (about 20–100 S; results not shown) which is different from the size range of their RNA moieties (8–30 S; fig.2), again suggesting that the particles are nucleoproteins. However, differences in their buoyant densities, their A_{260}/A_{280} ratios and the SDS–gel electrophoretic patterns of their protein moieties indicate that the nucleoprotein nature of the two types of mRNP is significantly different, the CmRNP being relatively protein rich.

Using the technique of thermal chromatography we have examined the mRNP particles from rat liver, rabbit reticulocytes and Ehrlich ascites tumor cells, selected as representative eukaryotic cells which are known to vary widely in their protein synthetic activity. The chromatographic elutions of mRNP in each case were very similar to those shown in fig.1. CmRNP isolated from these cells contained a complex set of proteins with major proteins sized at mol. wt 44, 52, 58, 64 and 78 × 10^3 and several minor components (fig.4A). The relative amounts of the higher mol. wt proteins (80–100 × 10^3) varied among cell types. PmRNP from these cell types contained only 3 major proteins with mol. wt of 52, 58 and 78 × 10^3, the relative amounts of which also varied among cell types (fig.4B). These results suggest that a set of similar, possibly common, mRNA-associated proteins is present in a wide variety of eukaryotic cells. The variations in relative distribution of P52 and P78 proteins strongly suggest that not all eukaryotic mRNP contains equimolar amounts of these components as was previously thought [26].

The biological functions of the protein moieties of mRNP are not yet understood. The set of proteins present in CmRNP but absent in PmRNP, which we have observed in a variety of cell types, may regulate in vivo the entry of mRNA into the polysomes. With regard to the possible role of the P78 component, it has been suggested that it is involved in the transport of mRNA [15], and it remains associated with mRNA only during translation [6]. More recently we have demonstrated that P78 is associated with the 3′-poly(A) tracts of the RNA moieties of thermally eluted CmRNP and PmRNP of chick embryonic muscles [27]. In conjunction with other reports that P78 is associated with poly(A) in hnRNP and PmRNP [2,3,6,9,11–15,17] our results suggest that the P78 component remains always bound to the mRNA sequences in the cell independently of the translation process. In summary, the present results show that thermal chromatography is a useful technique for the isolation of purified preparations of poly(A)$^+$-mRNP from a wide variety of eukaryotic cells. Furthermore, the two types of mRNP described here contain several common proteins of identical size, yet their nucleoprotein nature is quite distinct.

Acknowledgements

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