EVIDENCE FOR A CYTOPLASMIC TRANSLATIONAL INHIBITOR RNA IN *ARTEMIA SALINA* GASTRULA EMBRYOS

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1. Introduction

In the regulation of eukaryotic protein biosynthesis, several translational control RNAs have been isolated from initiation factor preparations of rabbit reticulocytes [1], and from chicken muscle and erythroblasts [2]. These translational control RNAs have generally the low molecular weights (6000–12 000) and elicit either stimulatory or inhibitory effects presumably at the initiation step of protein synthesis [1–3].

Recently two classes of small translation control RNA have been isolated from both free mRNP particles and polysomes of embryonic chicken muscle [4]. In particular, mRNP-associated translational control RNA contains almost 50% uridylate and inhibits the translation of poly(A)$^+$-mRNA in vitro [4]. Since the existence of poly(A)$^+$-mRNP particles in PMS of the dormant gastrulae of the brine shrimp, *Artemia salina,* has been well established [5–7], and since the free native mRNPs are largely inactive in stimulating the in vitro translational system ([6], our unpublished observations), we have investigated in the present study the possible presence of such translational control RNA in the free poly(A)$^+$-mRNP particles of *Artemia* dormant gastrulae, transforming these particles into an inactive state. We report here our preliminary results demonstrating that poly(A)$^+$-mRNP prepared in the absence of EDTA and poly(A)$^+$-mRNA purified thereof are associated with translational inhibitor RNA which can be released by EDTA in vitro and that poly(A)$^+$-mRNA which is free from such inhibitor RNA becomes an excellent template in the wheat-germ protein synthesizing system.

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Abbreviations: Poly(A)$^+$-mRNA and poly(A)$^+$-mRNP, poly(A)-containing mRNA and mRNP, respectively; poly(A)$^-$-RNP, poly(A)-lacking RNP; PMS, postmitochondrial supernatant; EDTA, ethylene diamine tetraacetate disodium salt; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

2. Materials and methods

[^{35}S]Methionine (480–590 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, UK). All non-radioactive amino acids were purchased from Sigma Chemical Co. (St. Louis, USA). Sepharose-poly(U) and oligo(dT)-cellulose were from Pharmacia Fine Chemicals (Uppsala, Sweden) and Collaborative Research Inc. (Waltham, USA), respectively. Amicon UM-10 membrane filter was from Amicon (Oosterhout, Netherlands). Proteinase K was from Merck (Darmstadt, FRG). Untreated wheat grains were obtained from the local mills. All other materials were indicated in previous publications [5,8].

PMS from the encysted formant gastrulae (20–100 g, dry weight) was prepared in buffer C (10 mM sodium phosphate buffer [pH 6.8], 5 mM $MgCl_2$, 50 mM NaCl) or in buffer G (10 mM Tris, [pH 7.6], 100 mM NaCl) as described [5,8]. In both cases, 150 mM sucrose was included in the buffer.

Chromatography of PMS-RNP particles on oligo(dT)-cellulose column (bedvolume, 10 ml) was conducted in buffer C. After elution of poly(A)$^-$-RNP, poly(A)$^+$-RNP was eluted stepwise with 5–10 ml 10 mM sodium phosphate buffer, (pH 6.8), 1% SDS. The

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first 30–35 ml eluant contained all poly(A)$^+$-RNP as judged by hybridization of [^{3}H]poly(U) to RNA isolated from each fraction [5].

For Sepharose-poly(U) chromatography, PMS-RNA was obtained by digestion of PMS prepared in buffer G with proteinase K (50 μg/ml) in the presence of 0.5% SDS, followed by phenolization with a mixture of phenol–chloroform (1 : 1 in volume) in the presence of 1% SDS after adjusting the digest to buffer H (10 mM Tris pH 7.6], 500 mM NaCl, 10 mM EDTA) by addition of NaCl and EDTA. After through washing of the column (bed volume, ca. 10 ml) with 50 ml of buffer H, poly(A)$^+$-RNA was eluted first with 10 mM Tris (pH 7.6), at room temperature (fraction A of fig.2a) and then with the same buffer at 53°C (fraction B of fig.2a). Each pooled fraction was concentrated by Amicon ultrafiltration to 140 and 660 μg/ml, respectively. When PMS-RNA was isolated in the absence of EDTA from PMS prepared in buffer C, poly(A)$^+$-RNA was eluted from Sepharose-poly(U) as above with 10 mM sodium phosphate buffer (pH 6.8).

In vitro protein synthesizing system of wheat embryo and the determination of acid-insoluble [^{35}S]methionine radioactivity were according to the published method [8]. Electrophoresis of the in vitro translation products was carried out in 12.5% SDS-polyacrylamide gel in slab as described [9]. The radioactive products separated on SDS-gels were identified by fluorography [10].

3. Results

Poly(A)$^+$-RNP prepared in the absence of EDTA and fractionated by oligo(dT)-cellulose chromatography and poly(A)$^+$-RNA isolated thereof were totally inactive in the wheat embryo cell-free system (table 1). However, total unfractionated PMS-RNA prepared in the absence of EDTA could stimulate to a certain extent the same protein synthesizing system (fig.1). In this case, poly(A)$^-$-RNA in the PMS-RNA preparation appears to be responsible for the template activity, since a specific cytoplasmic 19 S RNP lacking poly(A)-residues as well as its RNA component, present rather abundantly in PMS of the dormant gastrulae, could stimulate the in vitro protein synthesis (manuscript in preparation) and since a negligible amount of poly(A)$^+$-mRNA was detected in PMS of the same gastrulae [11].

Experiments conducted in both laboratories employed the buffers containing none [5] or a very low concentration of EDTA (0.1 mM; [11,12]) to prepare either RNP or RNA from the dormant gastrulae. We have observed earlier that when PMS-RNA prepared in the absence of EDTA was fractionated on a sucrose density gradient in 10 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, the increased template activity in the wheat embryo system was obtained between 7 S and 14 S. Therefore we have tried buffer H containing 10 mM EDTA for the preparation of PMS-RNA (Materials and methods).

Table 1
Inability of poly(A)$^+$-RNA prepared in the absence of EDTA in directing in vitro translation system.

Template RNA	Amount (μg)	[^{35}S]Methionine incorporation (cpm)[a]
none		9110
Poly(A)$^+$	0.52	9880
Poly(A)$^+$	1.04	9360
Poly(A)$^+$	1.56	10 100
TMV[b]	9.0	121 980

[a] Average of duplicate runs; no background has been subtracted

[b] 50 μl reaction mixture was adjusted to 2.75 mM $Mg(OAc)_2$ and 75 mM KCl, and contained generally 10–20 μl wheat embryo S-30 extract and 2–5 μCi [^{35}S]methionine. Incubation was at 30°C first for 5 min without RNA template and then for 60 min after addition of template RNA. Poly(A)$^+$-RNA was purified from poly(A)$^+$-RNP prepared and prefractionated by oligo(dT)-cellulose in the absence of EDTA (Materials and methods)

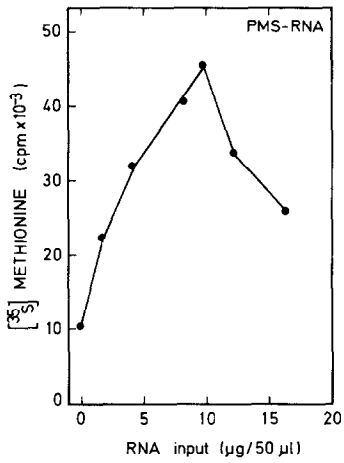

Fig.1. In vitro translation of total unfractionated PMS-RNA. PMS in buffer C plus 150 mM sucrose and 1% SDS was phenolized with an equal volume of phenol-chloroform and RNA was precipitated from the last aqueous fraction with 2% CH_3COONa and 2 vols of ethanol at −20°C. RNA precipitate was dissolved in distilled water and used in the wheat embryo protein synthesizing system as in table 1. No background was subtracted.

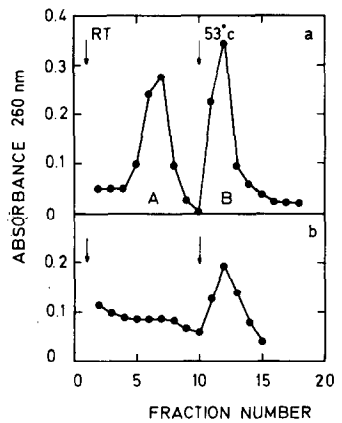

Fig.2. Separation of translational inhibitor RNA from poly(A)$^+$-mRNA by Sepharose-poly(U). (a) Proteinase K-digested PMS was phenolized and chromatographed on Sepharose-poly(U) as described in Materials and methods. (b) PMS was prepared in buffer C, poly(A)$^+$-RNP was isolated by oligo(dT)-cellulose, and poly(A)$^+$-RNA was further fractionated on Sepharose-poly(U) in buffer C followed by 10 mM sodium phosphate buffer (pH 6.8), at room temperature (20°C) and at 53°C.

Upon chromatography on Sepharose-poly(U), two fractions (A and B of fig.2a) were recovered from RNA absorbed to this column after extensive washing with buffer H. By contrast, when buffer C containing no EDTA but 5 mM $MgCl_2$ was employed throughout the preparation (Materials and methods), virtually no analogous A fraction was detected and only B fraction was recovered by Sepharose-poly(U) chromatography (fig.2b).

The template activity of these RNA fractions was tested in the wheat embryo translation systems. The fraction B purified in the presence of EDTA (fig.2a) was found to contain poly(A)$^+$-RNA having an excellent template activity reaching its maximum at about 2 µg in 50 µl incubation mixture (fig.3a), although the addition of this RNA beyond 2 µg resulted in a gradual inhibition, reaching about 50% of the maximal value at 6.6 µg (now shown). On the other hand, fraction A was completely inactive by itself and even depressed the residual endogeneous activity of this translation system (fig.3a). Contrary to fraction B-RNA (fig.2a), poly(A)$^+$-RNA of the analogous fraction prepared in the absence of EDTA (fig.2b) lacked any template activity in both wheat embryo and

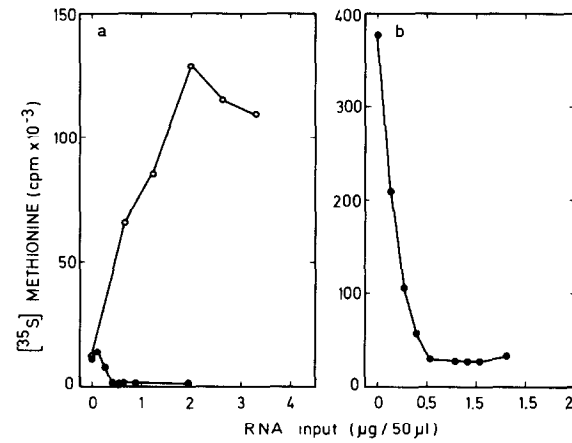

Fig.3. In vitro translation of free poly(A)$^+$-mRNA and its inhibition by translational inhibitor RNA. (a) RNA was tested for its template activity in the wheat embryo systems (table 1). (o—o), free poly(A)$^+$-mRNA (fraction B of fig.2a) and (•—•), RNA eluted in the fraction A (fig.2a). (b) Poly(A)$^+$-mRNA (2 µg) was then challenged by a various amount of fraction A-RNA as indicated in the figure for the translation in the wheat embryo system.

rabbit reticulocyte systems (not shown), as was the case for poly(A)$^+$-RNA isolated from the prefractionated RNP by oligo(dT)-cellulose in the absence of EDTA (table 1).

Hence it appears probable that poly(A)$^+$-RNA and -RNP were in an inactive form due to the binding of some translational inhibitor. The RNA eluted in the A fraction (fig.2a) seems to be a good candidate for such an inhibitor, because when poly(A)$^+$-mRNA is separated from this RNA, it becomes an excellent template (fig.3a), and because when no apparent dissocation occurs between these two fractions in the absence of EDTA, poly(A)$^+$-mRNA remains inactive in directing the in vitro protein synthesizing system. In order to test a presumptive inhibitory effect of the fraction A-RNA, the mixing experiment was carried out in which an optimal amount of poly(A)$^+$-mRNA (2 μg) was challenged by an increasing amount of the fraction A-RNA (0.13–1.3 μg). As can be seen in fig.3b, the fraction A-RNA was found to exert a very potent inhibition in the translation of poly(A)$^+$-mRNA (50% and 100% inhibition at 0.13 μg and 0.5 μg, respectively), even when added exogeneously in the in vitro system. Further, the linear sucrose density gradient analysis of these RNA fractions revealed that whereas low molecular-weight RNA was a significant species in the fraction A, the fraction B lacked such RNA and sedimented mostly between 8 S and 16 S.

Figure 4 shows one of the autoradiograms of the in vitro translation products of poly(A)$^+$-RNA analyzed by SDS-gels. Although the electrophoretical pattern was quite heterogeneous in size, the majority being smaller than 40 000 daltons, some distinct proteins up to and over 100 000 daltons were synthesized.

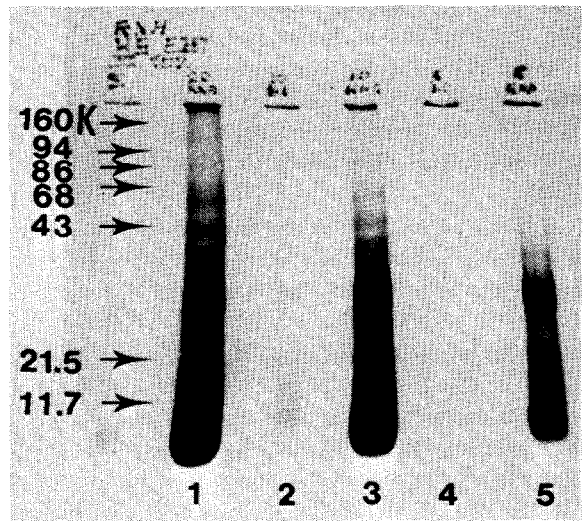

Fig.4. SDS-polyacrylamide gel analysis of in vitro protein products. 100 μl reaction mixture containing 4 μg of poly(A)$^+$-mRNA (fraction B of fig.2a) was incubated for 90 min at 30°C. After incubation, the reaction mixture was treated with pancreatic RNAase A [14] and the ^{35}S-products were precipitated with 10% TCA and dissolved in the electrophoresis buffer [15]. Electrophoresis was at room temperature for 4 h at 125 mV. Slots 1, 3 en 5 were the protein products directed by poly(A)$^+$-mRNA containing radioactivity of 1.7×10^6, 8.5×10^5 and 4.2×10^5 cpm, respectively. Slots 2 and 4 were the endogeneous products in the absence of added template containing radioactivity of 4.4×10^4 and 2.2×10^4 cpm, respectively. Molecular weights of the protein markers are indicated on the left; β',β-subunits of *E. coli* RNA polymerase (160 000), phosphorylase *a* (94 000), ovalbumin dimer (86 000), bovine serum albumin (68 000), ovalbumin monomer (43 000), soybean trypsin inhibitor (21 500) and cytochrome *c* (11 700).

4. Discussion

The experiments described here establish the presence of translational inhibitor RNA in *Artemia* dormant gastrulae. This RNA exerts a potent inhibitory action on the translation of poly(A)$^+$-mRNA in the wheat embryo cell-free system, when added exogeneously. In vitro it appears to be stably bound to free cytoplasmic poly(A)$^+$-mRNP and prevents from its being translated. Poly(A)$^+$-mRNP or mRNA isolated from animal in the absence of EDTA could not be translated also in vitro, unless translational inhibitor RNA was removed from poly(A)$^+$-mRNA by EDTA treatment. Poly(A)$^+$-mRNP (at least for 19 S mRNP) seems not to be bound with this translational inhibitor RNA, because isolated 19 S RNP was able to direct the in vitro protein synthesis.

When mRNA activity in PMS of *Artemia* dormant gastrulae was detected in the in vitro systems, RNA was always extracted with the buffers containing 1 mM [6] or 10 mM [7] EDTA, without Mg^{2+}, as was shown in this study. Moreover, translational inhibitor RNA isolated from mRNP of embryonic chicken muscle was also dissociated from mRNP by

dialysis against 50 mM potassium phosphate buffer (pH 6.8), 5 mM EDTA before isolation on DEAE-cellulose [4]. All available data suggest that a stable interaction of translational inhibitor RNA with poly(A)$^+$-mRNP requires divalent cation, most likely Mg^{2+}, and can be disrupted by a chelating agent, EDTA.

Although this translational inhibitor RNA of *A. salina* has not been fully characterized yet, it is tempting to suggest that this inhibitor RNA might be involved in general in the regulation of two alternative states (active or inactive) of cytoplasmic poly(A)$^+$-mRNP in vivo. Further, since an inactive mRNP has to be reactivated prior to its translation, one would anticipate a possible existence of a dissociation factor(s) which catalizes the conversion of the inhibitor-bound mRNP to free active one. Experiments to characterize this inhibitor RNA and its interaction with mRNP are now in progress.

Finally, a recent detection of very tightly bound translational inhibitor to globin 20 S mRNP of duck erythroblasts [13] might be in similar nature to one described here in *Artemia salina.*

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