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Selection of prosomes and prosomal RNA by immobilized viral RNAs

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Viral messengers were used to select and purify prosomes and prosomal RNA from subribosomal fractions of HeLa cells and mouse erythroblasts. Adenovirus mRNA immobilized on oligo(dT)-cellulose and tobacco mosaic virus RNA (TMV) sedimenting in sucrose gradients associated strongly with prosomes at high salt conditions forming intermolecular RNA-RNA hybrids between prosomal RNA and viral RNA. Hybrid selection of small cytoplasmic RNAs with immobilized TMV-RNA revealed a RNA species migrating at the same position as prosomal RNA. The possible existence of a box-like sequence involved in hybridization will be discussed.

Prosome; Prosomal RNA; Hybrid selection; Viral mRNA; Affinity chromatography

1. INTRODUCTION

Many previous reports have shown the existence of prosomes in various cell systems. They have been isolated from the cytoplasm of vertebrate and invertebrate cells, from tissues of plants and even from archaeobacteria [1-9]. In all cases they consist of a specific set of proteins with molecular masses ranging from 19 kDa to 35 kDa. Some of them were highly conserved during evolution, while others vary from species to species. A matter of controversy is the presence and number of RNA molecules as integral part of prosomes. While proteasomes which are homologous to prosomes were considered to be protein complexes without RNA components [10,11], small RNA with a length of about 70 to 100 nucleotides was isolated from purified prosomes of mouse and duck erythroblasts, HeLa cells, calf hepatocytes, drosophila and plant tissue [1-4,6]. All cell systems we investigated so far contain a prosomal RNA with a size of about 80 nucleotides. This RNA is an intrinsic part of prosomes and prosomal proteins cover and protect almost its whole length against nuclease digestion [12]. We recently have shown that prosomes and prosomal RNA inhibit the in-vitro translation of adenovirus mRNA while protein synthesis of cellular mRNA was not affected [13]. Dot blot analysis revealed that prosomal RNA hybridized strongly with viral mRNA which suggested that prosomes (19S RNP particles) will associate with adenovirus mRNA during cell free translation. Thus we were interested to investigate the interaction of prosomes with viral mRNA more closely. For this approach we tested the affinity of prosomes for viral RNA isolated from homologous and heterologous cell systems. Our results implicate that prosomes have no specific affinity for a certain viral RNA and demonstrate that chromatography or hybrid selection with immobilized viral RNA may be useful techniques to isolate and purify prosomes or prosomal RNA.

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2. MATERIAL AND METHODS

2.1. *HeLa cell culture and infection with adenovirus*

HeLa cells were grown in suspension at 4×10^5 cells per ml with a generation time of about 24 h in Eagle's spinner medium supplemented with 10% newborn calf serum. Propagation of adenovirus type 2 in suspension cultures, as well as the extraction and purification of the virus, has been described [14-15].

2.2. *Cell fractionation and isolation of prosomes*

The preparation of postmitochondrial supernatants of HeLa cells and mouse erythroblasts was as previously described [16]. Differential ultracentrifugation was used to separate polysomes and ribosomal particles. Postribosomal particles were fractionated by centrifugation in linear 10-25% (w/w) sucrose gradients (Beckman Rotor SW 27, 22 000 rpm, 4°C, 18 h) in TBNa 100 (20 mM Tris-HCl (pH 7.4); 100 mM NaCl; 3 mM $MgCl_2$; 7 mM 2-mercaptoethanol). Particles sedimenting in the range of 15-30S were pooled and concentrated by high speed centrifugation (Beckmann Rotor Ti 60, 48 000 rpm, 4°C, 18 h). The pellets were dissolved in TBNa 500 (20 mM Tris-HCl (pH 7.4); 500 mM NaCl, 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol) and 5-10 A_{260} units were loaded on 10-50% (w/w) sucrose gradients in the same buffer. After centrifugation (Beckman Rotor SW 40, 36 000 rpm, 4°C, 18 h) peak fractions in the range of 19S were pooled and exposed to 1% lauroylsarcosinate-Na (final concentration) and 2-5 A_{260} units were loaded on 10-50% (w/w) sucrose gradients in detergent buffer (1) and then centrifuged again in a Beckman Rotor SW 40 at 36 000 rpm at 4°C for 18 h. Peak fractions in the range of 19S (i.e. prosomes) were pooled and precipitated with ethanol. Prosomal RNA was extracted with chloroform/phenol as described [17] and the RNA was precipitated with ethanol and stored at −20°C.

2.3. *Isolation of TMV-RNA and CPMV-RNA*

TMV-RNA strain vulgare was purified from a virus suspension of 20 mg virus/ml by adding 0.2 ml cacodylate buffer (20 mM Na-

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cacodylate, 2 mM EDTA, 1 N NaOH, pH 7.2), 0.2 ml SDS (20%), 0.08 ml 2-mercaptoethanol to 1 ml virus suspension [18]. The milky suspension was incubated 1–2 min at 60°C in a waterbath and after addition of 2.52 ml cacodylate buffer, TMV-RNA was extracted three times with phenol (saturated with 0.05 M Tris-HCl (pH 7.2), and precipitated with 2.5 volumes of 95% ethanol and 300 mM Na-acetate, pH 5.2. TMV-RNA was stored at −20°C in ethanol or at −70°C in water. CPMV-RNA was isolated as described in [19] and stored at −20°C.

2.4. *Labeling techniques*

Protein labeling: HeLa cells were resuspended at 4 × 10^6 cells per ml in methionine-free medium and incubated for 3 h at 37°C with ^{35}S-methionine (10 μCi/ml; 1 Ci = 37 GBq).

RNA labeling: Prosomal RNA was labeled at the 3′end with ^{32}P-cytidine-3′,5′-biphosphate (^{32}P-pCp) in a reaction catalyzed by T4-ligase (BRL). After labeling, the RNA was precipitated several times with cold 70% ethanol to eliminate free ^{32}P-pCp [20].

2.5. *Polyacrylamide gel electrophoresis (PAGE)*

Electrophoresis of protein was performed in one-dimensional SDS-polyacrylamide gels [21] RNA electrophoresis in 11% polyacrylamide gels containing 7M urea according to [1]. Markerproteins were phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soy bean trypsin inhibitor (20 kDa) and lactalbumine (14 kDa).

2.6. *Dot-blot-hybridization and hybrid selection*

RNA samples were spotted on 'Gene Screen' nylon membranes (NEN, FRG) and baked at 80°C for 2 h. After pre-hybridization at 42°C, hybridization was carried out with 3 × 10^6 cpm of ^{32}P-labeled RNA for 24 h. The pre-hybridization mixtures and hybridization mixtures contained 50% formamide, 0.04% each of polyvinylpyrrolidine, bovine serum albumin, ficoll (2× Denhardt solution); 0.75 M NaCl, 0.075 M Na-citrate (5× SSC); 1% SDS and 100 μg/ml denaturated salmon sperm DNA. After hybridization the filters were washed twice in 2× SSC, 1% SDS at 60°C for 20 min and finally twice in 1× SSC at 60°C for 20 min. Thereafter the filters were dried and exposed to Fuji X-Ray film.

For hybrid selection TMV-RNA was spotted on 'Gene Screen' nylon membranes and maintained as described above. After hybridization with ^{32}P-labeled prosomal RNA (same procedure as above) radioactive spots were excised and transferred to Eppendorf-tubes. Excised pieces of membranes weer incubated with 400 μl elution buffer (90% formamide, 1 mM EDTA, 0.5% SDS, 20 mM Pipes (pH 6.4),) at 50°C. After 10 min of continuous stirring the eluated RNA was precipitated with 40 μl 300 mM Na-acetate (pH 5.2), 800 μl 95% ethanol and 10 μl carrier tRNA (1 mg/ml) at −20°C.

After sedimentation the RNA was washed several times with cold ethanol and analyzed by UREA-PAGE containing 11% polyacrylamide.

3. RESULTS

To demonstrate the association of prosomes with viral messengers two different techniques were applied. Poly(A)$^+$ mRNA isolated from adenovirus-infected HeLa cells was immobilized on oligo(dT)-cellulose or TMV-RNA was sedimented in sucrose gradients in the presence of prosomes.

For affinity chromatography equal amounts of polysomal mRNA isolated from infected and uninfected HeLa cells [13] were incubated with a crude 19S fraction of HeLa cells labeled with [^{35}S]methionine. The mixture was passed through oligo(dT)-cellulose equilibrated in high salt buffer. Thereafter the two columns were washed with high salt buffer and eluted with low salt buffer. Washed and eluted fractions were probed for TCA precipitable radioactivity.

Only little radioactivity was bound to the column with cellular mRNA while a pronounced peak of radioactivity was observed in the second fraction eluted from the column (Fig. 1). To identify the particles which were bound to the column we analyzed washed and eluted fractions by Laemmli-PAGE. Analysis of loaded and washed fractions revealed multiple protein bands migrating between 20 kDa and 94 kDa (Fig. 2,A,B,E).

No proteins could be detected in the fractions eluted from the column with cellular mRNA (Fig. 2C,D). However almost pure prosomes eluted from the column containing viral mRNA (Fig. 2F,G). Only traces of others than prosomal proteins were bound to the column, confirming the specific interaction of prosomes with viral mRNA.

We were furthermore interested if prosomes of HeLa cells would associate with viral messengers from other cell systems.

For this approach we used tobacco virus RNA; the large genomic TMV-RNA is known as excellent model messenger for cell free protein synthesis with a well defined size of 6400 nucleotides corresponding to a sedimentation rate of ^{32}S.

Unfortunately it lacks a poly(A) sequence, therefore we studied the possible association of [^{35}S]methionine labeled HeLa prosomes with TMV-RNA by ultracen-

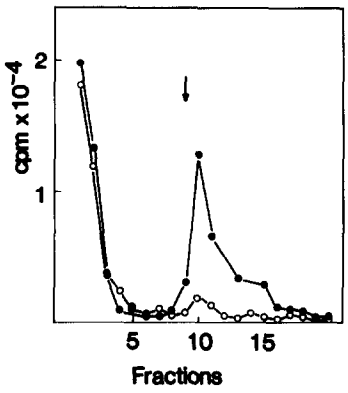

Fig. 1. Interaction of prosomes with poly(A)$^+$ mRNA. Free mRNPs of ^{35}S-labeled HeLa-cells were dissociated in TBNa-500 and sedimented through 10–50% sucrose gradients (SW 40 rotor, 36 000 rpm, 18 h, 4°C). 19S fractions were pooled and incubated with polysomal poly(A)$^+$ mRNA of infected and uninfected HeLa cells. This mixture was loaded on an oligo(dT)-cellulose column, with a bed volume of 1 ml equilibrated in TBNa-500 (high salt buffer). After washing with 5 ml TBNa-500 the column was eluted with low salt buffer (20 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol). 100 μl of each fraction was incubated with 10% trichloracetic acid (final concentration) and the precipitated radioactivity was measured in a liquid scintillation counter. Elution profile of ^{35}S-labeled 19S fractions incubated with polysomal poly(A)$^+$ mRNA of : (-•-) adenovirus-infected cells and (-o-) uninfected cells.

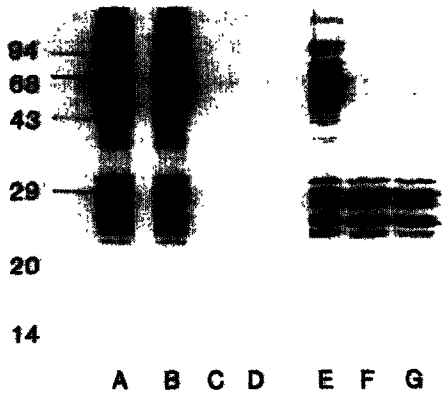

Fig. 2. SDS-PAGE of fractions eluted from oligo(dT)-cellulose. Crude [^{35}S]methionine labeled 19S fractions were incubated with poly(A)$^+$ mRNA of infected and uninfected cells and passed through oligo(dT)-cellulose (see legend Fig. 1). Washed and eluted fractions were analyzed by Laemmli-PAGE and visualized by autoradiography. Markerproteins: see section 2. (A) Crude 19S fraction loaded on oligo(dT)-cellulose, with poly(A)$^+$ mRNA of uninfected cells. (B) washed fractions. (C,D) eluted fractions 10 and 11, with poly(A)$^+$ mRNA of adenovirus infected cells, (E) washed fractions, (F,G) eluted fractions 10 and 11.

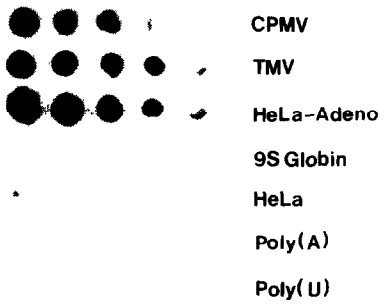

Fig. 4. Interaction of prosomal RNA with various RNAs and homopolynucleotides. Decreasing amounts of RNAs were spotted to a 'Gene Screen' membrane (a): 1 μg, (b): 0.75 μg, (c): 0.5 μg, (d): 0.25 μg, (e): 0.1 μg. The RNAs were allowed to hybridize with ^{32}P-labeled prosomal RNA as described in section 2. CPMV, cow pea mosaic virus, TMV, tobacco mosaic virus; HeLa-Adeno, poly(A)$^+$ mRNA extracted from polysomes of HeLa cells inoculated with adenovirus; 9S Globin, commercial rabbit globin RNA (BRL); HeLa, poly(A)$^+$ polysomal mRNA from uninfected cells; Poly(A), Poly(U), commercial homopolynucleotides (Sigma).

trifugation on sucrose gradients at different salt conditions (Fig. 3).

At a concentration of 100 mM KCl 10–15% of prosomes sedimented with TMV RNA near the bottom of the gradient (Fig. 3A). At 300 mM and 500 mM KCl increasing amounts of prosomes (up to 80%) were found associated with TMV-RNA (Fig. 3B). As control ^{32}P-labeled 9S globin mRNA was incubated with unlabeled HeLa prosomes at 500 mM KCl and analyzed under the same conditions. Fig. 3C demonstrates that globin RNA, a cellular RNA has no affinity for prosomes, which corresponds well to the dot blot hybridization analysis with prosomal RNA and different cellular RNAs (Fig. 4 and [13]).

These experiments demonstrate clearly that HeLa prosomes fail specific affinity for certain viral messengers. Furthermore prosomes associate with viral RNA by RNA-RNA interaction via prosomal RNA since high salt stabilizes nucleic acid duplex formation.

This was confirmed by the following experiments in which we investigated the interaction of prosomal RNA from mouse erythroblasts with various RNA species immobilized on nylon membranes. As shown in Fig. 4 we were able to hybridize ^{32}P-labeled prosomal RNA with poly(A)$^+$ mRNA from adenovirus infected HeLa cells, with TMV-RNA and with cow-pea mosaic virus RNA. Rabbit globin mRNA, poly(A)$^+$ mRNA from uninfected HeLa cells, as well as poly(A) and poly(U) were found negative.

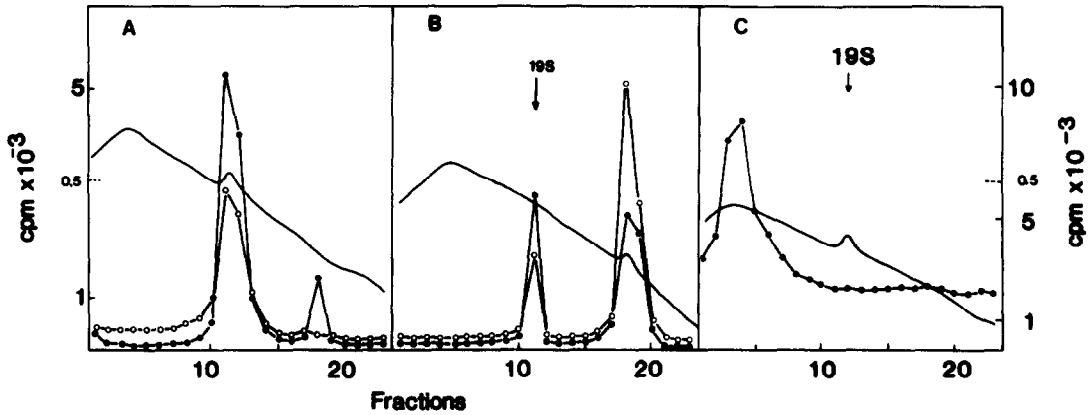

Fig. 3. Interaction of prosomes with tobacco mosaic virus RNA or globin mRNA. TMV-RNA was incubated with [^{35}S]methionine labeled prosomes. The samples were analyzed by centrifugation in 10–50% sucrose gradients under low and high salt conditions. Fractions were collected and precipitated with 10% TCA (final concentration). (A) Sedimentation profiles in gradients containing 100 mM KCl (-•-) TMV-RNA + prosomes, (-○-) prosomes (control). (B) Sedimentation profiles of prosomes + TMV-RNA in gradients containing 300 mM KCl (-•-) or 500 mM KCl (-○-). (C) Sedimentation profile of unlabeled prosomes + rabbit globin mRNA (end-labeled with ^{32}P-pCp) in gradients containing 500 mM KCl (-•-). (—) Absorbance at 254 nm.

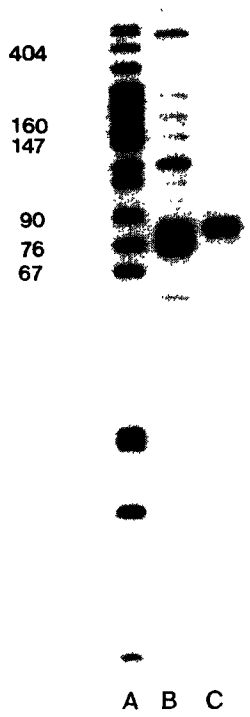

Fig. 5. Hybrid selection of prosomal RNA. TMV-RNA was spotted on a 'Gene Screen'-nylon membrane and hybridized with ^{32}P-labeled RNA isolated from a crude 19S fraction. After hybridization (see Material and Methods) bound RNA was eluted with a buffer containing 90% formamide at 50°C. After precipitation with cold ethanol the RNA was analyzed by UREA-PAGE. The RNA was visualized by autoradiography. (A) Marker nucleotides: pBR 322 digested with *Msp*I and labeled with γ-^{32}P-ATP in the presence of T4-polynucleotide kinase [22]. (B) total RNA from crude 19S fractions. (C) RNA eluted from immobilized TMV-RNA.

In a last experiment we tested if viral RNA selects prosomal RNA from a fraction containing different small RNAs. For this purpose crude 19 S fractions of mouse erythroblasts, corresponding to these we used for oligo(dT)-cellulose chromatography, were deproteinized with chlorophorm/phenol and the ^{32}P-labeled RNAs were hybridized with TMV-RNA immobilized on a nylon membrane. After hybridization the nylon membrane was washed several times with SSC-buffer and bound RNA was eluted with a buffer containing 90% formamide and analyzed by RNA-gel electrophoresis (Fig. 5). With this procedure we selected a distinct RNA species of about 80 nucleotides from a bulk of small RNAs migrating between 70 and 500 nucleotides (Fig. 5B,C). The selected RNA migrated at the same position as highly purified prosomal RNA [1,3,12].

4. DISCUSSION

In this report we demonstrate that viral RNAs are useful probes to select prosomes or prosomal RNA from subribosomal fractions. The association of prosomes with viral RNAs is stabilized by high ionic strength which is due to a RNA-RNA duplex formation between viral RNA and a 80 nucleotide long RNA which is an integral part of prosomes. However, the interaction is not virus specific since CPMV-RNA, TMV-RNA and adenovirus mRNA hybridized with the same efficiency. This suggests the existence of a common, box-like sequence on viral RNAs. Our hypothesis is reinforced by the following facts:

(i) Prosomes and prosomal RNA of mouse erythroblasts, calf-liver hepatocytes, tobacco tissue substituted completely for HeLa prosomes & prosomal RNA in cell free translation systems by blocking translation of viral mRNAs with the same efficiency ([13] and unpublished results).

(ii) Prosomes isolated from the cell systems described above contain a small RNA of about 80 nucleotides [1–9].

(iii) Preliminary experiments revealed that the genes for prosomal RNA are single copy genes, which favors the existence of a consensus sequence (Dineva, unpublished results).

Based on our results presented here we can exclude the poly(A) sequence since hybridization with prosomal RNA was negative and TMV-RNA which lacks poly(A) hybridized readily with prosomal RNA. The methylated cap as well seems not to be involved directly in the association since cow-pea mosaic virus fails this structure. Any way we assume such a consensus sequence in the 5′ region of viral RNAs since prosomes block the in-vitro translation of viral messengers by preventing their initiation (Horsch et al., submitted). This problem is under investigation.

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