POLY(A)-CONTAINING CYTOPLASMIC RNA IN DORMANT CYSTS OF *ARTEMIA SALINA**

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1. Introduction

Encysted embryos of *Artemia salina* Leach contain a fraction of heterodisperse RNA with high stimulatory activity in an *E. coli* polypeptide synthesizing system [1,2]. The active material has an average sedimentation coefficient of 17–20S, and is concentrated in a cytoplasmic membrane fraction, sedimenting at 15 000 *g* and banding in a sucrose gradient at a density of 1.17 g/ml.

Eukaryotic mRNA is in typical cases provided with a poly(A) sequence of somewhat variable size [3–12]. In order to further elucidate the question, whether dormant *Artemia* cysts contain a store of latent mRNA, the poly(A) content of the active RNA fractions was determined. The experiments indicated that RNA prepared from the membrane fraction mentioned above included poly(A)-containing components mainly sedimenting at 14–17S. The estimated chain-length of the poly(A) sequences was 45–65 nucleotides.

2. Materials and methods

A 15 000 *g* cytoplasmic fraction from *Artemia* cysts (Carolina Biological Supply Co., Burlington, North Carolina) was treated with buffered 20 mM EDTA and purified by banding in 0.5–1.8 M sucrose gradients as previously described [2]. The coloured band at about 1.3 M sucrose was aspirated, diluted with sucrose-free buffer and sedimented by centrifugation. RNA was extracted with SDS and phenol [2,13]. Fractionation by sucrose gradient centrifugation was carried out as described previously [1,2].

The poly(A) content of RNA fractions was determined by hybridization with [^{3}H] poly(U) according to Sullivan and Roberts [14]. Poly(A) and [^{3}H] poly(U) were from the Miles Laboratories. For the isolation of poly(A) fragments RNA was dissolved in STE-buffer (100 mM NaCl, 50 mM Tris–HCl, pH 7.4, and 1 mM EDTA [14]), and incubated for 60 min (37°C) with a mixture of pancreatic (2 μg/ml) and T_1 (10 units/ml) RNases [5]. For the removal of RNase the digest (0.4–1.0 ml) was passed through a 5 ml column of CM Sephadex C-50 (Pharmacia), prepared in a plastic syringe and equilibrated with STE-buffer (table 1). After elution with STE-buffer, the polynucleotides were precipitated with 2 vol of ethanol (–20°C). For gel electrophoresis 1.0 A_{260} unit of *E. coli* B tRNA (Calbiochem) was added as a carrier. The size of the poly(A) fragments was determined by two independent methods:
a) By centrifugation for 23 hr at 40 000 rev/min (Spinco rotor SW 41) in a 0.3–1.5 M sucrose gradient, using *E. coli* tRNA as a marker. Fractions were precipitated with ethanol in the presence of 100 μg tRNA carrier, and used for poly(A) determinations [14].
b) By electrophoresis [15,16] in 5 X 155 mm columns of 2.4% polyacrylamide gel, using diallyltardiamide for crosslinking [17]. The tRNA band was located by UV-illumination against a fluorescent screen [16], or by staining with toluidine blue. The gel was cut in 3 mm sections, which were dissolved in 0.1 M HIO_4. After elimination of the HIO_4 with 50 μl ethylene glycol, hybridization with [^{3}H] poly(U) was carried out as above, but in the presence of 50 μg tRNA.

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Table 1
Binding of RNase to CM-Sephadex

Fraction	[^{3}H]poly (U) hybridized cpm
1	6628
2*	6657
3*	6706
4*	6706
Control	6604

A mixture of 2 μg pancreatic RNase and 0.5 μg poly(A) in 1.0 ml STE-medium was placed on 5 ml CM-Sephadex C-50 columns, and eluted with the same medium (1.0 ml fractions). The added poly(A) was recovered in fraction 1 (collected after a void vol of 1.6 ml). No RNase activity that interfered with the hybridization assay appeared in fractions 1–4.

* 0.5 μg poly(A) was added after elution.

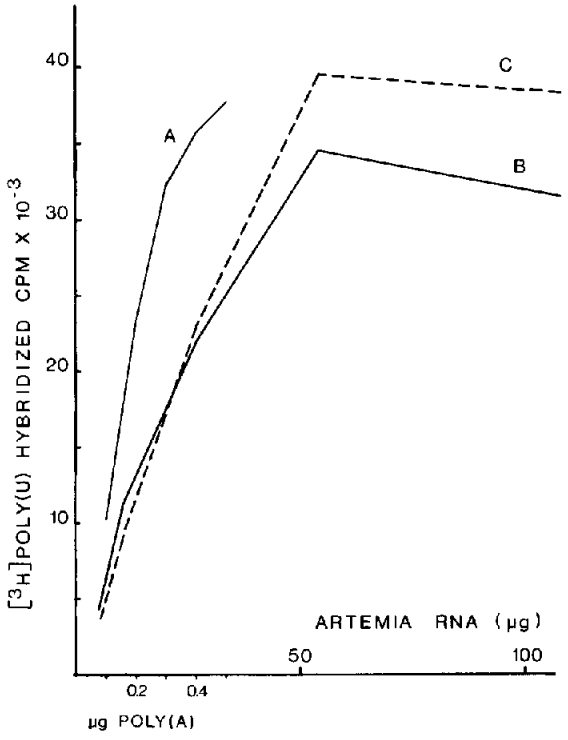

Fig.1. Hybridization assay of a) standard poly(A), b) RNA from the purified 15 000 *g* sediment, c) same RNA, but degraded with T_1 RNase (10 units/ml). Each tube contained 0.5 μg [^{3}H]poly(U) (47 Ci per mol polynucleotide phosphate).

3. Results

RNA from the purified membrane fraction of *Artemia* cysts gave linear hybridization curves with [^{3}H]poly(U) up to a distinct saturation level (fig.1). Comparison with the poly(A) standard curve included in each experiment indicated a poly(A) content of about 1%. Ribosomal and transfer RNA had negligible activity [14]. The unexpectedly high poly(U)-binding capacity of the preparation raised the question, whether a co-operative shielding of added [^{3}H]poly(U) by intermittent adenylic acid-rich regions might

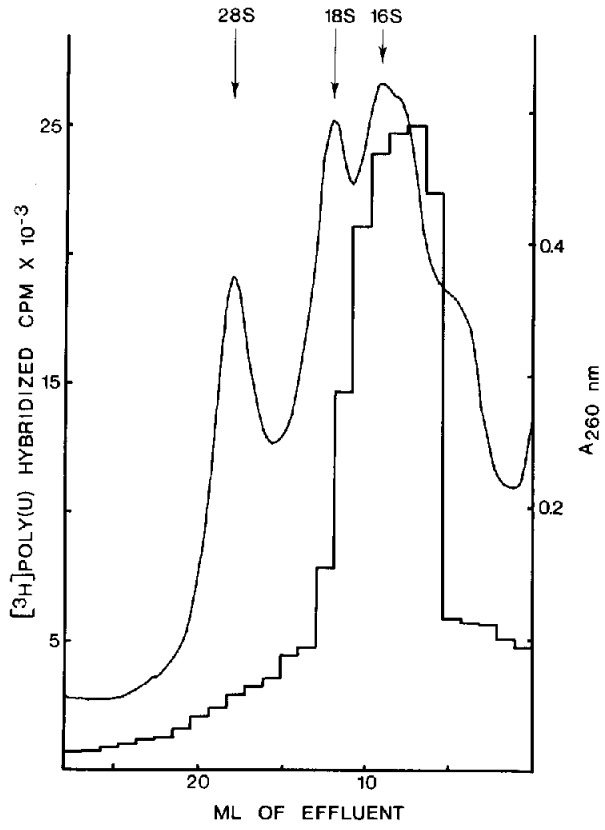

Fig.2. Sedimentation analysis of poly(A)-containing RNA. RNA (14.0 A_{260} units) from the purified 15 000 *g* sediment was layered on a 10–30% sucrose gradient in 20 mM NaOAc, 40 mM Tris–HCl pH 7.8 (25°C), 5 mM EDTA pH 7.8 and 0.1% SDS, and centrifuged for 22.5 hr (24 000 rev/min) in Spinco rotor SW 25 at 2°C. Consecutive 1.1 ml fractions were collected, and precipitated with ethanol in the presence of 100 μg yeast tRNA carrier. Aliquots were assayed for poly(A) by hybridization with 0.5 μg [^{3}H]poly(U) (9.4 Ci per mol polynucleotide phosphate).

contribute to the result. This possibility was disfavoured both by the strict linearity of the reaction, and by the fact that no inhibition was observed after extensive degradation of the RNA with T_1 RNase (fig.1c).

The distribution of poly(A) among different sedimentation classes of the RNA preparation was analyzed by gradient centrifugation (fig.2). The highest poly(A) content was around 16S.

Since the RNA could not be labelled in vivo, the chain-length of the RNase-resistent sequences had to

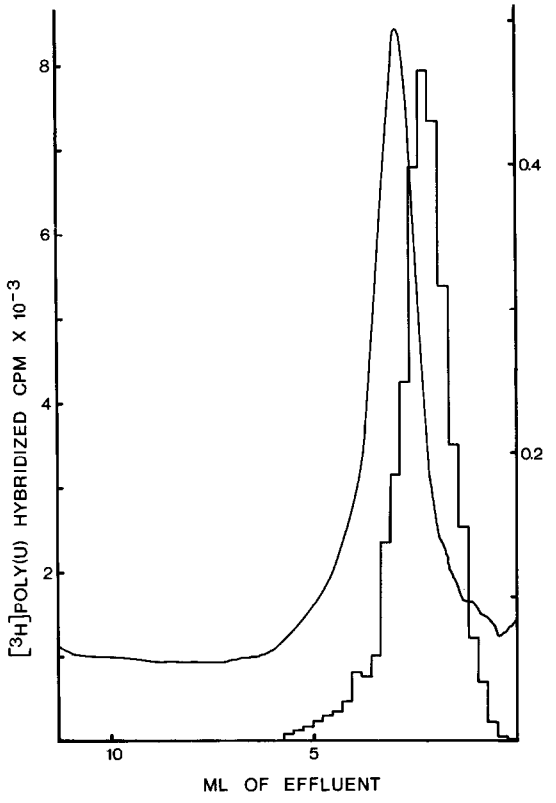

Fig.3. Sedimentation analysis of RNase-resistant fragments of poly(A)-containing RNA from the purified 15 000 *g* sediment. The digest was freed from RNase (table 1), and 0.84 A_{260} units were placed on a 0.3–1.5 M sucrose gradient in 150 mM NaCl, 50 mM Tris–HCl pH 7.4 (25°C), 1 mM EDTA pH 7.4 and 0.1% SDS. Centrifugation was for 23 hr (40 000 rev/min) in Spinco rotor SW 41 at 4°C. From the upper half of the gradient 24 consecutive 0.24 ml fractions were collected, precipitated with ethanol in the presence of 100 μg yeast tRNA carrier, and assayed for poly(A) as described in fig.2. A gradient with 3.42 A_{260} units of *E. coli* B tRNA was run in parallel as a marker.

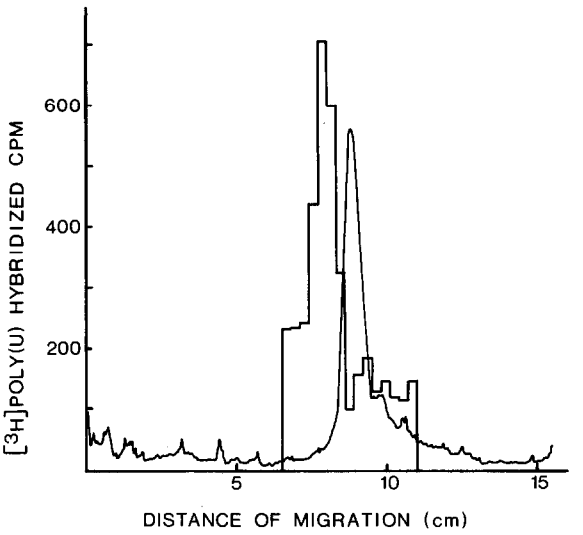

Fig.4. Electrophoretic analysis of poly(A)-containing fragments. The RNA digest was mixed with 1.0 A_{260} unit of *E. coli* B tRNA. Electrophoresis in 2.4% polyacrylamide gel (crosslinked with *N,N'*-diallyltartardiamide) was for 150 min at 200 V. Gels were stained in 0.2% toluidine blue; 3 mm sections were solubilized in 0.1 N HIO_4 and mixed with 50 μg tRNA before poly(A) determination as described in fig.2.

be determined using poly(U) as the labelled component. A method for complete removal of (pancreatic) RNase from the digest had therefore to be developed. This was achieved by use of a CM-Sephadex C-50 column (table 1). The size of the poly(A) fragments was determined by gradient centrifugation (fig.3) and gel electrophoresis (fig.4). In the former case the fragments sedimented as a uniform fraction slightly after the 4S marker. In polyacrylamide gels they migrated slightly behind the 4S RNA. This anomaly is consistent with previous observations [4–11]. From the available data a chain-length of 45–65 nucleotides was estimated, cf. [11].

4. Discussion

The present hybridization experiments, as well as introductory experiments using a centrifugation technique [2,18], confirmed that the annealing of [^{3}H] poly(U) to poly(A)-containing RNA is unaffected by rRNA and tRNA [14]. It is therefore concluded that the RNA from the heavy cytoplasmic fraction of

Artemia cysts is characterized by a high poly(A) content. Thus, in addition to its template activity in a RNA-dependent *E. coli* system [1,2], RNA from this fraction fulfills a second, independent criterium of stored, latent mRNA.

A closer comparison of the template activity [2] and the poly(A) content points to a slight difference in the sedimentation profiles. On the average, the poly(A)-containing RNA formed a fairly narrow fraction with a peak at 14–17S, while the template activity showed a more heterodisperse distribution with a maximum at 17–20S. The possibility that poly(A)-deficient, messengerlike RNA with template activity occurs in the same cytoplasmic fraction is currently investigated.

The 15 000 *g* cytoplasmic fraction contains mitochondria and rough endoplasmic vesicles, which are very difficult to separate [2]. Although in mammalian cells the poly(A) sequences of polysomal and mitochondrial mRNA may differ markedly in size [3–8], recent data indicate that this is not always the case [9–12]. Little information is available about the corresponding poly(A) sequences in invertebrates. Because of the intermediate size of the poly(A) sequences in the latent *Artemia* mRNA, the origin of this mRNA can therefore not, as anticipated, be deduced from chain-length determinations.

Acknowledgements

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