Messenger ribonucleoprotein complexes of cryptobiotic embryos of Artemia salina

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ABSTRACT

Poly(A)-containing ribonucleoprotein (poly(A)$^+$RNP) particles in the post-mitochondrial supernatant of cryptobiotic embryos of Artemia salina were characterized by hybridization to [^{3}H]-poly(U). By sucrose isopycnic centrifugation, approximately 2/3 of poly(A)$^+$RNPs was found to band at 1.27-1.30 (g/cm^3) and the rest 1/3 at 1.20-1.23 (g/cm^3) and below 1.20 (g/cm^3). The 1.27-1.30 RNPs could be separated into two density classes, 1.27-1.28 and 1.30 (g/cm^3) respectively. The latter RNP class was apparently complexed with ribosomal components because they were completely converted to the former RNP class (free RNPs) by 25 mM EDTA treatment. Further, the 1.30 (g/cm^3) RNPs were resolved into several RNP species having sedimentation coefficients above 50 S, which were transformed mostly to 20-30 S RNPs in the presence of 25 mM EDTA. The free 20-30 S RNPs contained 8-14 S poly(A)$^+$RNAs, having the highest template activity in a wheat embryo cell-free system, whereas the 1.20-1.23 poly(A)$^+$RNPs consisted of 10 S and 16 S RNPs, both of which contained 4 S poly(A)-containing sequences without any template activity.

INTRODUCTION.

Under certain conditions in nature, embryos of the brine shrimp, Artemia salina (L.), are forced to enter a cryptobiotic phase at an early embryonic development in the ovisac of the female and released into the environment after being encysted with a protective shell. When redevelopment of the cryptobiotic embryos is allowed by rehydration at 30°C [2,3], a rapid formation of polysomes in the cytoplasm and an increased transcriptional activity in the nucleus have been observed [4,5]. Since practically no polysomal structures have been found in dormant embryo[6], it might be plausible to assume that a rapid formation of polysomes after resumption of metabolism is due to utilization of the stored stabilized messenger RNP complexes in these desiccated embryos [7-12].

By taking advantage of the fact that messenger RNA molecules in eukaryotes contain a sequence of polyadenylic acid residues in varied length at the 3'-termini [13-15], perhaps with an exception of histone mRNA [16], we have investigated systematically poly(A)$^+$RNPs present in the postmitochondrial supernatant of cryptobiotic embryos of A. salina by the technique of [^{3}H]-poly(U) hybridization [11, 17, 18]. The present results indicate the existence of poly(A)$^+$RNPs with messenger activity sedimenting predominantly at an apparent buoyant density of 1.27-1.30(g/cm^3) when analyzed by sucrose isopycnic centrifugation[19]. On the other hand, poly(A)$^+$RNPs without messenger activity have a lighter buoyant density of 1.20-1.23 (g/cm^3) under the same conditions. We tentatively propose that the messenger RNPs identified in dormant embryos are possibly the stored genetic information which is immediately translated at the onset of resumption of embryonic development. A similar observation in other cytoplasmic fractions was reported from several laboratories[20-23].

EXPERIMENTAL PROCEDURES.

Preparation of PMS. Dried cryptobiotic embryos of *A. salina* (20 g, dry weight) were sterilized with 5 % NaClO as described earlier[24]. The treated embryos were processed according to Slegers and Kondo[25] in buffer C (pH 6.8) containing 10 mM sodium phosphate (equimolar primary and secondary salts), 5 mM $MgCl_2$ and 50 mM NaCl or buffer A (pH 7.6) containing 35 mM Tris/HCl, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), 70 mM KCl and 9 mM $MgCl_2$ (with 150 mM sucrose) as indicated in the figure legends.

Extraction of RNA. Samples (PMS or gradient fractions) were made 2 % sodium dodecyl sulfate. After 15 min at room temperature they were placed in an ice-bath and mixed with 0.5 volume of redistilled phenol. Then 0.5 volume of chloroform was added approximately 5 min later, and the mixture was centrifuged for 10 min at 5,000 x g for phase separation. The aqueous phase was directly used for [^{3}H]-poly(U) hybridization assay (see below) in the case of gradient fractions, whereas the same aqueous fraction was further extracted twice with phenol and chloroform in the case of PMS samples. RNA was recovered from the last aqueous fraction by precipitating overnight with two volumes of ethanol and 0.1 volume of 20 % potassium acetate (pH 5.0) at -20°C. Concentrations of RNA were determined spectrophotometrically assuming $E_{260}^{0.1\%}$ values of 24, 30.1 and 31.3 at pH 7.0 for PMS RNA, poly(U) and poly(A), respectively[18].

Sucrose Density Gradient Centrifugation. PMS was usually analyzed by centrifugation at 4°C through a linear 5-20 or 10-30 % (w/v) sucrose gradient in buffer A or C. Centrifugation conditions are indicated in the figure legends. The gradient fractions were collected from the bottom of the gradients by giving an appropriate air pressure on top and the absorption profiles at 254 nm were recorded by LKB Uvicord II. Each fraction of the PMS gradients was assayed for its poly(A) content by $[^3H]$-poly(U) hybridization after extraction of RNA with phenol and chloroform as described above. RNA isolated from either total PMS or fractionated RNP complexes was analyzed by centrifugation at 4° C through 5-20 % (w/v) sucrose gradient in buffer A in a Spinco SW 41 rotor for 15 hr at 24,500 rpm. Fractionation of RNA gradients was as above.

Sucrose Isopycnic Centrifugation. For analytical runs, PMS was placed on three layers of sucrose solutions (3 ml each) in buffer C or E (pH 6.8) containing 10 mM sodiumphosphate (equimolar primary and secondary salts), 2 mM $MgCl_2$ and 50 mM NaCl. Two different layer systems were used with sucrose concentrations of 34, 62 and 94 % (w/v) and 28, 41 and 87 % (w/v) respectively. Centrifugation was at 4°C in a Spinco SW 41 rotor for 92 hr at 35,000 rpm. For preparative runs, PMS was placed over the same layers of sucrose solutions in buffer A or E (9 ml each), but centrifuged in a Spinco R 60 rotor for 60 hr at 50,000 rpm. The fractions were collected by sucking through a stainless steal needles from the bottom of the gradients. Densities were determined by weighing samples in a 100 μl constriction pipette.

Hybridization. Hybridization assay was usually carried out in a reaction mixture (a final volume of 0.5 or 1.0 ml) composed of equal volumes of RNA sample and 4 x SSC (1 x SSC ; 0.15 M NaCl, 0.015 M sodium citrate), and 5 μl of $[^3H]$-poly(U) (1.2 x 10^5 cpm/ μg ; 10^4 cpm/ μl), resulting in 17.5 mM Tris-HCl, 10 mM HEPES, 35 mM KCl, 4.5 mM $MgCl_2$, 300 mM NaCl, and 30 mM sodium citrate as final concentrations. After incubation at 41°C for 16-24 hr, the reaction mixtures were cooled to 0°C, digested with 25 μg/ml pancreatic RNAase A for 20 min. RNAase resistant $[^3H]$-radioactivity was precipitated with 6.5 % (w/v) TCA (trichloroacetic acid) at 0°C, collected on glass fiber discs (Whatman GF/C), and counted in a toluene based scintillant (5 g PPO (2,5-diphenyloxazole) and 0.15 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene in 1l toluene) using a Packard Tricarb 2450 scintillation spectrometer. Only 0.1 and 1 % of the input $[^3H]$-poly(U) were acid-insoluble, in either presence or absence of 1 % BSA (bovine serum albumin), with and without RNAase treatment, respectively, when $[^3H]$-poly(U) alone was processed. The corresponding background with RNAase treatment was subtracted from all hybridization experiments. Under our conditions, a triple stranded structure is formed from which only one poly(U) strand is digested by RNAase [18]. Thus the observed maximal resistance (40 %) to RNAase of the commercial $[^3H]$-poly(U) after hybridization is close to the theoretical maximal value (50 %).

Materials. [3H]-poly(U), nonradioactive poly(U) and poly(A) were obtained from Miles laboratories. Bovine pancreatic RNAase A (type I A) and BSA (fatty acid free) were purchased from Sigma Chemical Co. HEPES was from Calbiochem AG. PPO and POPOP were from Merck. Encysted cryptobiotic embryos of A. salina were originally collected in Utah salterns and distributed by Division of Sterno Industries, Inc.

RESULTS.

Basic Hybridization Experiments. In order to characterise poly(A)$^+$RNPs and RNAs in the postmitochondrial supernatant (PMS) of cryptobiotic embryos of A. salina, hybridization technique utilizing [3H]-poly(U) has been extensively employed in the present studies[11, 17, 18]. Under our experimental conditions as described in Experimental Procedures, the amount of [3H]-poly(U) becoming resistant to pancreatic RNAse treatment (25 μg/ml) after hybridization with poly(A) was proportional to that of poly(A) added to the hybridization assay mixture until about 40 % saturation of the input poly(U) was obtained as shown in Figure 1a. When RNA purified from total PMS was hybridized with poly(U) under idential conditions, a similar linear relationship between the ribonuclease resistant [3H]-radioactivity and the amount of PMS RNA added was obtained as shown in Figure 1b, but because it was in this case still below 40 % saturation of the input poly(U), no plateau was yet observed.

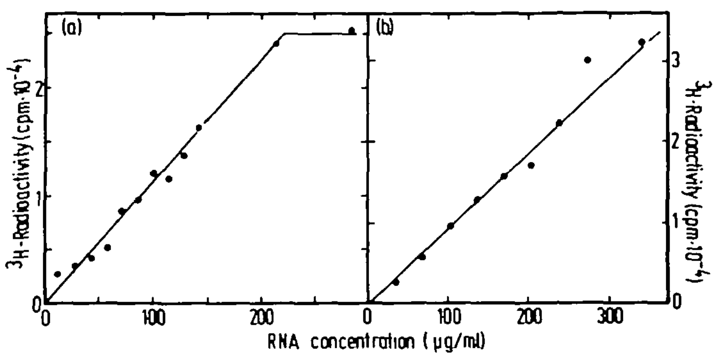

Figure 1 : Hybridization of Poly(U) with Poly(A) and PMS RNA. (a) A constant amount of [3H]-poly(U) (140μg, 5 x 10^2 cpm/ μg) was hybridized for 21 hr at 41°C with various quantitities of poly(A) as indicated. The reaction mixtures (1 ml) were prepared as described in Experimental Procedures and digested with 25 μg/ml pancreatic RNAase A for 20 min at 0°C. The RNAase resistant radioactivity was precipitated and collected on glass fiber discs (GF/C). (b) A constant amount of [3H]-poly(U) (0.7μg, 1.2 x 10^5 cpm/ μg) was hybridized with various amounts of PMS RNA as indicated. The reaction mixtures were treated exactly in the same way as in (a).

From the results of Figure 1, it was estimated that approximately 0.08 % of total PMS RNA constituted the poly(A) sequences being detectable by the present technique.

That a RNA complex formed between [^{3}H]-poly(U) and PMS RNA was due to formation of a real helical structure, but not due to other forms of aggregation, is illustrated by the melting experiments of these complexes as shown in Figure 2. The estimated Tm values for both types of RNA hybrids (e.g., PMS RNA-poly(U) and poly(A)-poly(U)) in 2 x SSC are 67°C and 70°C respectively. A slightly higher Tm value obtained for the poly(A)-poly(U) hybridization is presumably due to an incompletness of the direct assay used in this experiment as discussed by Bishop et al[18]. The Tm value of the PMS RNA-poly(U) hybrids is comparable to those obtained for the frog oocyte RNA-poly(U) complex[11] and the duck hemoglobin mRNA-poly(U) complex[18] under similar experimental conditions.

Characterization of Poly(A)$^+$RNPs by Sucrose Isopycnic Centrifugation. Although buoyant densities of cytoplasmic particles are determined commonly by isopycnic centrifugation in CsCl[8,26] the separated particles may not be suitable for further experimentation due to the necessary fixation of these particles with formaldehyde. Metrizamide, a triiodinated benzamido derivatives of glucose, has frequently been used for radioactive particles[27, 28], but it could not be employed for nonradioactive material because of its strong ultraviolet absorption with a maximum at 242 nm.

However, sucrose seems to be very useful for recovering cytoplasmic particles in native state, avoiding a problem of ulraviolet absorption, despite

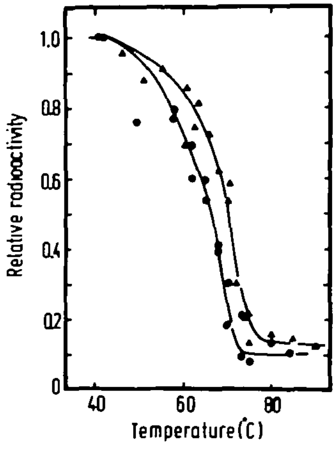

Figure 2 : Thermal Denaturation of Poly(A)-Poly(U) and PMS RNA-Poly(U) Complexes. The hybridization mixtures (1 ml) contained either 6.8 or 13.6μg of [^{3}H]-poly(U) (1.2×10^5 cpm/ μg) and either 20 μg of poly(A) or 280 μg of PMS RNA respectively in 2 x SSC and are incubated for 2 hr at 41°C. The reaction mixtures were immediately diluted with 150 μg nonradioactive poly(U) and the temperature was raised. After equilibration for 5 min at the indicated temperatures, 100 μl aliquots were removed for determination of the RNAase resistant radioactivity as in Figure 1. (▲ - ▲) for poly(U)-poly(A) complex and 1.0 equals 7980 cpm ; (● - ●) for poly(U)-PMS RNA complex and 1.0 equals 1650 cpm.

of its high viscosity. In fact, sucrose-glucose and sucrose-D_2O solutions have been used for isopycnic centrifugation[29,30]. A sucrose solution of 94 % (w/w) with a buoyant density of 1.347 (g/cm^3) at 5°C can be prepared without serious difficulty and after isopycnic centrifugation in fixed angle rotors at high centrifugal forces (or even in swinging bucket rotors for longer centrifugation time) the buoyant density of sucrose increases up to 1.39-1.40 (g/cm^3) at the bottom of the tubes (Figures 3 and 4). The range of sucrose buoyant density formed after isopycnic centrifugation is sufficient to cover the buoyant densities of cytoplasmic particles, due to the fact that sucrose reduces only slightly the degree of hydration of these particles in contrast to CsCl. Therefore the buoyant densities of RNPs in sucrose are lower than those in CsCl, but are more comparable to those in metrizamide (Table 1).

Figure 3 shows the distribution of poly(A)$^+$RNP particles obtained by a 92 hr centrifugation in a SW 41 rotor in the layer system 28, 41 and 87 % (a) or in the layer system 34, 62 and 94 % (b), under which ribosomes are at a buoyant density of 1.35-1.36 (g/cm^3). The same density value is also found

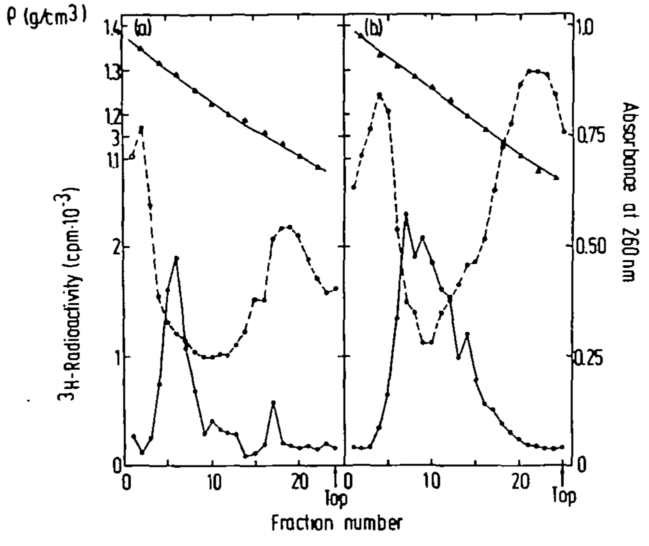

Figure 3 : Separation of Poly(A)$^+$RNP by Isopycnic Centrifugation. The buoyant density distribution of poly(A)$^+$RNP was analyzed by isopycnic centrifugation of PMS in sucrose using a SW 41 rotor. Centrifugation was at 4°C for 92 hr and 35,000 rpm. The poly(A) sequences were determined by hybridization with [^{3}H]-poly(U) as described in Experimental Procedures. (a) PMS analyzed on three layers (3 ml each) of 28, 41 and 87 % (w/v) of sucrose in buffer E respectively. (b) PMS analyzed on three layers (3 ml each) of 34, 62 94 % (w/v) sucrose in buffer E respectively. (● - ●), radioactivity ; (O - O), absorbance at 260 nm.

TABLE 1. Buoyant densities of ribosomes and RNPs banded in CsCl, metrizamide and sucrose.

Cytoplasmic Particles	Buoyant Density (g/cm^3) Sucrose [a] 2 mM Mg^{2+}	Metrizamide [b] 3 mM Mg^{2+}	CsCl [b]
Free RNPs	1.27-1.28	1.205	1.39
80 S Ribosomes	1.35-1.36	1.305	1.56

[a] Artemia cytoplasmic particles based on the present study.

[b] Taken from Buckingham and Gros[28]. Source of cytoplasmic particles is the skeletal muscle of foetal calf.

when centrifugation is carried out for 92 hr in a fixed angle R 60 rotor at 50,000 rpm and is in agreement with the results obtained in sucrose-D_2O solutions by Kempf et al[30]. An assymetrical peak sedimenting at 1.27-1.30 (g/cm^3) was seen after hybridization with [^3H]-poly(U) just behind the leading ultraviolet absorption peak of free ribosomes and amounted approximately to 64 % of the total poly(A) sequences present in PMS (Figure 3a). This peak was resolved into a more complex pattern with several peaks, which were separated into two classes centered at approximately 1.27 - 1.28 and 1.30 (g/cm^3) by a different layer system (Figures 3b). The 1.27-1.28 (g/cm^3) class coinciding with a valley of ultraviolet absorption seemed to be free RNPs, whereas the 1.30 (g/cm^3) class RNPs were complexed with ribosomal components (Figures 3b), because all of the poly(A)$^+$·RNPs of 1.30 (g/cm^3) could be converted to the 1.27-1.28 (g/cm^3) RNPs by treatment with 25 mM EDTA (Figure 4). The presence of 25 mM EDTA results in a lowering of the density of the ribosomal subunits to 1.28 for 40 S and to 1.30 (g/cm^3) for 60 S respectively. A similar decrease in density of ribosomal particles was already observed in D_2O-metrizamide isopycnic centrifugation in the absence of Mg^{2+} [28].

A minor fraction (20-30 %) of poly(A)$^+$RNPs was banded at a buoyant density of 1.20-1.23 (g/cm^3) and 1.14-1.15 (g/cm^3), separated in this region by a less steep gradient (Figure 3a). The 1.20-1.23 (g/cm^3) class included predominantly poly(A)$^+$·RNPs sedimenting at 10 S and 16 S[25] and they appeared to be the extensive degradation products of intact 1.27-1.30 (g/cm^3) RNPs, since isolated 1.27-1.30 (g/cm^3) RNPs purified further by oligo(dT)-cellulose chromatography followed by a second isopycnic centrifugation in sucrose resulted sometimes in producing a varied amount of 1.20-1.23 (g/cm^3) RNPs.

The degradation of mRNPs seemed to occur already in vivo, because the presence of the RNAse inhibitor bentonite (20 mg/ml) in buffers used during the preparation of RNP did not eliminate the 1.20-1.23 (g/cm^3) RNPs in PMS (Slegers and Kondo, unpublished results).

Characterization of Poly(A)$^+$ RNPs by Sucrose Density Gradient Centrifugation. When PMS was analyzed by a linear sucrose density gradient, more than 50 % of the poly(A) sequences present in PMS sedimented faster than 50 S, exhibiting several discrete reproducible peaks approximately at 65, 75 and 90 S (Figure 5). Under the condition used, no large aggregation of poly(A)$^+$RNPs was observed, as judged by the amount of poly(A) sedimented to the bottom of the gradient (Figure 5). The 20-30 S poly(A)$^+$RNPs were not well separated in this case (Figure 5) from 10-16 S RNPs of the 1.20-1.23 (g/cm^3) class, but they could be distinguished from the lighter RNPs in other sucrose density gradient condition[25]. As discussed above, the 1.30 (g/cm^3)

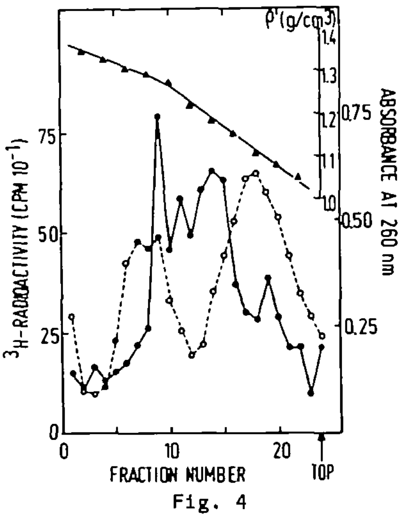

Fig. 4

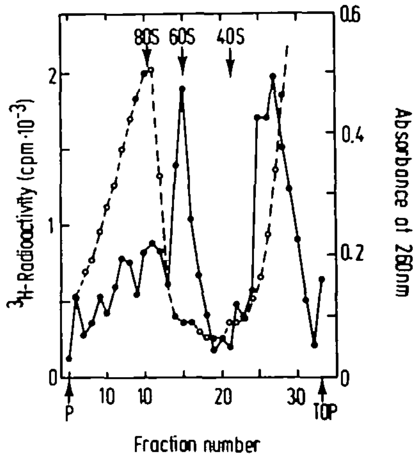

Fig. 5

Figure 4 : Effect of EDTA on the Buoyant Density of PMS Poly(A)$^+$RNP Complexes. PMS was analyzed by sucorse isopycnic centrifugation in buffer A concentration 25 mM EDTA (pH 7.0) in a Spinco SW 41 rotor as described in Experimental Procedures. Each fraction was hybridized with [^{3}H]-poly(U) as usual. (● - ●) radioactivity ; (O - O) absorbance at 260 nm ; (▲ - ▲) buoyant density.

Figure 5 : Sucrose Gradient Analysis of Poly(A)$^+$RNP of PMS. PMS was analyzed on a 10-30 % sucrose gradient in buffer E. Centrifugation was in a SW 27 rotor for 17 hr at 15,000 rpm. RNA was extracted from each fraction of the gradient and its poly(A) content was determined by hybridization with [^{3}H]-poly(U). (● - ●), radioactivity ; (O - O), absorbance at 260 nm.

class was suggested to be ribosome-bound RNPs, while the 1.27-1.28 (g/cm^3) class to be free ones. In agreement with this observation, the presence of 25 mM EDTA in the sucrose density gradient converted virtually all poly(A)$^+$ RNPs, sedimenting faster than 50 S present in the isolated 1.27-1.30 (g/cm^3) density peak, to free 20-30 S RNP with a concomitant transformation of ribosomal subunits to slow sedimenting particles[31]. By shortened contact of these RNP complexes with EDTA during centrifugation, some faster sedimenting poly(A)$^+$RNPs could still be recognized (Figure 6a). Such a conversion of the complexed RNPs did not occur in the presence of 2 % Triton X-100. Therefore, we conclude that in the PMS of cryptobiotic embryos of A. salina over 50 % of poly(A)$^+$RNP was in a complexed form with either ribosomes or their subunits which could be converted to free 20-30 S RNPs by EDTA treatment with a concomitant shift in buoyant density from 1.30 to 1.27-1.28 (g/cm^3) (Figures 4 and 6).

Analysis of Poly(A)$^+$RNA Components. Poly(A)$^+$RNA isolated from total PMS (Figure 7a) and 1.27-1.30 (g/cm^3) (Figure 7b) and also 1.20-1.23 (g/cm^3)

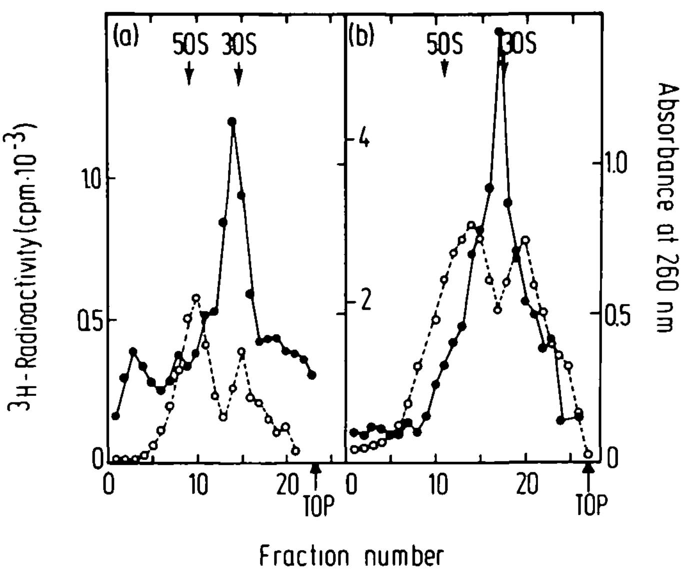

Figure 6 : Effect of EDTA on the Sedimentation Behaviour of the 1.27-1.30 (g/cm^3)Poly(A)$^+$RNP Complexes. The RNP complexes banding at 1.27-1.30 (g/cm^3) were analyzed by 5-20 % sucrose gradients in buffer A containing 25 mM EDTA (pH 7.0). Poly(A) sequences were determined as usual by hybridization with [^{3}H]-poly(U). (a) Centrifugation was carried out at 41,000 rpm for 120 min at 4°C in a Spinco SW 41 rotor. (b) Centrifugation was at 25,000 rpm for 16 hr at 4°C in a Spinco SW 27 rotor. (● - ●) radioactivity ; (O - O) absorbance at 260 nm.

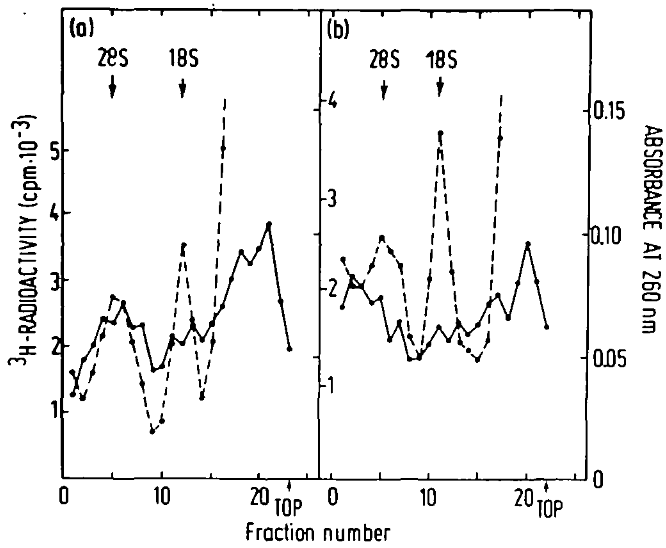

Figure 7 : Sucrose Density Gradient Analysis of Poly(A)$^+$RNA. RNA from RNP complexes of PMS (a) and the 1.27-1.30 (g/cm^3) class (b) was analyzed by 5-20 % sucrose gradient centrifugation in buffer A and the poly(A) contents determined as described in Experimental Procedures. (● - ●) radioactivity ; (O - O) absorbance at 260 nm.

(Figure 8a) was analyzed by a linear sucrose density gradient. The size of poly(A)$^+$RNA derived from PMS as well as the heavier density class RNPs was heterogeneous, ranging from 4 S up to 33-34 S (Figure 7), whereas the lighter density class RNPs exhibited essentially only one species of 4 S RNA (Figure 8a). Further, poly(A)$^+$RNA from narrowly pooled 20-30, 65, and 75 S RNPs was similarly analyzed (Figure 8). Free RNPs (20-30S species) were found to contain 8-14 S RNA (Figure 8b) and this RNA species was also detected in the 65 and 75 S RNP complexes (Figures 8c and 8d), supporting further the conclusion that free 20-30 S RNPs were complexed with ribosomal components, sedimenting faster than 50 S.

Finally template activity of RNA isolated from Artemia PMS was examined by a cell-free system of wheat embryos. The highest template activity was obtained by 9 and 14 S poly(A)$^+$RNAs eluted from a Sepharose 4B-poly(U) column at 53°C as judged by incorporation of [^{35}S] -methionine into protein, whereas 4 S RNA had practically no template activity [25].

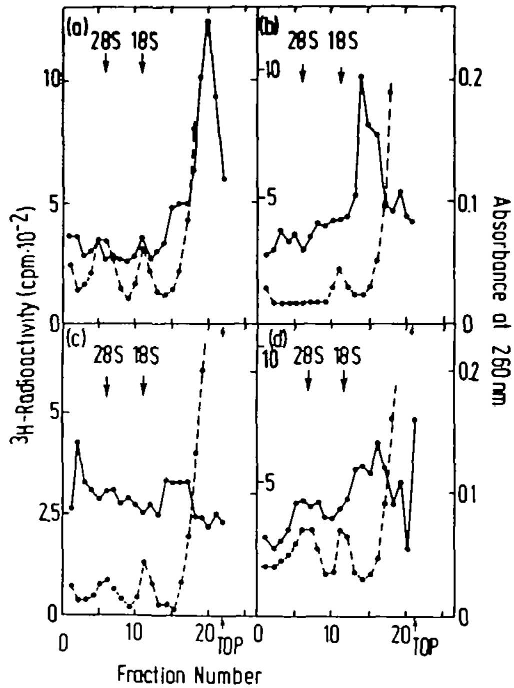

Figure 8 : Analysis of Poly(A)⁺RNA Components Extracted from Fractionated RNP Complexes. RNP complexes banding at 1.20-1.23 and 1.27-1.30 (g/cm^3) during preparative sucrose isopycnic centrifugation was precipitated with 10 % polyethylene glycol, dissolved in buffer A, and fractionated by 5-20 % sucrose gradient centrifugation using a Spinco SW 27 rotor at 14,200 rpm for 15 hr. After localizing the individual RNP complexes on the sucrose gradients by [^{3}H]-poly(U) hybridization, RNA was purified from pools of 1.20-1.23 (g/cm^3) RNPs (a), and of 20-30 S (b), 64-65 S (c) and 75-77 S (d) RNPs of 1.27-1.30 (g/cm^3) class and centrifuged through 5-20 % sucrose gradients in a Spinco SW 41 rotor at 24,500 rpm for 15 hr. Each gradient fraction was hybridized with poly(U) as usual. (● - ●) radioactivity ; (O - O) absorbance at 260 nm.

DISCUSSION.

Cytoplasmic mRNA in eukaryotes is found in a form of particle complexed with specific proteins[32-35] whose buoyant density is generally lower (1.4 (g/cm^3)) than those of ribosomes and polysomes (ca. 1.55 (g/cm^3)) when analyzed by CsCl isopycnic centrifugation[8, 36-38]. Although the exact nature of the mRNP particles has not been elucidated, available evidence indicates that genetic information is often preserved as a form of stable mRNP particle in undifferentiated embryonic cells[9, 11, 12]. The present study was therefore undertaken to pursue the possibility that completely desiccated ametabolic embryos of *A. salina* might have preserved some important mRNA whose genetic information would immediately be required for a continuation of the gastrulation process when the cryptobiotic state of the embryos is broken. This is further supported by observation that *in vitro* capability of transcription in isolated nuclei from cryptobiotic embryos is found almost negligible as compared with that of nauplius larvae[5,39] and that polysomes are immediately detected in the cytoplasm as soon as redevelopment is resumed in cryptobiotic embryos[4].

Nilsson and Hultin[20, 40] reported that a heterodispersely distributed RNA (17-20 S) isolated from the 15,000 x g sediment (mitochondrial fraction) of cryptobiotic embryos exhibited messenger activity when translated in a cell-free system of *E. coli* and that poly(A) sequences of 45-65 nucleotides long were also present in the same RNA preparation sedimenting at 14-17 S. On the other hand, since the most template active RNA in the 17,600 x g supernatant (non-mitochondrial fraction) was found to be 8-14 S, the stored mRNA in two cell fractions should possibly be distinct in coding for proteins. This is presently investigated in this laboratory.

In the PMS of cryptobiotic embryos of *A. salina*, two classes of poly(A)$^+$ RNP complexes are detected with different buoyant densities (Figure 3). The 1.27-1.30 (g/cm^3) class exhibits multiple RNP components sedimenting from 20-30 to 100 S (Figure 5), whereas the 1.20-1.23 (g/cm^3) class consisted essentially of 10 and 16 S[25]. Similar RNP components have already been demonstrated in sea urchin (*Lytechinus pictus* and *Strongilocentrotus purpuratus*) embryos[7], loach (*Misgrunus fosilis*) embryos[8], wheat (*Triticum vulgare*) embryos[9], and frog oocytes[11]. The heavier class RNPs could be separated into free RNPs (1.27-1.28 (g/cm^3)) and ribosome-bound RNPs (1.30 (g/cm^3)) by a less steep buoyant density sucrose gradient (Figure 3b). The latter RNPs were readily converted to the former ones by treatment with 25 mM EDTA (Figures 4 and 6). The free RNPs were found to be predominantly 20-30 S particles with several minor species sedimenting at 35-50 S (Figures 6). Analysis of RNA components of these RNPs supports this (Figures 7 and 8). Thus, it can be concluded that the larger poly(A)$^+$RNPs above 50 S are in a complexed form composed of free RNPs bound to either ribosomes or their subunits. A similar situation can be observed with RNP complexes of loach embryos, in which some of the larger RNPs (50-90 S) band at the intermediate buoyant densities between free RNP and free ribsome in a CsCl density gradient, while the smaller RNPs (28-38 S) band predominantly at a buoyant density of free RNP[8].

It has been shown that a complex of mRNP and the ribosomal subunit is active *in vitro* protein synthesis, functioning as an intermediate, in the case of rabbit globin mRNA[41, 42]. However, poly(A)$^{+}$RNP complexes of cryptobiotic embryos of *A. salina* were totally inactive in a S-30 extract of wheat embryos[22], while purified RNA could direct *in vitro* protein synthesis. Therefore, it appears that these RNP complexes of *A. salina* embryos seem to be in a repressed or inactivated state and must be reactivated by yet unknown meachnism before they could support active protein synthesis upon cessation of cryptobiosis by rehydration. In fact, it has been suggested that the stored mRNA seems inactive and must be activated to a translatable state by germination in wheat germ[43, 44] and in cotton seeds[45] or by fertilization of sea urchin eggs[46].

On the other hand, the 1.20-1.23 (g/cm^3) class having 10 S and 16 S RNPs, seemed to be *in vivo* degradation products of the larger poly(A)$^{+}$mRNPs and contained only poly(A)-containing sequences. However, it is not known whether an accumulation of these small poly(A)$^{+}$RNPs in the cryptobiotic *Artemia* embryos was a result of an active process required for entering a cryptobiotic state in an early embryogenesis. Moreover such 12-15 S complex, containing a 4 -5 S poly(A) stretch can be isolated by *in vitro* ribonuclease degradation of polysomes in mouse sarcoma 180 ascites cells[47]. The small poly(A) complex of this sort is not restricted to the cytoplasm, but also exist in nucleus. Quinlan, Billings and Martin[48] have found a nuclear 15 S particle containing poly(A) sequences in Taper hepatoma ascites cells. Moreover, an analogous RNP particle of 14 S in Ehrlich ascites carcinoma cells and rat liver has also been analyzed to have a high adenine content (over 70 %). Recently, Felicetti et al.[21] independently reported the presence of poly(A)$^{+}$ mRNP complexes sedimenting between 4 and 80 S whose RNA components exhibited an heterogeneous distribution from 4 to 28 S in PMS of cryptobiotic embryos of *A. salina* using a different cell fractionation method.

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