ISOLATION OF PROTEINS WITH HIGH AFFINITY FOR dRNA

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1. Introduction

Many recent data have shown that dRNA† or mRNA is associated in mRNP particles or 'informosomes" [1] with specific protein whose functions are as yet poorly understood [2–4]. In addition to structural and functional studies on these proteins, their binding to mRNA or dRNA in vitro is of great interest. This could be studied either by the nitrocellulose filter technique, or by density gradient centrifugation, as in the case of protein–DNA interactions [5,6]. In looking for a more flexible and versatile method, we have attempted to use affinity chromatography for isolating and purifying proteins which bind to nucleic acids, and for studying the interactions occurring between mRNA and defined proteins.

Several procedures have been developed for binding or retention of natural nucleic acids to insoluble supports [7–11]. In this work, a crude dRNA fraction was covalently bound to agarose by the method of Wagner et al. [12] and tested for its binding capacity for proteins derived from a cytoplasmic fraction, highly enriched in *informosomal-type structures* [13]. Similar experiments have been reported by Tsai and Green [14] on the affinity of cell proteins for agarose-bound DNA.

† *Abbriviations*: rRNA, ribosomal RNA; dRNA, DNA-like RNA; mRNA, messenger RNA; mRNP, messenger RNA-protein particles; SDS, sodium dodecyl sulfate.

Materials and methods

Solid transplantable plasma cell tumor RPC5, grown on Balb/c mice were used. Proteins were labelled by 3 intraperitoneal injections of 8 μCi of ^{14}C-labelled protein hydrolysate, 6, 4 and 2 hr before killing. RNA was labelled by one injection of tritiated uridine 20 hr before killing. The tumors were homogenized at 0–4°C in a glass homogenizer with a teflon pestle in 2.4 vol of ice-cold standard buffer: 0.02 M triethanolamine–HCl buffer (pH 7.6); 0.15 M KCl; 0.04 M Mg acetate; 0.006 M β-mercaptoethanol; 1.1 M sucrose.

2.1. *Isolation of free informosomes*

Ribonucleoprotein particles from a post-mitochondrial supernatant (S_{20}) were fractionated on a discontinuous D_2O-sucrose gradient as described elsewhere [13] by centrifugation at 4°C for 20 hr at 180 000 *g* av. After centrifugation the heavy D_2O-sucrose layer of density 1.29 g/cm^3 was carefully removed with a syringe and used as the free, informosome-rich, ribonucleoprotein fraction.

2.2. *Dissociation of ribonucleoprotein complexes and separation of proteins from nucleic acids*

The fraction highly enriched in informosomes was first dialysed for 48 hr against a 1 mM phosphate buffer (pH 6.5), containing 6 M urea. The dialysed material was mixed with DEAE-cellulose equilibrated with the dialysis buffer, and then added to a column

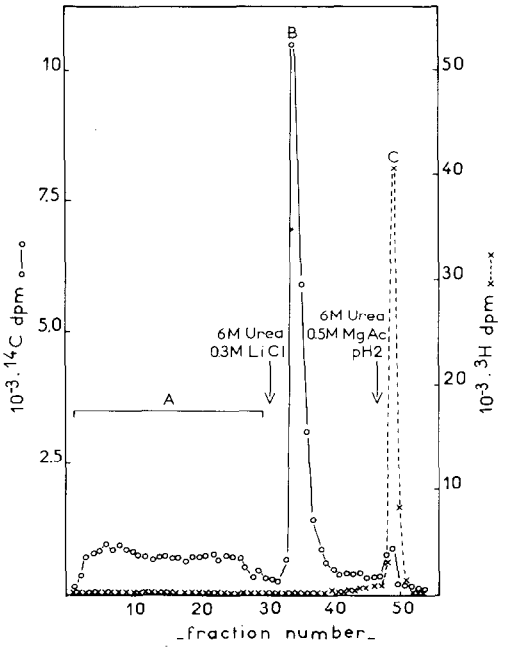

Fig. 1. Separation of proteins and nucleic acid from the ribonucleoprotein complexes by chromatography on DEAE-cellulose. The dialysed informosome-rich fraction (approximately 200 mg protein) was applied on a DEAE-cellulose column (12 × 1.6 cm) as indicated in Materials and methods. A: effluent; B: fraction eluted with 0.3 M LiCl; C: fraction eluted with 0.5 M Mg acetate (pH 2). Solutions contained 6 M urea throughout. 5 ml fractions were collected. Radioactivity was measured on 0.3 ml aliquots on filter papers washed with cold 10% TCA, ethanol, ethanol–ether and ether. (o—o—o) Radioactivity of ^{14}C-labelled protein. (X—X—X) Radioactivity of ^{3}H-labelled RNA.

which already contained a small amount of DEAE-cellulose. The effluent and the washes (in the same buffer) were recovered, then the column was eluted discontinuously as indicated on fig. 1.

2.3. *Preparation of RNAs*

As a rule, labelled RNA was used to facilitate quantitative control. Tumor-bearing mice were injected intraperitoneally with 1 mCi of tritiated uridine and sacrificed 12 hr later. RNA was extracted from plasma cell tumors by phenol-SDS at three temperature steps: 0°, 45° and 60°C [15]. The extracted RNAs were precipitated first with 2.5 vol ethanol, redissolved in water and reprecipitated with 1.5 M NaCl. RNAs were dissolved in water. The fraction extracted at 0°C was shown to contain over 99% pure ribosomal RNA (called thereafter the 'rRNA' fraction). The fraction extracted at 45°C was discarded, but the fraction extracted at 60°C was shown to contain between 70 and 80% of dRNA as determined by base composition, the rest of the RNA being rRNA (the 'dRNA' fraction). The yield of RNA was approximately 400 mg 'rRNA' and 20 mg 'dRNA'/100 g wet tumor.

2.4. *Covalent binding of RNA to Sepharose 2B*

'rRNA' or 'dRNA' were bound to Sepharose 2B following the technique of Wagner et al. [12]. Sepharose 2B was first activated with CNBr and then the RNA was coupled by incubation in a 0.2 M morpholine ethane sulfonic acid buffer (pH 6) for 20 hr at 4°C with slight stirring. The Sepharose was then exhaustively washed with the two following solutions, until there was no absorption at 260 nm : 1) 10 mM phosphate buffer (pH 7.5); 10 mM EDTA; 0.2% N-lauryl sarcosine; 90% formamide. 2) 50 mM Tris–HCl buffer (pH 7.5); 0.7 M NaCl; 10 mM EDTA; 25% formamide. The Sepharose–RNA could be stored in phenolsaturated water at 4°C for several weeks. Before use, the Sepharose–RNA was washed and equilibrated with the binding buffer. Protein binding took place under the conditions given in fig. 2. The amount of RNA bound to Sepharose, and the loss of RNA during the experiments was controlled by ^{3}H radioactivity measurement.

2.5. *Polyacrylamide gel electrophoresis of proteins*

The protein sample (50–100 μg protein) was precipitated with 10% TCA. The obtained pellet was suspended in 0.1 ml of a solution 10 mM Tris–HCl (pH 7.6) 0.3% SDS and incubated at 37°C for 90 min. Protein was measured by a microbiuret procedure.

Disk electrophoresis was carried out by a modification of the method of Waehneldt [16] in columns of polyacrylamide gel consisting of two sections: a 8% gel in the upper part, and a 10% gel in the lower part of a glass tube (8 × 0.6 cm internal diameter). Concentration of SDS in the tray buffer and in the gel was 0.1%. A current of 1.5 mA per tube was applied for 8 hr. The gels are fixed and stained in a solution of 0.25% Coomassie Brilliant Blue R. and destained with an acetic acid–methanol–water mixture. Inten-

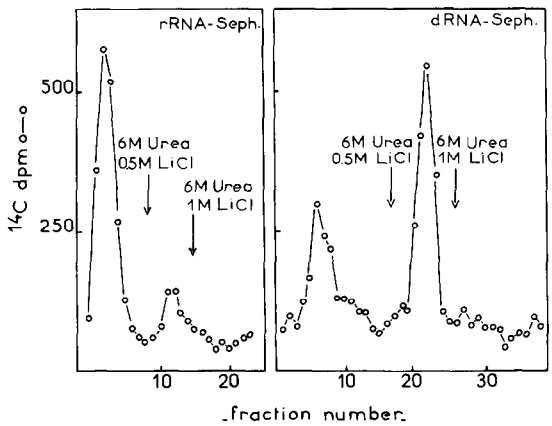

Fig. 2. Affinity chromatography of fraction B proteins on Sepharose–rRNA (left panel) and Sepharose–dRNA (right panel). 10 ml of Sepharose containing 0.35 mg/ml bound RNA, prepared and washed as described in methods, were charged in a glass column of 1 cm internal diameter, and equilibrated with a buffered solution 20 mM Tris–HCl (pH 7.6), 150 mM KCl, 10 mM β-mercaptoethanol, 0.5 M sucrose and 1 M urea. Proteins from peak B eluted from DEAE-cellulose (approximately 30000 dpm 2 mg protein) were slowly filtered at 0–4°C through the Sepharose and recycled twice. Then 2.5 ml fractions were collected. Retained proteins were first eluted with the same buffered solution but 0.5 M LiCl replacing the KCl, and 6 M urea. Final elution was carried out with the buffered solution containing 1 M LiCl and 6 M urea. Recovery was 95–100% of applied radioactivity. (o–o–o) radioactivity of ^{14}C-labelled proteins, measured on 0.3 ml aliquots plated on filter paper and washed with 10% TCA, ethanol, ethanol–ether and ether.

sity of the coloured bands was recorded with a Vernon PH15 scanning densitometer system.

2.6. *Radioactivity determination*

Radioactivity was measured on filter paper squares by the method of Mans and Novelli [17] and computed in dpm in an Intertechnique ABAC SL40 scintillation spectrometer.

3. Results and discussion

Partially purified informosomes were isolated on a D_2O-sucrose gradient [13]. In the subsequent step, particles were dialysed against a low ionic strength–urea buffer. Proteins not bound to, or dissociated from, the RNP particles during dialysis were called

Table 1

Percentage of protein interacting with RNA.

Fraction	Expt. no.	Ptoteins retained on I Sepharose–dRNA p.cent	Ptoteins retained on II Sepharose–rRNA p.cent	Ratio I/II
A	1	28	40	0.70
	2	25	42	0.60
	3	23	35	0.65
B	1	29	13	2.23
	2	34	14.6	2.32
	3	36	18	2.00

A – fraction not retained on DEAE-cellulose. B – fraction absorbed on DEAE-cellulose, eluted by 0.3 M LiCl. Proteins were labelled by ^{14}C and determined by their radioactivity.

'free' proteins. Proteins remaining in nucleoprotein complexes were called 'bound' proteins. The latter were separated from the RNA after adsorption onto DEAE-cellulose by salt elution [18] as shown in fig. 1. There was a first peak consisting of 'free' basic proteins in the effluent (fraction A); 0.3 M LiCl eluted 'bound' basic and acid proteins, and 'free' acid proteins (fraction B). These fractions were free of RNA, which was eluted with some residual protein, by the magnesium acetate step (fraction C).

Isolated protein fractions were chromatographed on Sepharose-RNA columns. The specificity of protein binding was controlled by running in parallel three columns: a) CNBr-treated Sepharose, inactivated by ethanolamine; b) Sepharose-rRNA and c) Sepharose-dRNA. The background of retained proteins shown by the CNBr-treated, ethanolamine-inactivated Sepharose was low and could be neglected. Urea-containing buffer was needed for complete solubilization of the protein fractions, which showed a marked tendency to aggregate. The amounts of protein bound to Sepharose–rRNA and Sepharose–dRNA were reproducible (table 1). The Sepharose–rRNA bound more protein from fraction A than from fraction B and thus fraction A probably contains a higher percentage of ribosomal protein species. Under our binding conditions, 25% of A proteins and 33% of B proteins were bound by Sepharose–dRNA, as calculated from the radioactivity in the peak of protein eluted by 0.5 M LiCl.

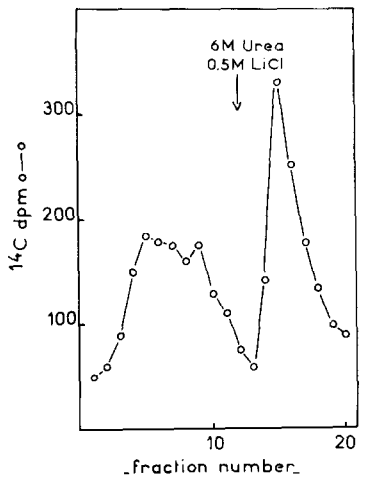

Fig. 3. Affinity chromatography on Sepharose–dRNA of proteins not retained on rRNA. Fraction B proteins were first chromatographed on Sepharose–rRNA. Proteins from the effluent were then chromatographed on Sepharose–dRNA. Binding and elution conditions were identical as in fig. 2. (o–o–o) TCA precipitable ^{14}C-radioactivity from 0.3 M aliquots.

The elution profiles of proteins from fraction B, adsorbed respectively on Sepharose–rRNA (left panel) and Sepharose–dRNA (right panel) are shown in fig. 2. If fraction B proteins were first chromatographed on Sepharose–rRNA, then the non-retained proteins rechromatographed on Sepharose–dRNA, more than 40% of the proteins having no affinity for rRNA bound to dRNA (fig. 3). Proteins not adsorbed, either to rRNA nor to dRNA could be contaminating soluble proteins, or could simply not be bound under our conditions. For example, non-electrostatic interactions are considerably reduced in the presence of urea, and thus the reaction with RNA will mainly be due to electrostatic forces. It has been found that elution by 6 M urea without salt did not detach the bound proteins. Considering our experimental system we cannot exclude the possibility that some of the protein components might have interaction sites for both rRNA and dRNA.

The SDS-polyacrylamide gel electrophoresis profiles of the proteins of fraction B retained by Sepharose–dRNA are shown in fig. 4. Four well-defined protein bands were observed, together with several minor or dubious components. Compared with standard protein, they had molecular weights of 52000, 61000, 79000 and 94000.

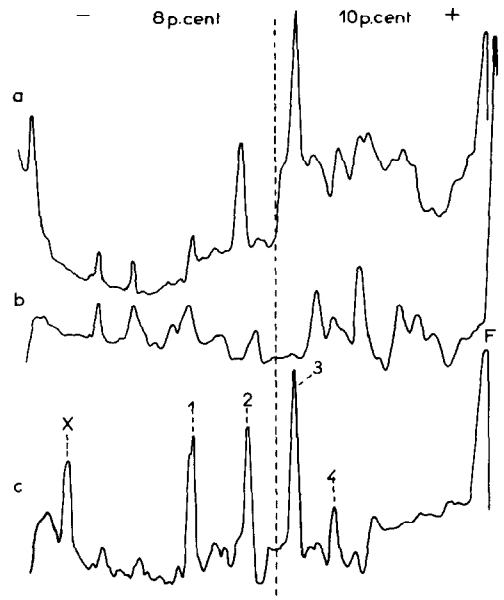

Fig. 4. SDS-polyacrylamide gel electrophoresis of proteins obtained from the informosome-rich fraction. Densitogram of the Coomassie Blue stained gels: a) proteins contained in the peak B eluted from DEAE-cellulose; b) proteins from peak B, not retained on Sepharose–dRNA; c) proteins from peak B, retained on Sepharose dRNA and eluted by 0.5 M LiCl, 6 M urea. X: inconstant, high-molecular weight protein. Estimated molecular weights of protein bands: 1, 94000; 2, 79000; 3, 61000; 4, 52000. F, bromophenol blue front.

These results are in partial agreement with those of Bryan and Hayashi [19], who found 2 proteins bound to mRNA from reticulocytes of molecular weight 48400 and 78500, and those of Blobel [3], who found two proteins of 52000 and 78000 bound to mRNA of L cell and rat liver polysomes. Lebleu et al. [20] found a heavier component, of molecular weight 130000 together with a protein of 68000 in reticulocyte mRNP-protein particles. We also detected a heavy band near the top of the gel (noted X in fig. 4), but it was not always observed. The minor irregularities in the densitometric curve (mainly in the 10% gel) probably are due to small amounts of ribosomal proteins retained by the rRNA contaminating the dRNA fraction. A monomer–polymer relationship between the 4 detected proteins could not be demonstrated. With this method, the preparative separation of the proteins can now be envisaged, which should enable more information on these proteins to be obtained.

Acknowledgements

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