THE POLYRIBOSOMAL POLY(A)-BINDING PROTEIN IS HIGHLY CONSERVED IN VERTEBRATE SPECIES

Comparison in duck, mouse and rabbit

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1. Introduction

In eukaryotic cells mRNA is present in the form of messenger ribonucleoprotein complexes (mRNP particles). When polyribosomes, from a variety of cell types and tissues, are dissociated with EDTA or puromycin, the released mRNA is found tightly associated with 3 major proteins of $M_r \sim 43\,000$–$52\,000$ and $72\,000$–$78\,000$ (review [1]). The larger of these proteins was shown to interact with the 3′-polyadenylate sequence of mRNA [2,3] since it may be recovered with poly(A) following digestion of polyribosomes or purified mRNP with ribonucleases A and T_1. From density measurements of the poly(A) RNP particle it may be estimated that ~4–5 copies of this protein are associated with the average-sized poly(A) fragment [4]. The affinity of the poly(A)-binding protein for poly(A) is very high since it resists not only 0.5 M KCl [1] but also centrifugation through Cs_2SO_4 density gradients [5,6] and affords protection of poly(A) against ribonucleases and nucleases [7–10]. Furthermore, this protein binds not only poly(A) but also regions of poly(A) adjacent and non-adjacent to poly(A) [8].

A protein of the same M_r as the polyribosomal poly(A)-binding protein has been observed to be associated with the 3′-poly(A) region of nuclear pre-mRNA in HeLa cells [11] and with globin pre-mRNA sequences in nuclear matrix preparations [12]. Treatment of HeLa cells with cordycepin prevented not only the polyadenylation process in the nucleus, thus preventing the appearance of mRNA in the cytoplasm, but also the appearance of newly-labelled poly(A) binding protein in polyribosomal mRNP [13]. It was proposed that this protein was implicated in the transfer of mRNA from the nucleus to the cytoplasm [13]. However, in [14] the poly(A) binding protein immunologically cross-reacted with the nuclear form of poly(A) polymerase.

This polyribosomal mRNP protein may also play a part in translational control since it is absent from polyadenylated free cytoplasmic mRNP, i.e., mRNP not associated with ribosomes in vivo and untranslatable in vitro [15,16]. This absence is significant since the poly(A)-binding protein may be isolated from post-mRNP cytoplasm and hence is not a limiting factor in its association with poly(A)$^+$ mRNA [4].

In all these studies, this protein was characterised by its M_r which varied with the cell type and electrophoretic systems used, from 72 000–81 000 [1]. In view of its potential role(s) in the metabolism of poly(A), transport of mRNA and/or translational control, we decided to investigate the degree of conservation of the poly(A)-binding protein in taxonomically distant species.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; SDS, sodium dodecylsulfate; TEA, triethanolamine

Enzymes: T_1 ribonuclease (EC 3.1.4.8) was from Sankyo Co. (Japan) and ribonuclease A (EC 3.1.4.22) was from Sigma Chem. Co. (St Louis); *Staphylococcus aureus* V8 protease was from Miles Labs (UK)

2. Experimental procedures

2.1. *Isolation of polyribosomes*

Polyribosomes were isolated from duck erythro-

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blasts as in [6]. Mouse Ehrlich ascites cells were maintained and harvested according to [17] and polyribosomes were obtained from the post-mitochondrial supernatant by centrifugation for 1 h at 200 000 × *g* at 4°C following cell lysis in 1.5 cell vol. hypotonic buffer (10 mM Tris–HCl (pH 7.5), 10 mM KCl, 1.5 mM magnesium acetate) at 0°C [17]. Similarly, the post-mitochondrial supernatant of rabbit reticulocyte lysate [16] served as source of polyribosomes.

2.2. *Isolation of mRNP*

Pellets of polyribosomes were resuspended in 10 mM TEA–HCl (pH 7.4), 50 mM KCl, 5 mM 2-mercaptoethanol at final conc. of 50 A_{260} units/ml with the aid of a glass Dounce homogeniser and dissociated with EDTA prior to affinity chromatography on oligo(dT) cellulose as in [6]. Elution of bound material was achieved with 50% formamide containing 10 mM TEA–HCl (pH 7.4), 10 mM EDTA, 50 mM NaCl. On occasion polyribosomes were treated with ribonucleases A and T1 at final conc. 200 μg/ml for 30 min at 30°C prior to chromatography. The formamide eluates containing poly(A)$^+$ mRNP were dialysed at 4°C against 10 mM TEA–HCl (pH 7.4), 20 mM KCl and precipitated with 2.5 vol. ethanol at −20°C overnight.

2.3. *Gel electrophoresis of proteins*

Electrophoretic analysis of proteins in one-dimensional SDS–polyacrylamide gels was performed as in [18] and bidimensional gels (non-equilibrium pH gel electrophoresis/SDS polyacrylamide gel electrophoresis) as in [19]. M_r markers used were phosphorylase A (92 000), bovine serum albumin (68 000), catalase (60 000), ovalbumin (45 000), aldolase (39 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 000).

2.4. *Protease digestion*

Following brief staining of gels with 0.1% Coomassie blue in 50% methanol, 10% acetic acid, the gels were rinsed in distilled water and protein bands excised with a razor blade. The gel slices were crushed with a glass rod, incubated overnight in 0.125 M Tris–HCl (pH 6.8), 0.5% SDS at room temperature and the eluted proteins separated from acrylamide by centrifugation in siliconised tubes. Following precipitation with ethanol, the proteins (~1.0 μg) were resuspended in 3 μl 0.125 M Tris–HCl (pH 6.8), 0.2% SDS, 2 mM EDTA, 25% glycerol and digested with 0.1 μg highly pure *Staphylococcus aureus* V8 protease at 37°C for 30 min. The digestion reaction was terminated by the addition of 2% SDS, 2% 2-mercaptoethanol (final conc.) and the products analysed on short (5 cm), thin (0.3 mm) 16% polyacrylamide–SDS gels, following heating at 100°C for 2 min.

3. Results

As reported in [6,20] the proteins associated with duck globin mRNA in the 15 S polyribosomal mRNP include a major polypeptide of 73 000 M_r (fig.1 (2,5)) as well as numerous minor components. Comparison of duck mRNP proteins with those of mouse and rabbit mRNP (fig.1 (1,6)) shows that the major protein component from the 3 species displays the same M_r-value. In order to see whether, as in the case of duck polyribosomal mRNP, this protein was associated with the poly(A) fragment [6,8], duck and mouse polyribosomes were treated with ribonucleases A and T_1 and the proteins recovered in the poly(A)-RNP particles were compared (fig.1 (3,4)). In both cases the 73 000 M_r protein is the major protein which binds to poly(A). It is interesting to note that some but not all other proteins, in addition to the poly(A)-

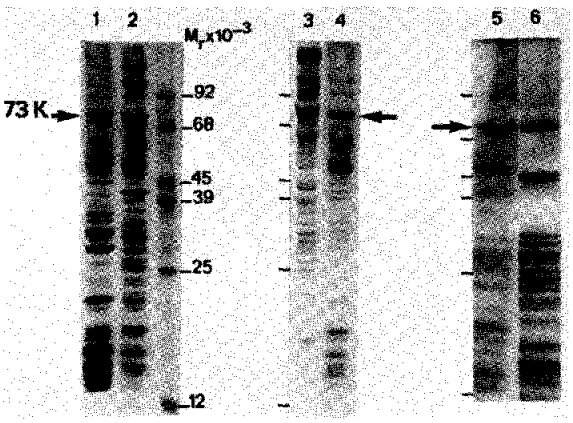

Fig.1. SDS gel electrophoretic analysis of proteins of polyribosomal mRNP: mRNP were isolated from EDTA-dissociated polyribosomes by chromatography on oligo(dT)-cellulose and their proteins analysed on 13% polyacrylamide–SDS gels: (1,2,5,6) proteins of total polyribosomal mRNP from duck (2,5), mouse (1) or rabbit (6); (3,4) proteins of poly(A)$^+$ RNP isolated from duck (4) or mouse (3) polyribosomes treated with ribonucleases A and T_1 prior to chromatography. Positions of migration of M_r protein markers are indicated by bars. The position of the 73 000 M_r poly(A)-binding protein is denoted by the arrow.

binding protein, are common to duck and mouse poly(A)-RNP.

We had noted that the poly(A)-binding protein of duck polyribosomal mRNP behaves characteristically when subjected to isotachophoresis where it migrates as a basic protein with a pI > 8 [6]. The 73 000 M_r protein from mouse and rabbit polyribosomal mRNP migrated in the same position as the duck mRNP protein when analysed by bidimensional gel electrophoresis (NEPHGE–SDS [19]) (fig.2). In all 3 cases the poly(A)-binding protein travelled near the ampholine front. Since precise alignment of protein positions between any 2 gels is not possible, mixed samples of duck and mouse, and duck and rabbit mRNP were analysed and the results obtained showed only 1 spot corresponding to the 73 000 M_r protein (fig.2E,F).

We investigated further homology between the poly(A)-binding protein from duck, mouse and rabbit polyribosomal mRNP by subjecting the purified protein to proteolytic digestion. The electrophoretically purified proteins were analysed on 13% polyacrylamide SDS gels (fig.3A) and the comigration of the 73 000 M_r duck, mouse and rabbit mRNP protein was thus confirmed. However, we noted that in all samples the protein suffered slight degradation, possibly during fixation and elution from the gel, resulting in 2–3 characteristic lower M_r bands. Such degradation also occurred when the gel was stained with ethidium bromide [21] thus avoiding the necessity for fixation, or when the pH of the elution buffer was raised from 6.8–8.0 (not shown). That the lower M_r bands present in the purified protein were due to contamination with other closely migrating proteins was excluded by the fact that they were also present (fig.3 (2)) when the protein was eluted from bidimensional gels where clearly no other proteins migrate near the position of the poly(A)-binding protein (fig.2).

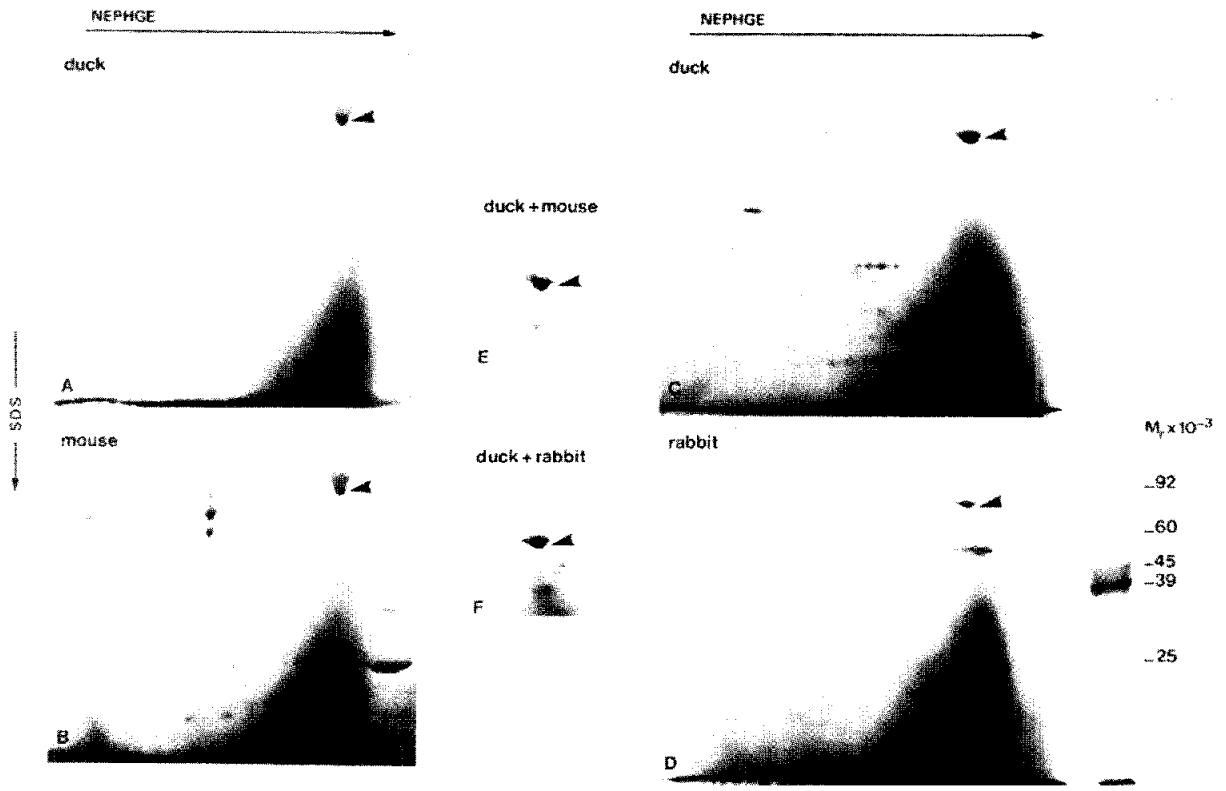

Fig.2. Two-dimensional gel electrophoretic analysis of polyribosomal mRNP proteins [19]. Proteins from polyribosomal mRNP were analysed by non-equilibrium pH gel electrophoresis (NEPHGE) in the first dimension followed by electrophoresis on 13% polyacrylamide SDS gels: duck (A,C), mouse (B), and rabbit (D) mRNP proteins; inset, mixed samples of duck and mouse (E) and duck and rabbit (F) mRNP proteins. The position of the 73 000 M_r poly(A)-binding protein is indicated on the gels by an arrow.

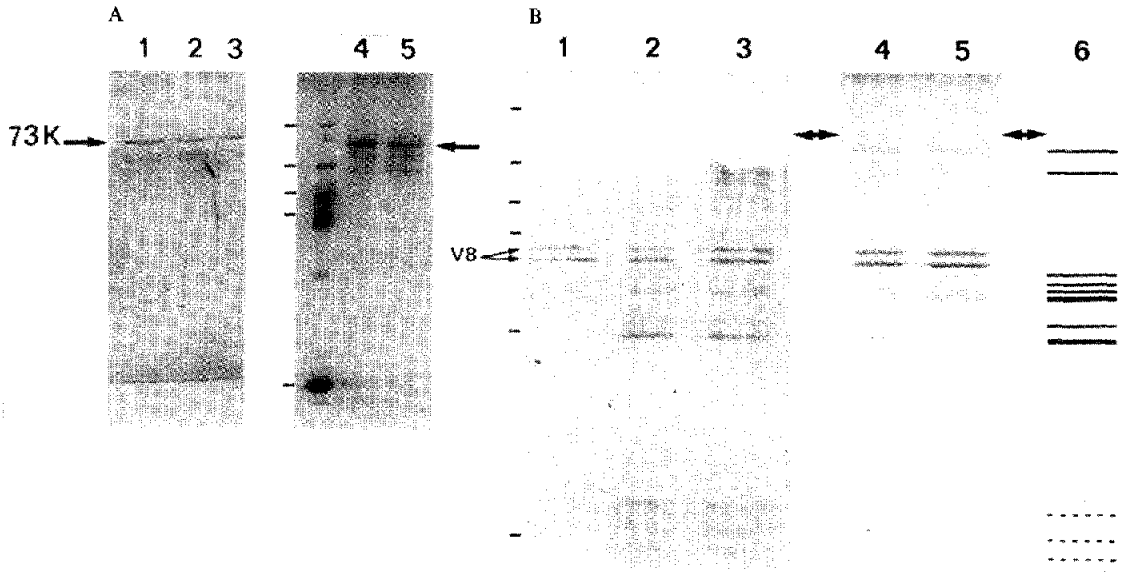

Fig.3. Partial peptide maps of poly(A)-binding proteins from duck, rabbit and mouse. The proteins were isolated as in fig.1 and further purified by electrophoresis in preparative gels. The proteins were eluted from the gel and portions were subjected to re-electrophoresis with (fig.3B) or without (fig.3A) digestion by protease V8: (A) duck (1) and mouse (2) proteins eluted from two-dimensional gels, duck (3,4) and rabbit (5) proteins eluted from one-dimensional gels; (B) *Staphylococcus aureus* V8 protease (1); partial peptide maps of duck (2,4), mouse (3), and rabbit (5) proteins; diagrammatic representation of the generated peptides (6). The position of migration of the 73 000 poly(A)-binding protein is indicated by the arrow.

The poly(A)-binding protein thus obtained was digested with *Staphylococcus aureus* V8 protease and the partial digest analysed by gel electrophoresis [22]. The parallel incubations of duck and mouse, and duck and rabbit protein were performed on separate occasions, with slightly different enzyme to substrate ratios. Thus some proteolysed bands display varying intensities in the two experiments (fig.3B (2–5)). However comparison of the digestion products in either experiment clearly shows their co-migration and, as indicated by the scheme (fig.3 (6)) the same partial products are generated by *Staphylococcus aureus* V8 protease digestion of duck, mouse and rabbit poly(A)-binding protein thus demonstrating that this protein contains the same cleavage sites in all 3 species.

4. Discussion

We have shown here that the major polyribosomal mRNP protein, which binds to the polyadenylate region of eukaryotic mRNA, is a protein which is highly conserved during evolution. We have compared mRNP proteins from the vertebrate species, duck, mouse and rabbit since in all 3 cases the composition of the proteins associated with polyribosomal mRNA was already established. In globin polyribosomal mRNP from duck erythroblasts the major component, which could be recovered in ribonuclease-resistant poly(A)-RNP, was a species of 73 000 M_r [6,20]. Characterisation of globin polyribosomal mRNP proteins of rabbit reticulocytes revealed the major component to have M_r 72 000 [23], 73 000 [24], 76 000 [25] or 78 000 [2]. This protein was shown to interact with the 3′-poly(A) region of rabbit globin mRNA [2,24]. In mouse Ehrlich ascites cells, the major polyribosomal mRNP protein displayed a size of 81 000 M_r and bound specifically to the poly(A) fragment of mouse mRNA [3].

Clearly, relying upon M_r data determined in different laboratories using a variety of SDS–polyacrylamide gel electrophoretic systems can be misleading. The poly(A)-binding protein of globin mRNP from duck erythroblasts and rabbit reticulocytes both of which displayed the same M_r of 73 000 were compared by one-dimensional gel electrophoresis [24]. In view of the possible general function of this protein,

which remains to be clarified, we were interested to extend these initial observations to the mRNP proteins from non-erythroid-type cells, mouse Ehrlich ascites cells, as well as duck and rabbit red blood cells, and by studying various parameters. On the basis of size, pI and common cleavage sites, the poly(A)-binding protein from all 3 species is identical. Interestingly, we have been so far unable to produce an antibody to the undenatured duck 73 000 M_r protein in either rabbits or mice.

The conservation of the poly(A)-binding protein implicates its essential role in mRNA metabolism and argues against a function of this protein in a selective control of mRNA translation or transport from the nucleus to the cytoplasm. In view of the high degree of evolutionary conservation observed for other nucleic acid-binding proteins, such as some ribosomal proteins [26] or histones [27], one may suggest a mainly structural role for the poly(A) binding protein.

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