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Prosomes discriminate between mRNA of adenovirus-infected and uninfected HeLa cells

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Prosomes are small cytoplasmic RNP complexes associated with repressed mRNA. In in vitro translation, they discriminate between the mRNA of adenovirus-infected HeLa cells and those of uninfected cells grown under normal conditions. Prosomes as well as their RNA constituents interact much more strongly with poly(A)+ mRNA of infected cells and inhibit their translation in vitro preferentially. A possible role of prosomes in the differential regulation of translation is discussed.

Prosome, ScRNP complex; mRNA translation; Inhibition

1. INTRODUCTION

The cytoplasm of eukaryotic cells contains various small ribonucleoprotein particles, which appear to be involved in different steps of protein synthesis. Notable is the signal recognition particle (SRP) which contains a 7 S RNA and is required for the translation of newly synthesized proteins across the endoplasmic reticulum [1]. A group of 4 S RNAs isolated from chick embryonic muscles has been shown to occur in 10 S particles which inhibit the translation of mRNA in a cell-free system [2]. Other small ribonucleoproteins from chick embryonic muscles containing so-called translational control RNA were reported to act more specifically by inhibiting the in vitro translation of muscle-derived but not heterologous messengers [3]. On the other hand, stimulation of protein synthesis by small ribonucleoproteins is known to occur in some cell systems. For example, the virus-associated VA I RNPs, found in adenovirus-infected cells, were reported to associate with viral mRNA [4] and are indispensable for efficient translation of adenoviral mRNA in the late phase of infection. Adenoviruses with deletions in the VA I coding sequence grow only poorly in host cells [5]. In addition, VA I RNPs were reported to inactivate the PI kinase which phosphorylates initiation factor eIF_2 [6,7].

A new group of small cytoplasmic RNP complexes called prosomes were recently discovered in association with repressed free mRNP complexes in the cytoplasm of duck and mouse erythroblasts and HeLa cells [8]. Here, we present evidence that prosomes are able to discriminate in vitro between the mRNA of adenovirus-infected HeLa cells and those of uninfected cells grown under normal conditions. After 20 h of infection most of the polysomal messengers consist of adenovirus mRNA [9,10]. This system was ideal for comparing the influence of prosomes on different messenger populations of the same cell type.

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Abbreviations: TBNa-X, 20 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol, X = 100 or 500 mM NaCl; RNP(s), ribonucleoprotein(s); PAGE, polyacrylamide gel electrophoresis; scRNPs, small cytoplasmic ribonucleoproteins

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2. MATERIALS 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 virus and viral DNA, has been described [9,10]. All restriction enzymes were obtained from BRL.

2.2. *Cell fractionation and isolation of prosomes*

The preparation of postmitochondrial supernatants of HeLa cells and mouse erythroblasts was performed as in [11]. Differential ultracentrifugation was used to prepare polyribosomes and ribosomal particles. Postribosomal ribonucleoprotein complexes were fractionated by sedimentation in linear 10–25% (w/w) sucrose gradients (Beckman SW 27 rotor, 16 h, 24000 rpm, 4°C) in TBNa-100. Particles sedimenting in the range 15–30 S were pooled and concentrated by high-speed centrifugation (Beckman Ti 60 rotor, 18 h, 48000 rpm, 4°C). 2–5 A_{260} units of the pellets were dissolved in TBNa-500 and loaded on 10–50% (w/w) sucrose gradients in the same buffer. After centrifugation (Beckman SW 40 rotor, 18 h, 38000 rpm, 4°C) peak fractions in the range of 19 S were pooled and exposed to 1% sodium lauroylsarcosinate (final concentration) and about 0.5–1 A_{260} units were loaded on 10–50% (w/w) sucrose gradients in detergent buffer and then centrifuged in a Beckman SW 40 rotor for 18 h at 36000 rpm at 4°C [8]. Peak fractions in the range of 19 S (prosomes) were pooled and precipitated with ethanol. For in vitro protein synthesis the pellets were washed several times with cold ethanol to eliminate the detergent. After this procedure they were dissolved in distilled water by gentle shaking for 2 h at 4°C. For RNA extraction prosomes were incubated with proteinase K and deproteinized with chloroform/phenol as described by Perry et al. [12], the RNA being precipitated with ethanol and stored at −20°C. Alternatively, prosomes were purified by FPLC [13].

2.3. *Isolation of polyribosomal mRNA from HeLa cells*

Polyribosomes of adenovirus-infected and uninfected HeLa cells were extracted with chloroform/phenol according to Perry et al. [12]; total polyribosomal RNA was cycled through oligo(dT)-cellulose equilibrated in high salt buffer. Bound poly(A)$^+$ mRNA was eluted with low salt buffer, precipitated with ethanol and stored at −20°C for further analysis [14].

2.4. *PAGE*

Electrophoresis of proteins was performed in one-dimensional SDS-polyacrylamide gels [15]. Marker proteins were: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). After proteinase K digestion prosomal RNAs were extracted from prosomes with chloroform/phenol and electrophoresis was performed in 12% polyacrylamide gels containing 7 M urea, 50 mM Tris/borate (pH 8.3) and 2 mM EDTA as described by Schmid et al. [8].

2.5. *RNA and DNA labeling*

Prosomal RNA or poly(U) was labeled at the 3′-end with [^{32}P]pCp in a reaction catalyzed by T_4 ligase according to Peattie [16]. After labeling, the RNA was precipitated several times with cold ethanol to eliminate free [^{32}P]pCp; adenoviral DNA was labeled via nick translation according to Maniatis et al. [17].

2.6. *Dot-blot hybridization*

RNA samples (0.1–1 μg) were spotted on 'gene screen' hybridization transfer membranes and baked at 80°C for 2 h. After prehybridization at 42°C, hybridization was carried out for 24 h with 3×10^6 cpm of ^{32}P-labeled RNA or DNA. The prehybridization mixtures and hybridization mixtures contained 50% formamide, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin, 0.04% ficoll, 750 mM NaCl, 75 mM Na citrate, 1% SDS and 100 μg/ml denatured salmon sperm DNA. After hybridization the filters were washed twice in 3 mM Na citrate, 30 mM NaCl for 5 min at room temperature, then three times in 30 mM NaCl, 3 mM Na citrate, 1% SDS for 20 min at 60°C and finally twice in 5 mM NaCl, 0.5 mM Na citrate for 20 min at 60°C. Thereafter, the filters were dried and exposed with Fuji X-ray film.

2.7. *In vitro protein synthesis*

Protein synthesis was performed in 25 μl reticulocyte lysate (New England Nuclear) containing 1 μg mRNA and different quantities of prosomes or prosomal RNA. After incubation at 37°C for 10, 30, 60 and 90 min, 1-μl aliquots were spotted on Whatman 3 MM filter paper (2 × 2 cm). Thereafter, the papers were subjected first to cold and then to boiling 10% trichloroacetic acid and washed twice in 5% trichloroacetic acid at room temperature. Finally, the papers were dried and the precipitated radioactivity was measured in a liquid scintillation counter.

3. RESULTS

Prosomes were first isolated as components of the untranslatable free mRNP fraction and considered to be essential factors involved in the inhibition of translation [8,18]. However, their role in the living cell is not yet understood. To elucidate their possible biological function, we have investigated the influence of prosomes on in vitro translation of different mRNA populations. For this purpose, prosomes were isolated from the postribosomal supernatants of HeLa cells [18]. To assess their purity the protein and RNA moieties were analyzed by PAGE. Ten major protein bands were observed within the range 20–35 kDa (fig.1A) and small RNA in the range 4 S (fig.1B). Here, we wish to point out that HeLa prosomes purified by centrifugation in detergent-containing sucrose gradients consist of 10–15% prosomal RNA. A more detailed comparative description of the prosomal constituents in several cell systems has been reported [18].

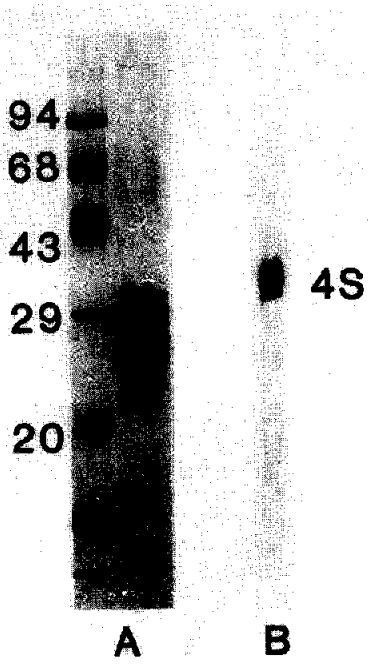

Fig.1. Gel electrophoresis of prosomal proteins and prosomal RNA. Prosomes were purified by sedimentation through lauroylsarcosyl containing 10–50% sucrose gradients (SW 40 rotor, 36000 rpm, 18 h). Prosomal proteins were separated by Laemmli PAGE and visualized by Coomassie blue stain (A). Marker proteins: see section 2. Prosomal RNA was extracted after digestion with proteinase K, with chloroform/phenol and separated by 7 M urea-containing PAGE (B).

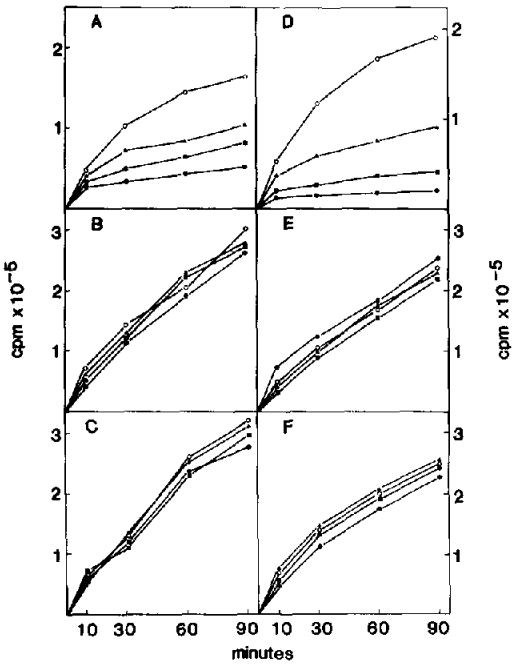

Fig.2. Influence of prosomes or prosomal RNA on translation of various mRNAs. Poly(A)$^+$ mRNA was incubated in 25 μl rabbit reticulocyte lysate (New England Nuclear) with 0.25–1 μg prosomes (A–C) or prosomal RNA isolated from HeLa cells (D–F). Reaction mixture contained: (A,D) 1 μg polysomal poly(A)$^+$ mRNA of adenovirus-infected HeLa cells; (B,E) 1 μg polysomal poly(A)$^+$ mRNA of uninfected HeLa cells; (C,F) 1 μg rabbit globin mRNA; (○—○) control, no addition; (▲—▲) + 0.25 μg prosomes/prosomal RNA, (■—■) + 0.5 μg prosomes/prosomal RNA, (●—●) + 1 μg prosomes/prosomal RNA.

Poly(A)$^+$ mRNAs were isolated from polysomes of HeLa cells grown under standard conditions and from adenovirus-infected HeLa cells 20 h after inoculation. These different mRNA populations were translated in a rabbit reticulocyte cell-free system. They were incubated together with varying quantities of HeLa cell prosomes or prosomal RNA. Under the chosen conditions, increasing amounts of prosomes (up to 1 μg) did not influence the translation rate of 1 μg HeLa mRNA or rabbit globin mRNA (fig.2B,C). In contrast, the translation efficiency of the mRNA from adenovirus-infected cells was remarkably reduced; the rate of inhibition was proportional to the quantity of prosomes added (fig.2A). Deproteinized prosomal RNA gave the same results, with even more pronounced inhibition of viral mRNA translation (fig.2D–F).

These experimental results were confirmed by analysis of the translation products. 1 μg prosomes or prosomal RNA added to the cell lysate in the presence of polysomal mRNA of infected cells dramatically reduced incorporation of [^{35}S]methionine into protein (fig.3A,B). However, prosomes or prosomal RNA did not influence the quality and quantity of the translation products of polysomal mRNAs from uninfected HeLa cells or globin mRNA (fig.3C–F) [15]. The 25 and 48 kDa bands in fig.3G are components of the reticulocyte system.

These results revealed that prosomes interfere selectively with the translational activity of polysomal messenger RNA from infected cells.

In the following experiment, we investigated whether prosomes interact directly with polysomal RNA of the infected cells. For this approach, equal

Fig.3. SDS-PAGE of in vitro translation products after 90 min incubation (see fig.2) visualized by autoradiography. Translation products of: (A) 1 μg mRNA of adenovirus-infected HeLa cells; (B) 1 μg mRNA of adenovirus-infected cells + 1 μg prosomal RNA; (C) 1 μg mRNA of uninfected HeLa cells; (D) 1 μg mRNA of uninfected HeLa cells + 1 μg prosomal RNA; (E) 1 μg rabbit globin mRNA; (F) 1 μg rabbit globin mRNA + 1 μg prosomal RNA; (G) reticulocyte system without exogenous mRNA (control).

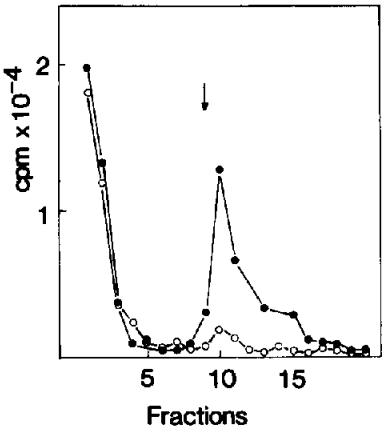

Fig.4. Interaction of prosomes with poly(A)$^+$ mRNA. 20 S free mRNPs of ^{35}S-labeled HeLa cells were dissociated in TBNa-500 and sedimented through 10–50% sucrose gradients (SW 40 rotor, 36000 rpm, 18 h). 19 S fractions were pooled and incubated with polysomal poly(A)$^+$ mRNA of infected and uninfected HeLa cells. This mixture was loaded on an oligo(dT)-cellulose column, with a bed volume of 1 ml equilibrated in TBNa-500. After washing with 5 ml TBNa-500 the column was eluted with 20 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol. 100 μl of each fraction was incubated with 10% trichloroacetic acid (final concentration) and the precipitated radioactivity was measured in a liquid scintillation counter. Elution profile of ^{35}S-labeled prosomes incubated with polysomal poly(A)$^+$ mRNA of: (●—●) adenovirus-infected cells and (○—○) uninfected cells.

amounts of polysomal mRNA isolated from infected and uninfected HeLa cells were bound to oligo(dT)-cellulose in the presence of a crude fraction of [^{35}S]methionine-labeled prosomes. Thereafter, the two columns were washed intensively with high-salt buffer and bound radioactivity was eluted with low-salt buffer (fig.4). The 5-fold higher radioactivity in the eluted fractions from the column containing viral mRNAs (fig.4) demonstrates that prosomes interacted preferentially with the mRNA of infected cells.

From this experiment one may assume that the association of prosomes with mRNA is mediated by prosomal RNAs. Therefore, they should hybridize directly with mRNAs of infected cells. To test this possibility, decreasing amounts of mRNAs were spotted onto gene screen membranes and hybridized with ^{32}P-labeled prosomal RNA. After incubation, membranes were carefully washed in buffers containing 0.1 × SSC and 1% SDS at 60°C. Under these rather stringent conditions, polysomal mRNA from adenovirus-infected cells hybridized readily with prosomal RNA (fig.5A, lane 1), while that of uninfected HeLa cells hybridized only weakly (fig.5A, lane 2). Poly(U), poly(A) and rabbit globin mRNA gave no reaction under the conditions chosen (fig.5A, lanes 3–5). As control, a second and a third gene screen sheet with the same pattern of spots was incubated with ^{32}P-labeled adenovirus DNA to prove the presence of adenovirus mRNA (fig.5B) and with [^{32}P]poly(U) (fig.5C) to show poly(A)$^+$ mRNA. Fig.5C (lanes 1,2) demonstrates further that approximately equal amounts of poly(A)$^+$ mRNA from infected and uninfected cells hybridized with poly(U).

4. DISCUSSION

Our results demonstrate that prosomes inhibit the in vitro translation of polysomal mRNA from adenovirus-infected HeLa cells, while protein synthesis of polysomal mRNA isolated from uninfected cells was not affected. This cannot be simply explained by RNase or proteinase activity of prosomes, since mRNAs from uninfected HeLa cells was readily translated under the same condi-

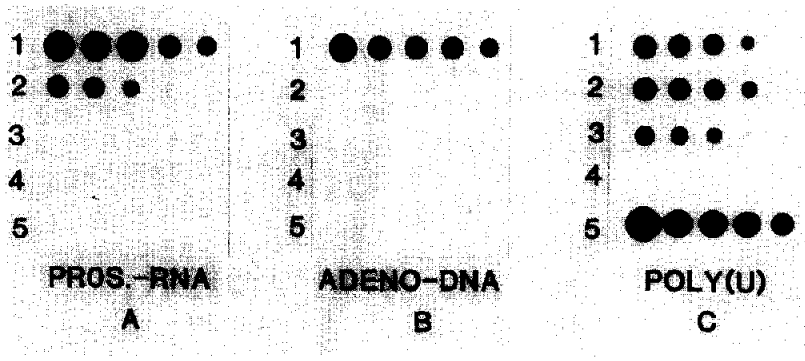

Fig.5. Interaction of prosomal RNA with various mRNAs. RNA or oligonucleotide samples (from left to right: 1, 0.75, 0.5, 0.25, 0.1 μg) were spotted on three gene screen hybridization membranes (A–C) and incubated with various ^{32}P-labeled RNA or DNA probes as described in section 2. Prosomal RNA or poly(U) was labeled at the 3′-end with [^{32}P]pCp by T_4 ligase. Adenovirus DNA was labeled via nick translation. (1) Poly(A)$^+$ mRNA of infected HeLa cells; (2) poly(A)$^+$ mRNA of uninfected HeLa cells; (3) rabbit globin mRNA; (4) poly(U); (5) poly(A); hybridization with: (A) ^{32}P-prosomal RNA, (B) ^{32}P-adenovirus DNA, (C) [^{32}P]poly(U).

tions and the newly synthesized proteins were not degraded. Oligo(dT)-cellulose chromatography suggested that the inhibition is caused by interaction of prosomes with some or all polysomal mRNAs of infected cells mediated via the prosomal RNA. Dot-blot analysis revealed that prosomal RNA has a much stronger affinity for mRNA of infected cells. Prosomal RNA and HeLa cell mRNAs hybridized with a low efficiency and duck globin mRNA was also reported to hybridize with prosomal RNA under less stringent conditions [18].

Based on the stringency of our hybridization conditions we suggest that more than 10 nucleotides are complementary to polysomal mRNAs of infected cells. The possible candidates are certainly adenoviral mRNAs which represent most of the HeLa cell messengers in the late phase of infection. Preliminary experiments revealed furthermore that prosomal RNA hybridize very strongly to adenovirus DNA (Dineva, not shown). We assume that the inhibition occurs in an early step of initiation, just before viral mRNAs bind to 40 S subunits, since prosomes were never found associated with polysomes and ribosomal subunits in adenovirus-infected and uninfected HeLa cells ([19] and Kreutzer, unpublished). Also initiation factors were not directly affected by prosomes, since in vitro translation of viral mRNAs was not recovered with additional lysate (not shown). According to these results we postulate that prosomes prevent the association of adenoviral mRNA with 40 S subunits by masking sequences translational factors shuttle on. However, how the adenovirus escapes the influence of prosomes in HeLa cells remains speculative. We suppose that the number of prosomes in uninfected cells is too low to repress the bulk of viral mRNAs transcribed after virus invasion. Possibly, the synthesis of prosomes increases after adenovirus infection in HeLa cells, an idea which correlates well with data recently obtained by Yazaki et al. [20], who reported that prosome-like particles accumulate in BHK cells after VSV infection. This problem is under investigation.

Finally, it is worth mentioning that prosomes of FLV-induced mouse erythroblasts substituted completely for HeLa cell prosomes in all experiments shown in figs 2, 3 and 5 (not shown), supporting the hypothesis that prosomes are of highly conserved structure and function [8,18].

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