MEMBRANE ASSOCIATED POLY(A)-CONTAINING RIBONUCLEOPROTEINS IN *ARTEMIA SALINA* GASTRULA EMBRYOS

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1. Introduction

The embryonic development of *Artemia salina* is often interrupted by a period of dormancy at the gastrula stage. Although no endogeneous protein synthesis could be detected in these encysted embryos [1], the presence of stored mRNA has been demonstrated [2–4]. Messenger activity is associated with both poly(A)$^+$-RNA and poly(A)$^-$-RNA extracted from the membrane fraction but only with the poly(A)$^-$-RNA of the free cytoplasmic ribonucleoproteins [3]. Poly(A)$^+$-RNA of the cytoplasmic RNP is complexed with a translational inhibitor RNA, and messenger activity is restored if the inhibitor RNA is dissociated from the mRNA by the use of EDTA during the fractionation procedure [5]. We have shown by sucrose gradient centrifugation that free cytoplasmic poly(A)$^+$-RNPs exhibited a heterogeneous distribution consisting of several discrete peaks ranging from 20–120 S [4]. Approximately 50% of the RNPs sedimented faster than 50 S and were complexed with ribosomes or ribosomal subunits. The presence of EDTA converted all these poly(A)$^+$-RNPs into free 20–40 S particles.

We demonstrate here that the poly(A)$^+$-RNPs of the membrane fraction exhibit a different size distribution on sucrose gradients as compared with the poly(A)$^+$-RNPs isolated from the free cytoplasmic fraction. The former fraction is devoid of poly(A)$^+$-RNPs complexed with ribosomes or ribosomal subunits. The lack of the latter complexes in the membrane fraction suggests differences in the regulation of translation of these mRNAs in both fractions.

Abbreviations: Poly(A)$^+$-RNP and poly(A)$^+$-RNA, poly(A)-containing RNP and RNA, respectively; poly(A)$^-$-RNP, poly (A)-lacking RNP; EDTA, ethylene diamine tetra acetate disodium salt; DOC, sodium deoxycholate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid

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2. Materials and methods

Encysted cryptobiotic embryos of *A. salina* were obtained from San Franscisco Bay, division of metaframe corporation, Newark, CA. 5–100 g (dry wt) were treated with 10% NaClO at room temp. for 10 min, extensively washed with distilled water and homogenized at 4°C in a mortar in the presence of a small amount (10 μl/g) of buffer A (35 mM Tris, 20 mM Hepes (pH 7.6) 70 mM KCl, 9 mM $MgCl_2$) or buffer C (10 mM sodiumphosphate (pH 6.8) 5 mM $MgCl_2$, 50 mM NaCl) and 150 mM sucrose. The homogenate diluted to 3 ml/g with buffer A or C was filtered through 4 layers of cheese cloth and centrifuged at 300 g for 10 min to remove nuclei.

The postnuclear supernatant was fractionated by centrifugation at 18 000 × *g* for 15 min and the membrane and mitochondrial pellet washed free of post-mitochondrial particles with buffer A or C. Membrane bound particles were released from the 18 000 × *g* sediment by resuspending in 1 ml buffer containing 1% Triton X-100. After 30 min at 4°C solutions were centrifuged at 18 000 × *g* for 20 min. The supernatant contained the RNP particles released from the membranes. Fractions to be analyzed by isopycnic sucrose density centrifugation were placed on 3 layers of

sucrose (3 ml each) in buffer C with respective sucrose concentrations of 70%, 50% and 30% (w/w). Centrifugation was in a Spinco SW 41 rotor for 40 h at 35 000 rev./min. Fractionation and density measurements have been described [4].

5–20% (w/v) linear sucrose gradient centrifugation in buffer C was in the SW 41 rotor. Centrifugation conditions are indicated in the figure legends. The absorbancy was measured in a Zeiss PMQ3 spectrophotometer.

The extraction of RNA from gradient fractions was described [4]. Hybridization assay was carried out in reaction mixtures (1.0 ml), composed of equal volumes of RNA in buffer A or buffer C, 4 × SSC (SSC, 0.15 M NaCl, 0.015 M sodium citrate) and 5 μl poly([^{3}H]U) (1.36 × 10^5 cpm/μg, 7542 cpm/μl) and were processed as in [4].

If necessary, pooled fractions were concentrated by ultrafiltration. An Amicon PM10 membrane filter (Amicon, Oosterhout) was used which had been pretreated with 5% formaldehyde.

3. Results

The postnuclear supernatant was separated into a membrane and a cytoplasmic fraction by differential centrifugation. RNA was extracted from both fractions by phenolization and was hybridized with poly([^{3}H]U). The results are presented in table 1. Approximately 65% of the acid-precipitable radioactivity was found in the cytoplasmic fraction independent of the initial amount of embryos homogenized or the buffers used in the homogenization and fractionation procedure. Membrane bound particles were released by treatment with Triton X-100 and/or DOC. A wash with 1% Triton X-100 resulted in a release of only 19% of the poly([^{3}H]U) hybridizable radioactivity of the postnuclear supernatant. An additional wash with 2% DOC released still 8%. Approximately 10% of the poly(A) sequences were not released from the membrane fraction with these detergents (table 1). Although mitochondria contaminate the membrane fraction, the contribution of mitochondrial mRNA seems to be negligible as evidenced by the low percentage of membrane extracted RNA hybridizable to mitochondrial DNA [6].

The cytoplasmic fraction and the membrane fraction were analyzed by isopycnic sucrose density centrifugation before and after treatment with 1% Triton X-100. Each fraction was assayed with poly([^{3}H]U) as described and the results presented in fig.1. The cytoplasmic poly(A)$^+$-RNPs band at a density of 1.29

Table 1
Distribution of poly(A) sequences among different fractions obtained from the postnuclear supernatant of *A. salina* embryos

	I		II		III	
	cpm	%	cpm	%	cpm	%
Cytoplasmic fraction	111 826	62	29 719	61	5162	69
Membrane fraction	68 670	38	18 736	39	2292	31
Released by detergent						
1% Triton X-100	34 571	19				
2% DOC	15 043	8				
Not released	19 056	11				

Homogenization of approx. 20 g embryos in buffer C (I), 10 g in buffer C (II) and 5 g in buffer A (III). Values are initial weights and are reduced in the washing procedures. RNA samples (500 μl) in homogenization buffer, were hybridized (section 2) and the total amount of acid precipitable radioactivity calculated after measurement of the volumes of the different fractions. Membrane bound particles were released from the membrane fraction by treatment with 1% Triton X-100 and additionally with 2% DOC. Hybridization in buffer A resulted in a decrease of 50% in acid precipitable radioactivity of poly([^{3}H]U) in comparison with buffer C (our own observations)

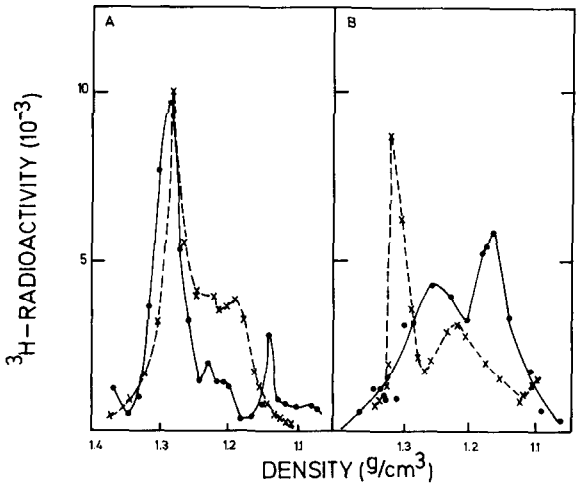

Fig.1. Analysis of the free cytoplasmic and membrane bound poly(A)$^+$-RNPs by sucrose isopycnic density centrifugation. (A) Localization of poly(A) sequences in the free cytoplasmic fraction before (•——•) and after treatment with 1% Triton X-100 (X– – –X). (B) Localization of poly(A) sequences in the membrane fraction before (•——•) and after treatment with 1% Triton X-100 (X– – –X). The poly(A) sequences were localized by hybridization with poly([^{3}H]U) as described in section 2. Acid precipitable radioactivity is plotted as a function of fraction density. Centrifugation was in a Spinco SW 41 rotor for 40 h at 35 000 rev./min and at 4°C.

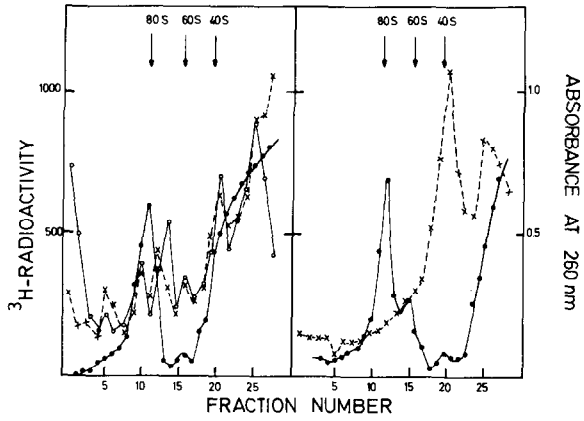

Fig.2. Analysis of free cytoplasmic and membrane bound poly(A)$^+$-RNPs by 5–20% (w/v) linear sucrose gradient centrifugation. (A) Sedimentation distribution of poly(A) sequences of the free cytoplasmic fraction before (o——o) and after treatment with 1% Triton X-100 (X– – –X). A_{260} (•——•). (B) Sedimentation distribution of poly(A)$^+$-RNPs released from the membrane fraction by 1% Triton X-100 (X– – –X). A_{260} (•——•). Centrifugation was in the Spinco SW 41 rotor at 24 500 rev./min for 15 h and at 4°C.

(g/cm^3) and the density distribution is practically unchanged by treatment with detergent although minor density peaks were observed as a consequence of RNP degradation [4]. The results indicated that the cytoplasmic fraction is not contaminated with membrane bound particles. It may be noted that ribosomes band at a density of 1.35–1.36 (g/cm^3) and that 18 S rRNA extracted therefrom does not interfere with the poly(A) assay in the conditions used [4]. The poly(A)-sequences of the membrane fraction are distributed into two regions with maxima at sucrose densities of 1.25 (g/cm^3) and 1.16 (g/cm^3). Treatment of the membrane fraction with 1% Triton X-100 resulted in a shift of the majority of the acid precipitable radioactivity to 1.3 (g/cm^3) which is a characteristic density of the free cytoplasmic poly(A)$^+$-RNP.

The poly(A)$^+$-RNPs were further analyzed by linear 5–20% sucrose gradient centrifugation (fig.2). Free cytoplasmic poly(A)$^+$-RNPs exhibited a heterogeneous distribution in which several discrete peaks were observed [4]. The distribution was only slightly changed if 1% Triton X-100 was added. Approximately the same discrete peaks were observed in the presence or absence of 1% Triton X-100.

We have previously shown that addition of 25 mM EDTA converted all the poly(A)$^+$-RNPs into free 30–40 S particles if RNPs with a density of 1.3 (g/cm^3) were used, i.e., if degraded material with a lower density was omitted [4]. This observation indicated that approx. 50% of the free cytoplasmic poly(A)$^+$-RNPs were complexed with ribosomes and ribosomal subunits.

The distribution of poly(A)$^+$-RNPs released from the membranes consisted of only two peaks well separated from ribosomes and ribosomal subunits (fig.2B) and sedimenting at 36 S and 16 S. These peaks are not a consequence of ribosome dissociation as indicated by the fact that 80 S ribosomes are not dissociated during the release from the membrane fraction by Triton X-100. As already mentioned, the 16 S peak resulted from RNP degradation by Triton X-100.

The similarity in size between EDTA dissociated free cytoplasmic poly(A)$^+$-RNPs and the Triton X-100 released membrane particles is obvious [4].

4. Discussion

In dormant embryos of *A. salina* approx. 65% of the poly(A) sequences of the postnuclear supernatant were located in the free cytoplasmic fraction and only 20% of the total acid precipitable radioactivity was washed from the membranes in mild detergent conditions and an additional 8% was released with 2% DOC. The presence of poly(A)$^+$-RNP classes in the membrane fraction which are loosely and tightly bound to membranes suggested the existence of different detergent-sensitive proteins located at or near the 3′-end of mRNA as was proposed [7].

Poly(A)$^+$-RNP of the free and the membrane fraction band at the same characteristic density of 1.30 ± 0.01 (g/cm^3) if the latter are released from the membrane fraction with detergents, otherwise these particles are located in lower density fractions and are distributed over a broad density range.

Analysis of the poly(A)$^+$-RNP of the free cytoplasmic fraction and the membrane fraction by linear sucrose gradient centrifugation demonstrated that these particles exhibited a completely different sedimentation distribution. Approximately 50% of the poly(A)$^+$-RNP of the free cytoplasmic fraction are complexed with ribosomes and ribosomal subunits. By contrast, the RNP particles released from membranes are devoid of such complexes.

In eukaryotic systems secretory proteins are synthesized on the membranes through which they pass [8–10]. The differences observed between the membrane associated and free cytoplasmic poly(A)$^+$-RNPs suggests differences in the regulation of translation of these mRNAs.

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