In vitro reconstitution of messenger ribonucleoprotein particles from globin messenger RNA and cytosol proteins

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Deproteinized globin poly(A)+mRNAs reassociate readily in vitro with soluble RNA-binding proteins of the cytosol; reconstituted messenger ribonucleoprotein complexes are obtained which are very similar to native globin polyribosomal-mRNP as far as bouyant density in Cs_2SO_4 and the composition of proteins which can be crosslinked to the mRNA are concerned. Proteins thus identified bind specifically to mRNA and not to ribosomal RNA or any synthetic oligonucleotides, with one exception: a 78-kDa protein could be cross-linked to poly(A).

Messenger ribonucleoprotein *Poly(A)-protein* *Ultraviolet-light cross-linking* *RNA-binding protein*

1. INTRODUCTION

Messenger RNAs have been isolated in the form of messenger ribonucleoprotein particles (mRNPs) from many eukaryotic cells (reviews [1–4]). The existence of mRNPs in the living cell and their functional importance have been questioned for a long time, since pure and protein-free mRNA stimulates cell-free translation systems with the same efficiency as mRNP [5–9]. Recently published data have, however, now erased any doubts that still remained. UV-irradiation which produces covalent linkages between nucleotides and amino acids in zero distance have been used in living cells, and demonstrated that mRNPs indeed exist in vivo [10–16]. The physiological role of the associated proteins has also been shown, when it was demonstrated that in a cell-free system, from which RNA-binding proteins had been removed, 'naked' mRNA was not translated. However, this system readily resumed translation when optimal quantities of cytosol RNA-binding proteins were added [17–19]. Additionally, published data show that some of the cytosol RNA-binding proteins, especially those which bind tightly to mRNA, have similar molecular masses, isoelectric points and even limited proteolytic digestion patterns to the proteins of the native mRNP [11,17,18,20–23]. Obviously some of the proteins interact with the protein-free mRNA forming an mRNP complex. Nonetheless, the question as to whether these reconstituted mRNA-protein complexes contain the same proteins as the native ones isolated from polysomes has not yet been answered.

Here, we use a more direct approach. Deproteinized mRNA was mixed with cytosol RNA-binding proteins. They were covalently linked in vitro by means of UV-irradiation and following RNase digestion; the nucleotides that remain attached to the cross-linked proteins were end-labeled with cytidine 3′,5′-[5′-^{32}P]biphosphate. The proteins cross-linked to mRNA were analyzed by PAGE and autoradiography. We demonstrate

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Abbreviations: mRNP, messenger RNA–ribonucleoprotein complex; Mes, *N*-morpholinoethanesulfonic acid; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; BP350, cytosol RNA-binding proteins eluted from the RNA–Sepharose column with buffered 350 mM KCl; BP1000, cytosol RNA-binding proteins eluted from the RNA–Sepharose column with buffered 1000 mM KCl

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that the binding reaction is very specific and results in structures comparable to the native mRNPs.

2. MATERIALS AND METHODS

2.1. *Cell fractionation*

20 BALB/c inbred mice were each inoculated with 0.2 ml of spleen extract from mice previously infected with Friend erythroleukemia virus. After 17–20 days the animals were killed to remove the hypertrophic spleens. These were rinsed twice with ice-cold buffered saline and homogenized as in [18]. Poly(A)$^+$mRNA was extracted from the polyribosomes with chloroform–phenol–isoamyl alcohol and purified by affinity chromatography on oligo(dT)-cellulose.

2.2. *Isolation of cross-linked poly(A)$^+$ polyribosomal mRNP, oligo(dT)-cellulose chromatography*

Polyribosomes were isolated as in [11], and resuspended in 10 mM Tris–HCl (pH 7.6), 3 mM $MgCl_2$, 100 mM NaCl, 7 mM mercaptoethanol, UV-irradiated at 2000 μW/cm^2 for 15 min in an ice bath; thereafter the suspension was made 0.5% in SDS and 0.5 M in NaCl and was heated at 60°C for 3 min. After cooling to 20–23°C, the sample was applied to an oligo(dT)-cellulose column, previously equilibrated in binding buffer [10 mM Tris–HCl (pH 7.6), 3 mM $MgCl_2$, 500 mM NaCl, 0.5% SDS, 7 mM mercaptoethanol]. The column was then washed extensively with 10 vols binding buffer and eluted with 5 ml elution buffer [10 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.5% SDS] to release poly(A)$^+$ polyribosomal mRNA together with cross-linked proteins. The eluted fractions were then made 0.5 M in NaCl and rechromatographed. Eluted poly(A)$^+$ polyribosomal mRNP fractions were precipitated with ethanol at −20°C and pelleted. They were then resuspended in 150 μl sarkosyl buffer [62.5 mM Tris–HCl (pH 6.5), 0.5% sarkosyl] and digested with RNase A and RNase T2 at 37°C for 1 h.

2.3. *Isolation of RNA-binding proteins, in vitro mRNP reconstitution and end-labeling with cytidine 3′,5′-[5′-^{32}P]biphosphate*

The method for the isolation of RNA-binding proteins has been described [17,18,21]. Briefly, 3 g CNBr-activated Sepharose and 30 mg polyribosomal RNA were resuspended in 0.2 M Mes (pH 6.0). The slurry was stirred gently at room temperature for 2 h, packed into a column and washed extensively, and thereafter equilibrated in binding buffer [10 mM Tris–HCl (pH 7.6), 100 mM NaCl, 3 mM $MgCl_2$, 7 mM mercaptoethanol]. Cytoplasmic post-ribosomal supernatant (S100) was passed through the column followed by washing extensively with the binding buffer. Proteins with affinity for RNA were eluted stepwise, first using a buffer containing 350 mM KCl, 20 mM Tris–HCl (pH 7.6), 3 mM $MgCl_2$, 7 mM mercaptoethanol and then with a buffer containing 1000 mM KCl, 20 mM Tris–HCl (pH 7.6), 3 mM $MgCl_2$, 7 mM mercaptoethanol; the protein eluates were hence called BP350 and BP1000. They were dialyzed against 20 mM Tris–HCl (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 7 mM mercaptoethanol.

For the in vitro mRNP reconstitution 10 μg RNA-binding proteins were added to 1 μg poly(A)$^+$mRNA in 10 mM Tris–HCl (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, and 10% glycerol [24]. The sample with a total volume of 25 μl was then UV-irradiated and chromatographed on oligo(dT)-cellulose to separate cross-linked mRNA-protein complexes from unbound proteins. The eluted fraction was digested with RNases as described above.

For end-labeling of the covalently linked, remaining nucleotides, the following were added to 4 μl of the digested samples: 1 μl RNA-ligase buffer [500 mM Hepes (pH 8.0), 30 mM dithioerythritol, 200 mM $MgCl_2$, 10 μg/ml albumin], 2 μl of 0.2 mM ATP, 1 μl of 100% DMSO, 1 μl (about 10 μCi) cytidine 3′,5′-[5′-^{32}P]biphosphate and lastly 1 μl T4 RNA-ligase (4 units/μl). This mixture was incubated at 0°C overnight [25]. The end-labeled samples were then analyzed by SDS–PAGE [26].

3. RESULTS AND DISCUSSION

Previous experiments have shown that pure mouse globin messenger RNA is not translated in a cell-free system which has been deprived of all RNA-binding proteins usually present in the lysate (supernatant after centrifugation at 30000 × *g*) [18,19]. Maximal translation was achieved, however, when RNA-binding protein fractions, called

BP350 and BP1000, were supplied in optimal quantities. These experiments are summarized in fig.1a. The translation efficiency is comparable to or even higher than that obtained with polyribosome-derived mRNPs (fig.1b, column 1) (for further discussion see [17–19]). It was therefore concluded that some RNA-binding proteins interact more or less tightly and specifically with mRNA in vitro and in vivo to allow initiations and subsequent complete rounds of translation.

To prove this, deproteinized globin mRNAs were incubated with saturating quantities of the two RNA-binding protein fractions, BP350 or BP1000. The mixtures were then irradiated to produce covalent cross-links between proteins and mRNA. The resulting complexes have a density of

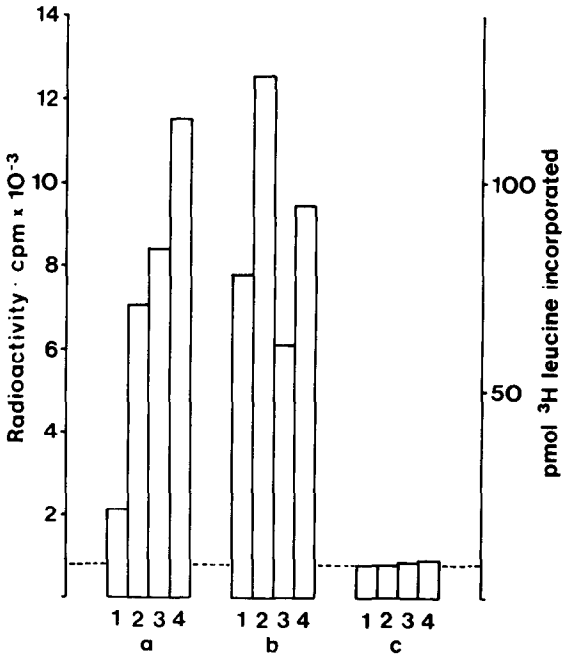

Fig.1. Synoptic presentation of a set of representative experiments concerning the translational efficiency of deproteinized globin mRNA compared with the globin polyribosomal mRNP in a preincubated lysate depleted of RNA-binding proteins [18]. Added template in: (a) purified and deproteinized globin mRNA (1 μg); (b) purified globin polyribosomal mRNP (containing the equivalent of 1 μg mRNA); (c) none. The RNA-binding proteins added in the assays were as follows: (1) no RNA-binding proteins added; (2) plus BP350; (3) plus BP1000; (4) plus BP350 and BP1000.

1.32 g/cm^3, indicating that mRNP were obtained (fig.2b,c). This is quite similar to the density of cross-linked polyribosomal mRNP (1.30 g/cm^3, fig.2a) and deviates from pure mRNA (1.60 g/cm^3, fig.2d) and RNA-binding proteins (1.20 g/cm^3, fig.2e), run as controls. The reconstituted mRNP complexes with their proteins 'frozen' to globin mRNA could of course not be tested for translation in vitro. However, we have analyzed the protein composition after digestion of the mRNA and end-labeling of the nucleotides remaining bound to the liberated proteins and shielded from digestion. PAGE in fig.3 shows that of the host of proteins in the BP350 fraction (fig.3, lane f) only a small number was cross-linked to nucleotide sequences of the mRNA (fig.3, lane g). Major bands are in the region of 110, 80 and 45 kDa. Among these and the minor bands we suspect several initiation factors (eIFs). We as well as others [17,18,22,27,28] have shown that the RNA-binding proteins indeed possess initiation factor activity. The importance of some initiation factors such as eIF-3, -4A, -4B, -4C for the mRNA binding to the 40 S ribosomal subunit to form the initiation complex has been reported [29,30]. Finally, by the UV-cross-linking method we have directly demonstrated that 3 subunits of eIF-3 (100, 95 and 65 kDa), as well as eIF-4A (45 kDa), -4B (80 kDa) and -4C (17.5 kDa) could be cross-linked to mRNA in vitro [24].

Especially interesting is the 28-kDa protein. This protein, which could be isolated from cytosol and from the polysomal salt wash, proved to be the cap-binding protein. It was isolated from the two fractions by affinity chromatography [31–33]. It is eluted by m^{7}GTP together with a minor component of 24 kDa which is also seen in fig.3e. The fact that the cap-binding proteins and initiation factors were usually isolated from the polysomal salt wash (which means that they are less tightly bound to the mRNA) does not contradict the UV-cross-linking results with the cytosol RNA-binding proteins. It rather confirms the hypothesis that by these protein/RNA interactions a dynamic structure is created in which proteins with affinity for mRNA can shuttle on and off [34].

PAGE of BP1000 shows fewer proteins with molecular masses between 100 and 30 kDa; but only about half of which could be cross-linked to mRNA. The protein pattern is similar to that re-

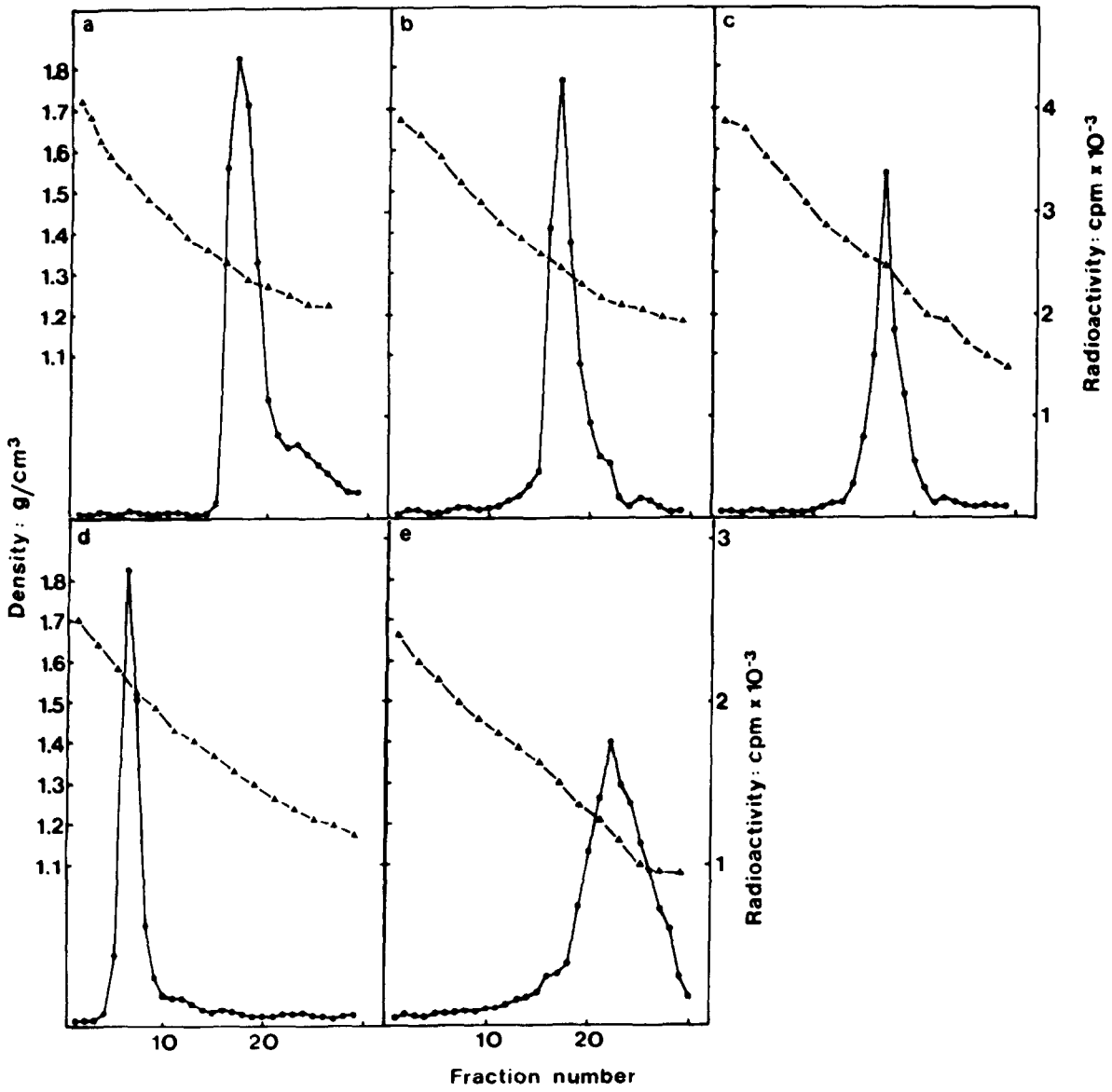

Fig.2. Cs_2SO_4–DMSO density gradient analysis of UV-cross-linked polyribosomal mRNPs, mixture of mRNA and BP350 and mRNA plus BP1000; as well as of deproteinized mRNA and of pure RNA-binding proteins as controls. Irradiated and control mixtures in 10 mM Tris–HCl, pH 7.6, 10 mM KCl, 0.01% Triton X-100, 15% (v/v) DMSO were layered onto 15–50% Cs_2SO_4–DMSO preformed gradients [17,24]. Centrifugation was performed in a Beckman SW56 rotor at 40000 rpm for 20 h at 20°C. Thereafter, 150-μl fractions were collected. The density of each fraction was determined by weighing 20-μl aliquots. The trichloroacetic acid-precipitable radioactivity was measured from 50 μl samples. (a) Polyribosomal mRNP; (b) BP350 and mRNA; (c) BP1000 and mRNA; (d) mRNA only; (e) BP only.

cently reported by Greenberg [10] and confirms earlier results of this laboratory [4,11]. Six protein bands, corresponding to 98, 78, 65, 52, 45, 42 and 41 kDa, could be resolved (fig.4, lane b). These proteins are also present in polyribosome-derived mRNPs (fig.4, lane c). Proteins below 40 kDa were not found in mRNPs reconstituted from BP1000. Earlier investigations have shown that

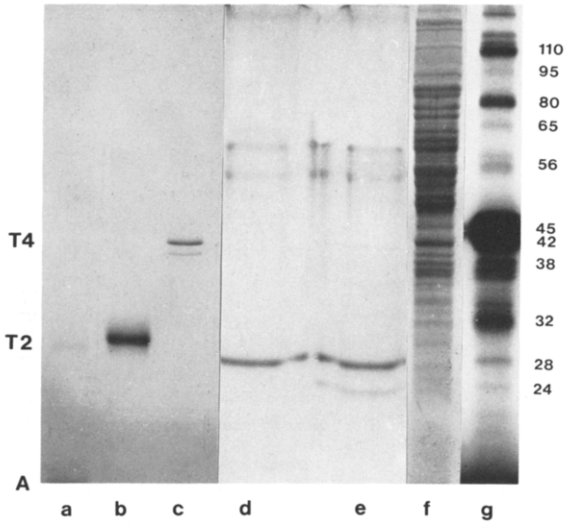

Fig.3. SDS–PAGE analysis of a mixture of deproteinized mRNA and BP350 after UV-cross-linking, RNase digestion and end-labeling. Lanes: (a) RNase A and RNase T2; (b) RNase T2; (c) T4 RNA-ligase; (d) cap-binding protein isolated from the polyribosomal salt wash as in [31–33]; (e) cytosol protein with affinity for cap-analogue m^7GTP; (f) cytosol RNA-binding proteins BP350; (g) autoradiograph of deproteinized mRNA plus BP350, UV-irradiated, RNase digested and end-labeled as described in section 2. All gels were Coomassie blue stained, unless otherwise indicated. Molecular mass range: $\times 10^{-3}$ Da.

several of the BP1000 proteins have the same M_r and isoelectric point as polyribosomal mRNP proteins [17,18,20–23]. They are tightly bound, which is in agreement with recently published data indicating that some of the polyribosomal mRNP-proteins remain attached to the mRNA throughout initiation and polyribosome formation [35,36].

To ensure that the UV-cross-linking results presented here were specific for mRNA, we performed control experiments such as that shown in fig.5. End-labeling of BP350 and BP1000 without any mRNA addition resulted only in the labeling of the T4 RNA-ligase (fig.5, lanes a,b). This is in fact the intermediate product of the end-labeling reactions [25]. This negative result, along with the buoyant density of 1.2 g/cm^3 in Cs_2SO_4 and the regularly determined $A_{280}:A_{260}$ ratio of 1.64 clearly showed that the RNA-binding proteins are not contaminated with RNA. When poly(A) was added to BP350 (fig.5, lane c) or BP1000 (fig.5, lane d) only the 78-kDa protein appeared in the autoradiographs. Neither poly(U) nor 18 S or 28 S ribosomal RNA could be cross-linked to any of the RNA-binding protein fractions. Recently published data from our group have clearly shown that this 78-kDa protein obtained from the cytosol's

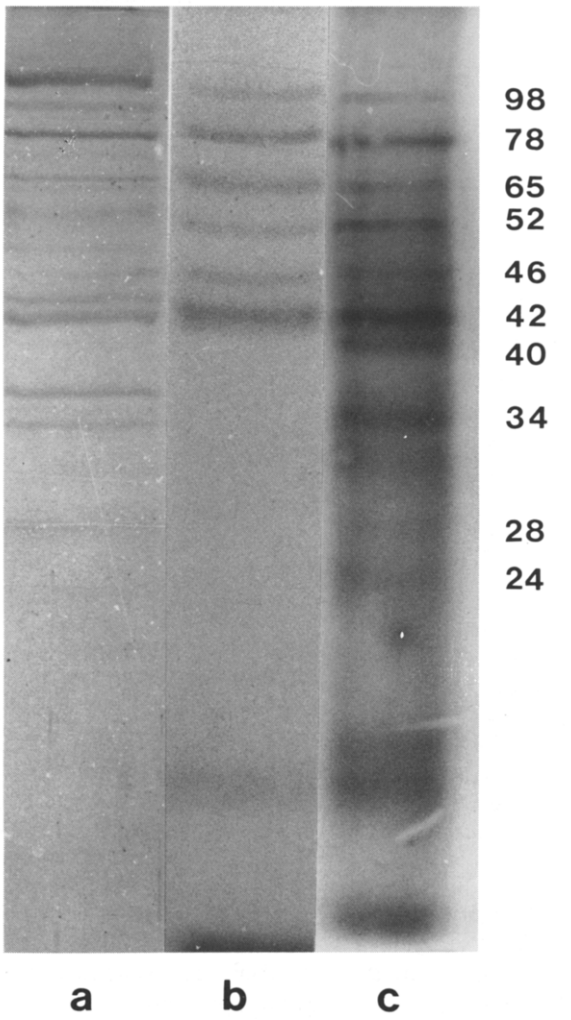

Fig.4. SDS–PAGE analysis of a mixture of mRNA plus BP1000 after UV-cross-linking RNase digestion and end-labeling. Lanes: (a) cytosol RNA-binding proteins BP1000; (b) autoradiograph of deproteinized mRNA plus BP1000, UV-irradiated, RNase digested and end-labeled as described in section 2; (c) autoradiograph of in situ UV-cross-linked globin polyribosomal mRNP, RNase digested and end-labeled.

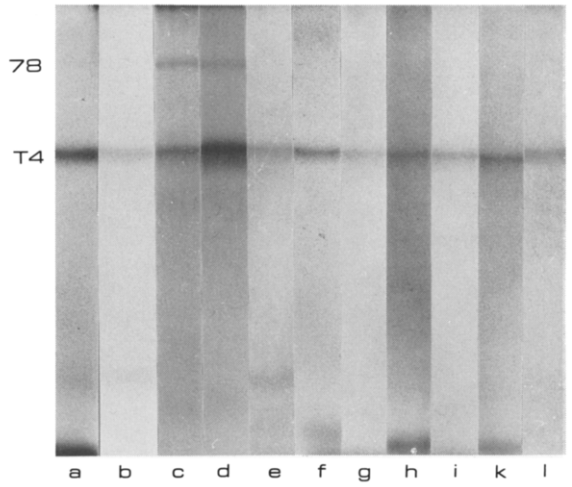

Fig.5. SDS–PAGE analysis of end-labeled control experiments described in the text. Lanes: (a) BP350 only; (b) BP1000 only; (c) poly(A) + BP350 (UV-cross-linked, end-labeled); (d) poly(A) + BP1000; (e) poly(U) + BP350; (f) poly(U) + BP1000; (g) 28 S ribosomal RNA + BP350; (h) 28 S ribosomal RNA + BP1000; (i) 18 S ribosomal RNA + BP350; (k) 18 S ribosomal RNA + BP1000; (l) T4 RNA-ligase only.

RNA-binding protein is identical to the 78-kDa protein of the mRNP as shown by their isoelectric points [17] and limited proteolytic digestion pattern [11,37].

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