

Isolation of Messenger Ribonucleoproteins from HeLa Cells by Affinity Chromatography on Poly (U) Sepharose

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Messenger Ribonucleoproteins

Polysomes of HeLa cells were adsorbed on Poly(U)-Sepharose columns. This adsorption is probably due to poly(A) sequences at 3' terminus of the messenger. Stepwise elution initially removed ribosomal subunits thereafter mRNA and a set of proteins.

These proteins are identical with the main components of the polysomal messenger ribonucleoprotein particles described previously. Thus, this method allows their rapid and easy separation from ribosomal and other proteins.

Introduction

Messenger RNAs with poly(A) sequences at their 3' ends have been isolated successfully from various tissues by column chromatography using poly(U) or oligo(dT) covalently bound to matrices^{1–4}.

Lindberg⁵ prepared polysomal mRNP particles by the same principle by passing cytoplasmic extracts through a column of oligo(dT) linked to cellulose. Since mRNPs are an integral part of the polysomal complexes, we have tried to fix whole polysomes to such a column, hoping that free 3' ends of mRNA would bind to the immobilized poly(U).

Preliminary experiments indicated that this could be accomplished. It was further possible to remove the main constituents of the bound polysomes by stepwise elution. We have characterized the eluted fractions and prove that mRNAs and their associated proteins can be obtained by this means.

Materials and Methods

Buffers

Buffer A: 10 mM Tris-HCl (pH 7.4); 10 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.

Buffer B: 10 mM Tris-HCl (pH 7.4); 500 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.

Buffer I (isotonic): 10 mM Tris-HCl (pH 7.4); 150 mM KCl; 3 mM MgCl₂; 7 mM mercaptoethanol.

Abbreviations: mRNPs, messenger ribonucleoproteins; EDTA, ethylenediaminetetraacetic; DOC, deoxycholate; poly(U), polyuridylic acid; poly(A), polyadenylic acid.

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Buffer W (wash buffer): 10 mM Tris-HCl (pH 7.4); 500 mM KCl; 3 mM MgCl₂; 1 M urea; 7 mM mercaptoethanol.

Buffer E (elution buffer): 20 mM Tris-HCl (pH 9.0); 6 M urea; 7 mM mercaptoethanol.

Buffer S (containing sarkosyl): 150 mM KCl; 0.5% sarkosyl; 0.2% NaN₃.

Cell culture and fractionation

HeLa cells (clone S3) were grown in Eagle's medium complemented with 5% calf serum. Cellular RNAs were labeled with 0.2 μ Ci/ml [³H]uridine for 12 hours incubation. A ten times concentrated cell suspension was grown in the presence of 0.05 μ g/ml actinomycin D for 20 min followed by the addition of [³H]uridine (1 μ Ci/ml) so that mRNA was preferentially labeled⁶. After 3 hours incubation the cells were sedimented by centrifugation, resuspended and washed with buffer A, and homogenized. Nuclei and mitochondria were removed by centrifugation. The post-mitochondrial supernatant was layered on a 1.5 ml 30% sucrose cushion in buffer B and centrifuged in a SW 50.1 rotor at 25 000 rpm for 17 hours. The pellet was immediately dissolved in buffer I or stored at –80 °C.

Affinity chromatography

This procedure was carried out at 4 °C. 1.5 g of poly(U)-Sepharose (obtained from Pharmacia) was allowed to swell in buffer E for 10 min, and then packed in a column of 1 cm diameter. The column was washed with 50 ml buffer E and equilibrated with 100 ml of buffer I. Samples of 8 mg of polysomes were prepared in 3 ml of buffer I and applied to the column. The column was washed with 50 ml buffer I, followed by 20 ml buffer W, and eluted with buffer E. The column was finally washed with buffer S, and stored in this buffer pro up to 1 week. Columns were used 3 times.



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Protein electrophoresis

To 1 ml of the solution for analysis 40 μ l of iodoacetamide and 5 μ l mercaptoethanol and 200 μ l of 50% TCA were added successively. Proteins were allowed to precipitate overnight at 4 °C, and the samples were centrifuged for 20 min at 3000 g at 4 °C. The pellets were washed in ethanol/ether (1/1), then in ether and dried at room temperature. Proteins were dissolved in appropriate amounts of buffer (20 mM Tris-HCl (pH 7.4), 21 mM mercaptoethanol, 2% SDS) and heated in boiling water for 2 min. 10% acrylamide gels with a bisacrylamide/acrylamide ratio of 0.013 were made according to Favre and Laemmli⁷. Acrylamide solution was poured in 0.6 cm diameter glass tubes and allowed to polymerize overnight. 1 cm of 3% acrylamide (bisacrylamide/acrylamide = 0.05) was deposited on the 8 cm long separating gel. Electrophoresis was run at room temperature at 3 mA/gel. The proteins were stained with Coomassie Brilliant Blue⁸ and molecular weights were estimated by comparison with standard proteins.

Centrifugation in CsCl

To a 100 μ l sample for analysis 10 μ l of 30% glutaraldehyde neutralised with 1 M Na₂CO₃⁹ were added. Fixation took place at 4 °C for 5 min, then the resulting solution was layered on to pre-formed CsCl gradients and immediately centrifuged.

RNA analysis

Two volumes of chloroform/phenol were added to one volume of RNA-containing sample. RNA was extracted at room temperature according to Perry *et al.*¹⁰. The RNA was analysed on 2.2% acrylamide gels containing 0.5% agarose as described by Thiollais *et al.*¹¹.

Results and Discussion

A set of preliminary experiments indicated that polysomes have a high affinity for poly(U)-Sepharose. This affinity is largely due to binding to poly(U) although other less specific binding cannot be excluded (Table I). From a variety of eluants a schedule of elution was established.

The most effective procedure is as follows: When the total ribosome/polysome fraction from a sucrose gradient is loaded on a poly(U)-Sepharose column, the bulk of it passes through unadsorbed. However, as much as 20% of the original fraction remains on the column and is not removed by extensive washing with isotonic or hypotonic buffer; buffered 0.5 M KCl releases only some proteins. When, however,

Table I. Retention of labeled mRNA containing material on different Sepharose columns. Polysomes with [³H]uridine labeled mRNA were fractionated on Sepharose as described in Fig. 1. "unbound" refers to fraction A plus B. "bound" refers to fraction C plus D.

Column matrix	"unbound"	"bound"	No. of experiments
poly (U)-Sepharose	28% \pm 3%	72% \pm 3%	8
poly (A)-Sepharose	71%	29%	3
mere Sepharose	74%	26%	3

1 M urea in 0.5 M KCl buffer is added to the column, ribosomal RNA and ribosomal proteins are quantitatively eluted. In a further step the column is flushed with buffered 6 M urea (Buffer E); which removes more proteins and heterogenous RNA. The remainder of the total RNA (about 2%) can be detached by means of detergents.

For a closer analysis of the fractions eluted with the various buffers, HeLa cells were allowed to grow in the presence of [³H]uridine and actinomycin D under conditions where predominantly mRNA and 5S RNA are labeled⁶. When a polysomal/ribosomal fraction from such cells is passed through the column (step 1) 85% of the radioactive RNA is retained; approximately 15% is not adsorbed and constitutes fraction A in Fig. 1. The

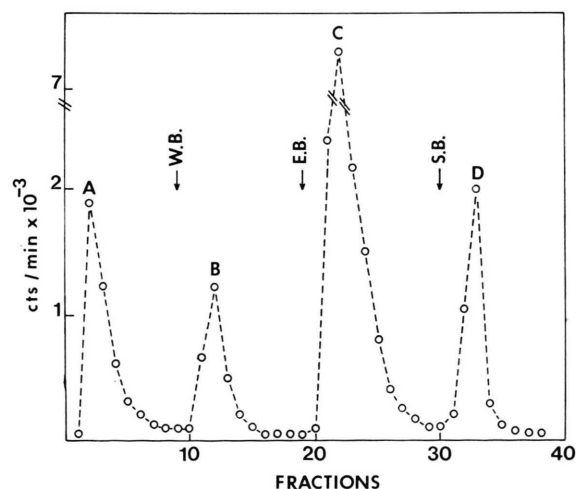


Fig. 1. Fractionation of polysomes on a poly(U)-Sepharose column. Approximately, 5×10^8 cells were grown in the presence of [³H]uridine and actinomycin D in order to label mRNA; poly(U)-Sepharose column chromatography was performed as described in Materials and Methods. Fractions of eluate were collected and precipitated with 10% TCA at 4 °C. Precipitates were adsorbed on glass fibre filters, rinsed, and dried thoroughly. Radioactivity was determined in a Packard Scintillation counter.

following wash (step 2) with buffered 0.5 M KCl and 1 M urea removes approximately 15% and gives fraction B. From these two fractions RNA was extracted. They contain 18S, 28S and 5S RNA as revealed by polyacrylamide gel electrophoresis (Fig. 2). The column was then rinsed with buffered 6 M urea (step 3) yielding fraction C and finally (step 4) treated with sarkosyl to remove all tenaciously bound radioactivity (fraction D). As shown in Fig. 2 fraction C contains heterogeneous RNA.

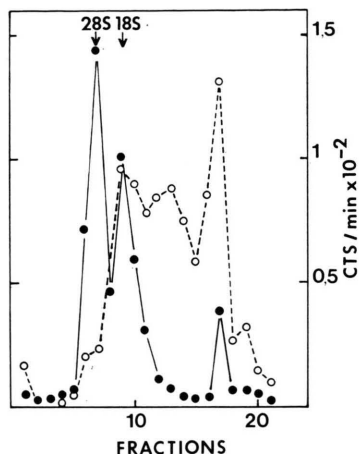


Fig. 2. Size distribution of RNAs obtained by chromatography on poly(U)-Sephacryl column. Approximately, 5×10^8 cells were incubated with [3 H]uridine for 12 hours in order to label RNAs uniformly; poly(U)-Sephacryl column chromatography and the RNA extraction procedure are described in Materials and Methods. ●—●, RNA from fraction B; ○—○, RNA from fraction C.

We have further characterized the protein composition of the four fractions. Fraction A and B contain the spectrum of ribosomal proteins. Some apparently nonribosomal proteins with molecular weights above 55 000 daltons are also present; they probably represent factors of the "salt wash" (Fig. 3 a and b). Fractions C and D reveal a set of protein bands with two major proteins of molecular weights of 51 000 and 78 000, of the same order as those found in mRNPs. There are, also several minor bands (above 50 000 daltons) which have been previously reported by many groups¹²⁻¹⁴ (Fig. 3 c and d). Other minor bands with lower molecular weights may represent contamination with ribosomal proteins as suggested by the split gel electrophoresis procedure shown in Fig. 4. The two major proteins in fraction C do not coincide with any ribosomal protein in fraction B or proteins of fraction A.

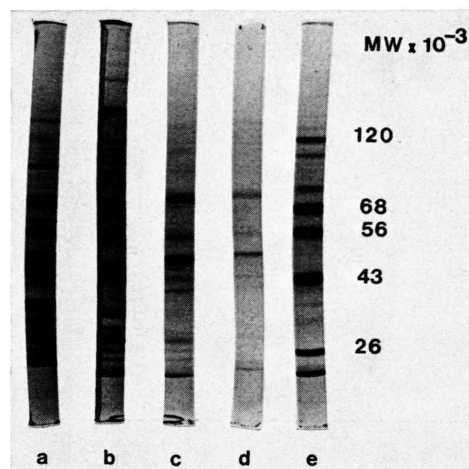


Fig. 3. From the poly(U)-Sephacryl column. Polysomes from 5×10^8 cells were fractionated as described in Fig. 1. The proteins were analysed on 10% acrylamide gel electrophoresis in presence of SDS, as indicated in Materials and Methods. a. Proteins of fraction A; b. proteins of fraction B; c. proteins of fraction C; d. proteins of fraction D; e. proteins standard: β -galactosidase M.W. 120 000; bovine serum albumin M.W. 68 000; catalase M.W. 50 000; ovalbumine M.W. 43 000; carboanhydrase M.W. 26 000.

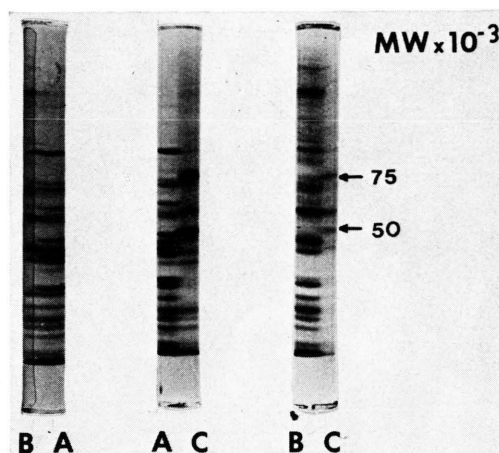


Fig. 4. Comparison of the proteins from fractions A, B and C by split gel electrophoresis. The proteins were prepared the same way as described Fig. 3. Likewise, electrophoresis was run in the same buffer system, but wider tubes (9 mm diameter) were used. Each tube was divided in two chambers in the upper 2 cm by a thin glass slide. A 10% acrylamide separating gel was filled in up to lower edge of the glass divider, and the stacking gel poured onto it. Equal amounts of protein were layered in each chamber. Electrophoresis was run at 5 mA/gel. A, proteins of fraction A; B, proteins of fraction B; C, proteins of fraction C.

It was of interest to know if the material eluted from the column is derived from defined, pre-existing particulate units. We have, therefore, analysed

the eluates by sucrose gradient centrifugation. Fraction A contains essentially ribosomes and ribosomal dimers or disomes (Fig. 5a). Fraction B contains

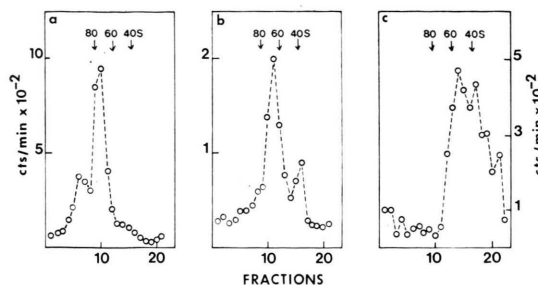


Fig. 5. Size distribution of particles in fractions A, B and C. The cells were labeled with [^3H]uridine for 12 hours, and polysomes were fractionated as described Fig. 1. The fractions were layered on 15–30% sucrose gradients made up in buffer A. Samples were centrifuged in a SW 40 rotor at 4 °C for 16 hours at 22 000 rpm. Radioactivity was monitored as described Fig. 1. Sedimentation coefficients were determined according to McEwen¹⁹. a. fraction A; b. fraction B; c. fraction C.

two particles with sedimentation rates of ribosomal subunits (Fig. 5b). The analysis of fraction C reveals a heterogeneous group of particles with sedimentation rates below 60S (Fig. 5c). However, since fraction C contains 6 M urea, it seems unlikely that the eluate consists of unaltered and integral particles; yet, since they appear upon gradient centrifugation, they have apparently been re-formed in the gradient. In order to test this, the eluate C was analysed by CsCl density gradient analysis after fixation with glutaraldehyde. By this procedure no ribonucleoprotein particles could be detected in fraction C (Fig. 6a), instead RNA was found (Fig. 6, fraction 1 to 3). If fraction C was first dialysed against buffer I, aggregates re-form from heterogeneous mRNA and proteins, yielding particles with a density in CsCl of 1.5 g cm⁻³ (Fig. 6b). The density of these particles is equal to the density of “salt washed” ps-mRNPs, as found earlier in our laboratory¹⁵.

When in step 3 of the elution schedule 6 M urea was replaced by a buffer containing DOC, a fraction C' containing several proteins was eluted. As seen from Fig. 7 the spectrum of proteins resembles the one obtained from ps-mRNPs, although only one of the two major proteins (75 000 daltons) was detected, together with several minor ones. The second major protein (50 000 daltons) was eluted only by the following ionic detergent treatment in

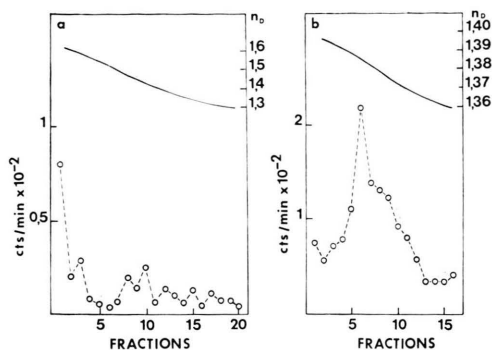


Fig. 6. Spontaneous reassociation of mRNA with proteins of fraction C in low salt buffer. Polysomes were prepared and fractionated as described Fig. 1. Particles from fraction C were layered on CsCl gradients and centrifuged (SW 56, 40 000 rpm, 20 hours, 20 °C). a. fraction C in buffer E (*i.e.* 6 M urea), not dialysed, fixed with glutaraldehyde; b. fraction C, dialysed 17 hours against buffer I and fixed thereafter with glutaraldehyde. ○—○, Radioactivity; —, refractive index.

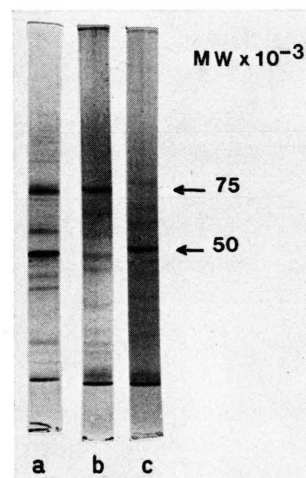


Fig. 7. Proteins released polysomes bound to poly(U)-Sephacrose by DOC treatment in step 3 (fraction C'). The ribosomes/polysomes fraction was prepared as described in Materials and Methods. The column was washed free from unabsorbed material with buffer I and from ribosomal subunits with buffer W. Then the column was eluted with 0.5% DOC (fraction C'), and finally with 0.5% sarkosyl in order to release the remaining proteins (fraction D'). Proteins were analysed by polyacrylamide gel electrophoresis (see: Fig. 3). a. proteins of fraction C'; b. Proteins of fraction D'.

step 4. This is in agreement with previous results from this laboratory, indicating that DOC removes protein from ps-mRNP, rendering them incapable of reassociating with ribosomes¹⁵; this is most likely due to the removal of the 75 000 daltons protein. EDTA, when used in step 3 (compare: Fig. 1), liberates no protein from the column^{16–18}.

The results reported above indicate that in an immobilized state polysomal particles and their constituents behave as they do in solution. The immobilized state is most likely due to a binding of poly(A) sequences to poly(U). The choice of the right sequence of eluants facilitates the stepwise

isolation of constituents of the functional polysomal particles including proteins of the ps-mRMP.

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