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Membrane associated cytoplasmic mRNA in Artemia salina; functional and physical changes during development

Haim Grosfeld, Hermona Soreq and Uriel Z. Littauer

Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

The physical and functional properties of the mRNA population from developing embryos of the brine shrimp Artemia salina were characterized. About 20% of the total poly(A)-rich mRNA in these embryos appears to be specifically associated with the membrane fraction throughout early development, and physically differs markedly from the free cytoplasmic mRNA. The membrane-associated mRNA fraction consists of two well-defined populations of molecular weight of 5.2×10^5 and 3.6×10^5, whose relative amount changes during the various stages of embryo development. The size of the poly(A) tail at the 3'-end of the mRNA molecules, as estimated by processive phosphorolysis, was found to consist of 180 and 210 adenosine residues for the two respective mRNA species. The in vitro translation products of the membrane-bound mRNA molecules are apparently similar to those of the free mRNA molecules.

INTRODUCTION

The developing embryos of the brine shrimp Artemia salina offer a useful system to investigate control mechanisms operating at the transcriptional and translational levels. The first 24 hours following rehydration are characterized by a rapid resumption of metabolic activity (1), and a simultaneous commencement of protein synthesis (2,3). Marked differences in the protein composition of cysts and nauplii have also been observed (4). Thus, low molecular weight protein species (around 25,000), characteristic of the undeveloped embryos, give way to those of higher molecular weight, among which tubulin (55,000) and actin (42,000) have been positively identified (5). We have also shown that dormant cysts are not devoid of messenger but that the majority of mRNA occurs in a masked 40S form (possibly mRNP) which disappears folloing rehydration (6). In addition, a small fraction of mRNA is present in polysomes, which increase dramatically with development. Recently, Nilsson and Hultin (7) observed that a portion of the poly(A)-containing RNA is associated with a membrane fraction.

The occurrence of the clearly defined transitions in the protein composition of cysts and nauplii, prompted us to examine the free and membrane-bound

mRNA population during embryogenesis, with respect to their physical and functional characteristics. Evidence is presented to support the hypothesis that the membrane-associated mRNA fraction is not of a mitochondrial origin, but originates in the nucleus.

MATERIALS AND METHODS

Isolation of membrane-bound mRNA

Artemia salina cysts, 5-10 g dry weight (Longlife Aquarium Products, Harrison, New Jersey), were separated from sand and other heavy impurities by flotation in cold saturated NaCl, followed by washing with cold water. The cysts were then incubated with agitation at 30° for the desired length of time in 4% NaCl. After the incubation period, cysts were collected on a No.1 sintered glass funnel, washed extensively with water and then with cold STKE buffer (0.44 M sucrose/50 mM Tris-HCl (pH 7.6)/0.2 M KCl/10 mM Na_2EDTA). The washed cysts were suspended in approximately 5 volumes of cold STKE buffer and homogenized with 10 strokes of a loose-fitting, motor-driven Potter-Elvhjen homogenizer. The suspension was filtered through Miracloth (Calbiochem) and centrifuged twice at 4° for 10 min at 1,500 x g. Finally, the supernatant was centrifuged for 10 min at 12,000 x g and the pellet membrane fraction washed twice with cold STKE buffer. For the preparation of the membrane fraction from nauplii, 5 g of cysts were incubated for 36 hrs in 1 liter of 4% NaCl with vigorous agitation at 30° in a 4-liter Erlenmeyer flask. The hatched nauplii were then separated from unhatched cysts by allowing them to settle on top of the latter in a separatory funnel at room temperature. Collected nauplii (30-40 ml) were transferred to a 250 ml measuring cylinder, diluted 5-fold in water, and then glycerol was added to a final concentration of 30% (V/V). The suspension was mixed by inverting the cylinder back and forth several times. After 5 min, all the nauplii floated to the top and were collected. The flotation procedure was repeated 3 more times, except that in the last cycle the nauplii were suspended in cold STKE buffer and 30% glycerol. Nauplii were diluted two-fold with STKE buffer and carefully homogenized in a glass Dounce homogenizer using five strokes each on an A and then a B pestle; the membrane fraction was isolated as described above. Membrane pellets were resuspended in 10 mM Tris-HCl (pH 7.6)/100 mM NaCl/ 2 mM Na_2EDTA buffer at room temperature, sodium dodecyl sulphate was added to a final concentration of 1%, and the lysed suspension was extracted twice with one volume of water-saturated phenol. The RNA was precipitated overnight from the separated aqueous phase by the addition of K-acetate buffer (pH 5.5) to 2% and two volumes of ethanol

at -20°. The RNA was then collected, washed three times with ethanol- 0.2 M NaCl (2:1 v/v), dried under vacuum, and subjected to oligo(dT)-cellulose column fractionation (8). The pooled mRNA fractions were precipitated with K-acetate and ethanol, washed twice with ethanol-0.2M NaCl, dried under vacuum, dissolved in water and kept in liquid air until use; the A_{260}/A_{280} absorbance value was found to be close to 2.00.

Analysis of RNA by electrophoresis in agarose-acrylamide composite gels and in acrylamide-formamide gels

Electrophoresis was carried out on 1.7% polyacrylamide - 0.5% agarose slab gels according to Peacock and Dingman (9) or on acrylamide-formamide gels according to Staynov et al (10). Gels were fixed and stained in the dark using 50% formamide containing 0.003% "Stains all". The photographic transparencies of the different slots of the stained gels were scanned at 540 nm in a Gilford 2400 Spectrophotometer equipped with a scanning attachment.

Cell-free protein synthesis and product analysis

Cell-free protein synthesis in a wheat-germ extract, product identification on 10%-18% sodium dodecyl sulfate polyacrylamide gels and fluorography of the dried gels were carried out as previously described (6).

Sucrose gradient fractionation of mRNA

Poly(A)-rich mRNA of the free or membrane-bound fractions were isolated from 3-hr incubated cysts, and then centrifuged for 4.0 hrs at 42,000 rpm on a 5-20% sucrose gradient (0.1 M K-acetate, 10 mM Hepes buffer (pH 7.5), 1 mM EDTA buffer) in a SW 50.1 Spinco rotor. Aliquots of 12.5 µl from each fraction were added to the wheat-germ reaction mixture in a final volume of 25 µl.

Removal of poly(A)-tail from mRNA with polynucleotide phosphorylase (PNPase)

Artemia nauplii were transferred into petri dishes and incubated for 10 hrs with carrier-free [^{32}P] (0.1 mCi/ml). mRNA was isolated from the membrane fraction and incubated in a reaction mixture containing 3-10 µg [^{32}P] mRNA (20,000 counts/min), 80 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM potassium phosphate (pH 8.0), 1 mM EDTA, 0.3 M NaCl and 3 µg purified polynucleotide phosphorylase in a final volume of 100 µl. The final salt concentration was 0.47 M. The mixture was incubated at 0° for various time intervals. Aliquots of 15 µl were removed and the liberated nucleoside diphosphates in which the α-phosphate was labeled with [^{32}P]were resolved by chromatography on DEAE-cellulose as previously described (11).

RESULTS

The membrane fraction used in this study was shown by electron microscopy to be rich in mitochondria (12). In addition acrylamide gel electrophoresis of RNA extracted from the EDTA-washed membrane fraction, purified by isopycnic gradient centrifugation, demonstrated that these preparations were free of cytoplasmic ribosomes (unpublished results). About 18% of the RNA extracted from the STKE buffer-washed membrane fraction was found to possess a poly(A) segment, as measured by specific adsorption to an oligo(dT)-cellulose column, whereas only 1.4% of RNA isolated from the post-mitochondrial supernatant was bound under these conditions. Acrylamide-agarose gel electrophoresis (Fig. 1) revealed that this membrane-associated poly(A)-rich mRNA fraction is

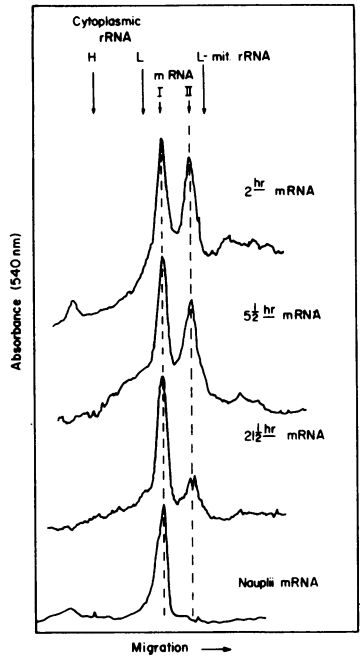

Fig.1: Densitometric traces of acrylamide-agarose gels after electrophoresis of membrane-associated mRNA isolated from developing embryos. 5 µg samples of membrane-associated mRNA preparations from A.salina cysts (incubated as indicated) were subjected to slab gel electrophoresis, staining and scanning as described in Materials and Methods.

comprised of two sharply defined species, of molecular weight of 5.2×10^5 ($mRNA_I$) and 3.6×10^5 ($mRNA_{II}$). The same pattern was also obtained upon electrophoresis in acrylamide gels in the presence of 98% formamide, ruling out the possibility that the two bands arise from RNA aggregation (Fig.2).

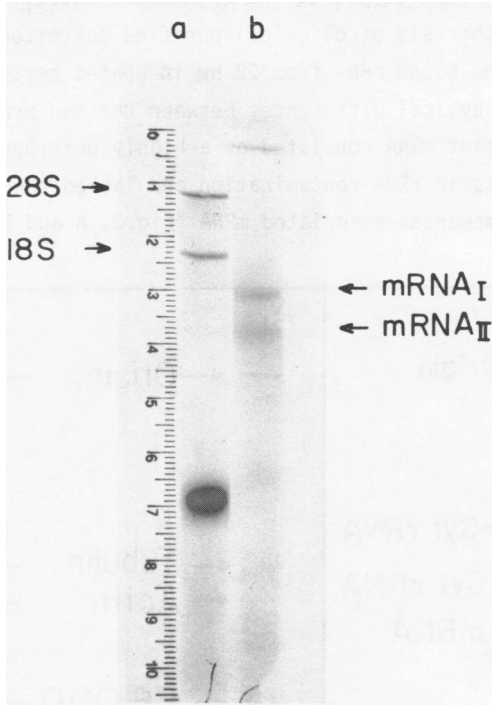

Fig.2: Analysis of membrane-bound mRNA on acrylamide-formamide gels. 20 µg of rat liver rRNA markers (a) were electrophoresed on 4% acrylamide-98% formamide gels, prepared and run according to Staynov et al (10), parallel to 20 µg of poly(A)-rich mRNA isolated from the membrane fraction of 2 hr incubated cysts(b). The gels were stained with "Stains-all".

During cyst development a pronounced change in the ratio between the two mRNA populations was observed. Whereas in 2 hr incubated cysts $mRNA_I$ and $mRNA_{II}$ were present in approximately equivalent amounts, the quantity of the lighter species ($mRNA_{II}$) steadily decreased during development, so that at the nauplius stage only $mRNA_I$ could be detected (Fig.1). When total RNA from the membrane-associated fraction was electrophoresed on an acrylamide-agarose gel, the heavy mitochondrial ribosomal RNA ($5.2x10^5$) (13) comigrated with $mRNA_I$. However, the poly(A)-rich mRNA could be freed of other membrane-associated RNA molecules, such as rRNA from mitochondrial mini-ribosomes. Thus, following oligo(dT)-cellulose chromatography mRNA could be analysed. The molecular weights of the mRNAs were determined both in acrylamide-agarose and acrylamide-formamide gels and a close agreement was obtained between both estimations. This indicates that the electrophoretic migration of $mRNA_I$ and $mRNA_{II}$ is not markedly affec-

ted by the secondary or tertiary structure of the polynucleotide chains.

When gel electrophoresis of oligo(dT)-purified postmitochnodrial-supernatant mRNA and membrane-bound mRNA from 22 hr incubated cysts was performed in parallel, striking physical differences between the two preparations became apparent. The supernatant mRNA consisted of a highly heterogeneous population containing some cytoplasmic rRNA contamination and lacked the two sharp bands characteristic of the membrane-associated mRNA (Fig.3, A and B).

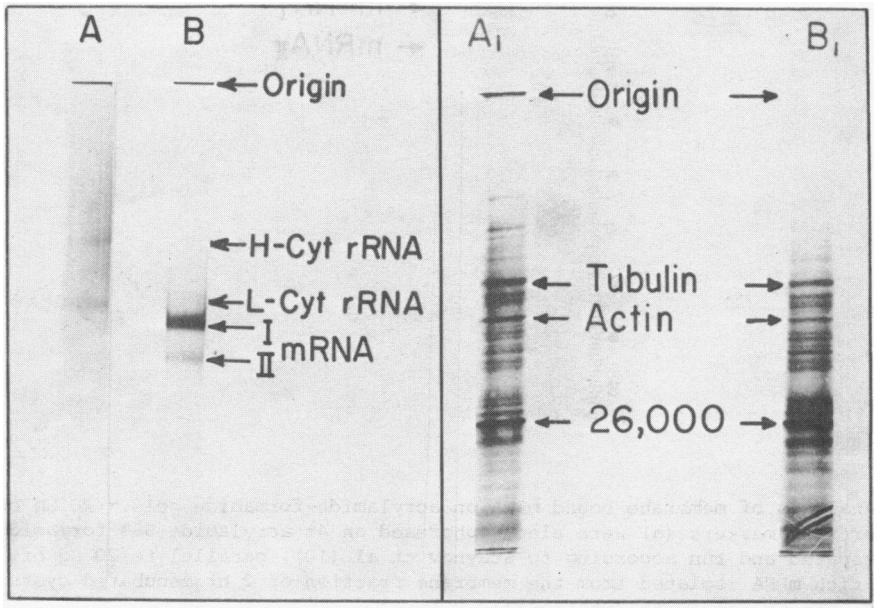

Fig. 3: Comparison of the electrophoretic mobilities and the translation products of post-mitochondrial mRNA and membrane-associated mRNA. Poly(A)-rich mRNA was isolated from 22-hr incubated cysts, either from the post-mitochondrial fraction (4), or from the membrane fraction (as in Fig. 1). A and B show the electrophoretic pattern of the postmitochondrial and membrane-bound mRNA, respectively. A_1 and B_1 are the autoradiographs of [^{35}S]-methionine labeled polypeptides directed by the mRNA preparations and separated by electrophoresis on 10-18% sodium dodecyl sulfate acrylamide gradient gels. The two mRNA preparations were translated in the wheat-germ cell-free system (5).

Nevertheless, cell-free translation of these two mRNA preparations showed that both yielded a qualitatively similar array of labeled polypeptide products (Fig. 3, A_1 and B_1). Thus, despite the physical homogeneity of the membrane-bound mRNA, its coding capacity resembled that of the more heterogeneous cytoplasmic species. In order to establish that the translational activity of the

bound mRNA indeed resided with $mRNA_I$ and $mRNA_{II}$, their ability to direct *in vitro* protein synthesis following fractionation on a sucrose gradient was assessed. Fig. 4 depicts the sucrose gradient analyses of membrane-associated mRNA before (A) and after (B) oligo(dT)-cellulose chromatography. In both preparations the translational activities appeared as a narrow peak, coinciding with the optical density tracings of the poly(A)-rich mRNA (Fig.4B). The *in vitro* synthesized [^{35}S]-labeled polypeptides were analyzed by SDS-acrylamide gel electrophoresis. Fig. 4C shows the autoradiograph of the *in vitro* products directed by the four mRNA peak fractions (Fig. 4B, 1-4).

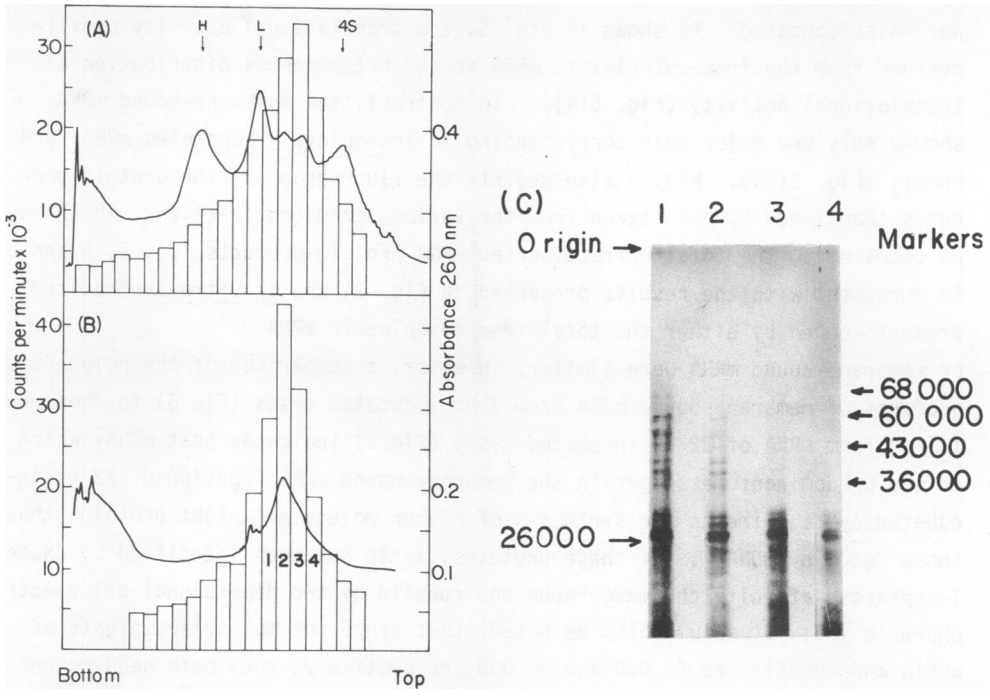

Fig. 4: Optical density tracings and translational activities of membrane-associated RNA fractionated on sucrose gradients. (A) Total RNA extracted from membrane fraction of 22 hr incubated cysts. (B) Poly(A)-rich mRNA isolated by oligo(dT)-cellulose fractionation from the same RNA preparation as in (A). Sucrose gradient centrifugation and wheat-germ cell-free protein synthesis were run as described in Materials and Methods. Hot trichloroacetic acid precipitable counts of the whole reaction mixtures are depicted by the bars. (C) Autoradiographs of the *in vitro* synthesized protein products, directed by the fractions 1-4 depicted in (B). The mRNA was separately precipitated from each tube by ethanol and K-acetate at -20°, collected by centrifugation, lyophilized and resuspended in 50 µl of H_2O. 25 µl of the resuspended mRNA solution was translated in a final volume of 50 µl of the wheat-germ cell-free system. 10 µl of each reaction mixture were then analyzed by SDS-gel electrophoresis and autoradiography.

Although not completely separated from $mRNA_{II}$, the heavy $mRNA_I$ (520,000 Mr) should be the predominant species on the left side of the optical density peak (fraction Number 1), while the lighter $mRNA_{II}$ (360,000 molecular weight) should be more abundant in the right side of the peak (fraction 4). A difference in the polypeptide products was indeed observed between fraction 1 and fraction 4 mRNAs. Fraction 4 mRNA directed the synthesis of low molecular weight products (26,000 and down), while the polypeptides synthesized with fraction 1 mRNA reach a molecular weight of over 60,000.

Poly(A)-rich mRNA isolated from the post-mitochondrial supernatant (free cytoplasmic mRNA) and from the membrane preparation of 3.5 hr incubated cysts were also compared. As shown in Fig. 5, the translational activity profile derived from the free-cytoplasmic mRNA showed heterogenous distribution of translational activity (Fig. 5(A)). In contrast, the membrane-bound mRNA showed only one major peak corresponding to incompletely separated $mRNA_I$ and $mRNA_{II}$ (Fig. 5(B)). Fig. 5 also depicts the fluorograph of the protein products translated by mRNA taken from the various gradient fractions, which may be compared to the total unfractionated mRNA protein products (Fig.5, a and b). In agreement with the results presented in Fig. 3, the *in vitro* synthesized products coded by either the total free cytoplasmic mRNA or membrane-bound mRNA were similar. However, a comparison of the polypeptide products of membrane-bound mRNA from 3 hr incubated cysts (Fig.5) to those obtained from mRNA of 22 hr incubated cysts (Fig.3) indicates that $mRNA_I$ which is the predominant messenger in the membrane-bound mRNA fraction of 22 hr incubated cysts, directs the synthesis of higher molecular weight proteins than those coded by $mRNA_{II}$. Of these proteins, actin has been identified by DNase 1-Sepharose affinity chromatography and tubulin by two dimensional gel electrophoresis (5). It should also be noted, that since the molecular weights of actin and tubulin are 42,000 and 55,000, respectively, they both need to be translated from mRNA having a molecular weight of about 0.5×10^6 in its translatable region. This theoretical consideration suggests that $mRNA_I$, and not the shorter $mRNA_{II}$, serves as mRNA for the high molecular weight products.

An additional point for consideration is the existence of a nontranslatable poly(A)-tail in both $mRNA_I$ and $mRNA_{II}$. The length and location of the poly(A) region in the membrane-bound mRNA from *Artemia* was therefore determined by sequential phosphorolysis of the poly(A) tail with polynucleotide phosphorylase. This was carried out under conditions in which the poly(A) region is removed without apparent degradation of the rest of the molecule (11). From the difference in molecular weights of the intact and the phosphorolyzed mRNA

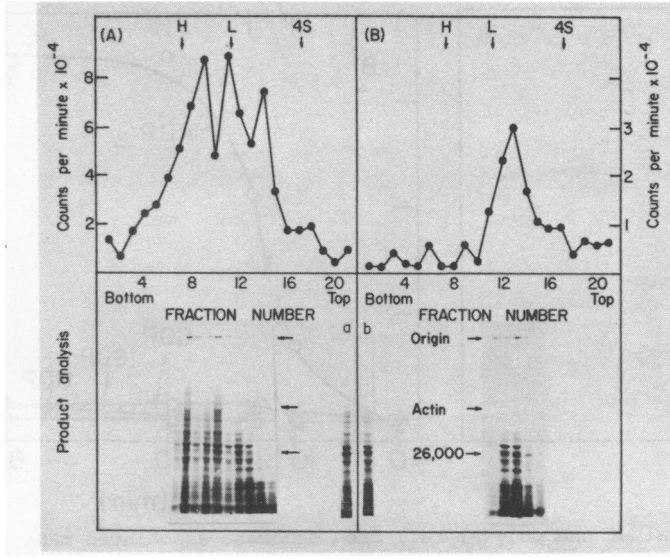

Fig. 5: Protein synthetic activity in fractions from sucrose gradients of post-mitochondrial and membrane-associated mRNA. (A) Post-mitochondrial supernatant mRNA (free cytoplasmic mRNA) and (B) membrane-associated mRNA were isolated from 3-hr incubated cysts (Fig. 1), and then centrifuged for 3.5 hrs on a 5-20% sucrose gradient (0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA buffer) in a SW 50.1 Spinco rotor. RNA was precipitated from each of the collected fractions by K-acetate and ethanol. Each mRNA fraction was dissolved in water and then translated in the wheat-germ cell-free system. The [^{35}S]-methionine labeled products were separated by electrophoresis on SDS-acrylamide gradient gels. The upper panel shows the distribution of total hot TCA-precipitable cpm per 5 µl of each reaction mixture, while the lower panel depicts the autoradiograph of the protein products coded by each mRNA fraction, as analyzed by SDS-polyacrylamide gradient gel electrophoresis (5). a and b depict the total, unfractionated mRNA protein products of the two RNA sources.

preparations, as determined by acrylamide-agarose gel electrophoresis (Fig.6A), values of about 180 and 210 adenosine residues were calculated for $mRNA_I$ and $mRNA_{II}$, respectively. A similar value of about 170 adenosine residues was obtained for $mRNA_I$ by analysis of the α-[^{32}P]-nucleoside diphosphates released from *in vivo* [^{32}P]-labeled $mRNA_I$ isolated from embryos at the nauplius stage (Fig. 6B). As expected from RNA containing a poly(A) tail at its 3'-OH end, the released nucleoside diphosphates were mainly composed of ADP molecules (Fig. 6B).

The close agreement between the percentage of the radioactivity released as nucleoside diphosphate and the difference in molecular weight calculated from gel electrophoresis further strengthens the estimate of the length of the poly(A) tail (11).

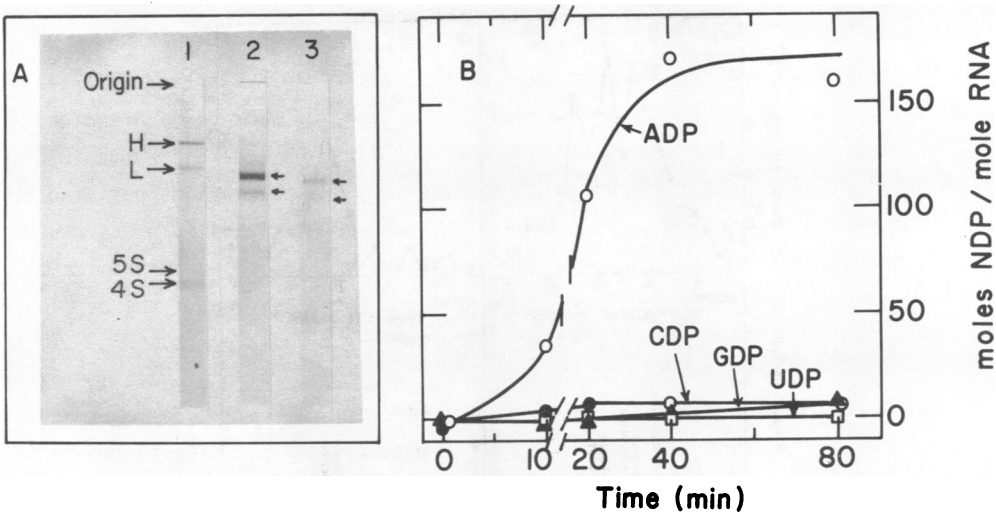

Fig. 6: Removal of the poly(A) tail from membrane-associated mRNA of A.salina cysts. A. Membrane-bound mRNA was isolated from 6-hr incubated cysts and digested by PNPase in a reaction mixture containing 10 µg membrane-bound mRNA, 30 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM potassium phosphate buffer (pH 8.0) 1 mM EDTA and 10 µg purified E.coli PNPase, in a final volume of 100 µl. The mixture was incubated at 0° for 30 min; the poly(A)-free mRNA was extracted from the reaction mixture with phenol/chloroform/isoamylalcohol (50:50:1), precipitated, and washed as described previously (11). Phosphorolyzed and intact mRNA samples (5-8 µg per slot) were subjected to electrophoresis on acrylamide-agarose slab gels and stained with "Stains all". The apparent molecular weights of the intact and phosphorolyzed mRNA were estimated to be 5.2×10^5 and 4.6×10^5 for $mRNA_I$ and 3.6×10^5 and 2.9×10^5 for $mRNA_{II}$, respectively, by comparing their electrophoretic migration to that of heavy (H) and light (L) cytoplasmic rRNA of Artemia (slot 1). B. 10 µg of [^{32}P]-labeled $mRNA_I$ (2×10^3 cpm/µg) from embryos at the nauplius stage were phosphorolyzed with 3 µg of E.coli PNPase as described in A., and α-labeled NDPs were determined in 15 µl aliquots as described in Materials and Methods.

Fig. 6A also shows that after phosphorolysis the poly(A)-free mRNA still migrated upon electrophoresis in agarose-acrylamide gels as two sharp bands. Similar results were obtained upon electrophoresis in the presence of formamide. These results indicate that the poly(A)-lacking mRNA preparation remained intact and that the non-poly(A) region in both mRNA preparations has a homogeneous length distribution.

Hybridization experiments of [^{125}I]-labeled mRNA with mitochondrial DNA, under conditions of excess DNA, showed that only 6% of the membrane-associated mRNA, and less than 1% of the cytoplasmic mRNA, could hybridize to the mitochondrial DNA. These observations, together with the similar protein products obtained with the membrane bound as well as the free cytoplasmic mRNA, indicate their common nuclear source.

DISCUSSION

The changes previously observed in the nature of the *in vitro* translation products directed by the mRNA isolated from developing embryos of *Artemia salina* (5) led us to expect changes in the mRNA population. We therefore examined the physical as well as the functional properties of mRNA populations purified from different developmental stages of these embryos. Physical differences could only be observed in the mRNA populations which were isolated from the 12,000 x g pellet, consisting of the mitochondrial and membrane fraction of the developing embryos. The differences observed in the profile of the electrophoretically separated membrane-associated mRNA populations (Fig. 1) were in good agreement with our previous results, namely - the increase in the relative amount of the high molecular weight mRNA species is accompanied by an increase in the formation of products of higher molecular weights, such as tubulin and actin (5). Thus the amount of these products synthesized by membrane-associated mRNA of developing embryos (Fig. 3) is markedly higher than that directed by mRNA isolated from undeveloped cysts (Fig. 5).

Is the origin of the membrane-associated mRNA population mitochondrial or cytoplasmic? We have tried several independent techniques in order to characterize more carefully the nature of the examined mRNA populations and to answer this question. The evidence found to support the hypothesis that the two membrane-bound mRNA species are derived from a cytoplasmic and not a mitochondrial source is four-fold: (a) the exceedingly high proportion (18%) of total membrane-associated RNA which they represent, (b) the diversity of the protein products and their close similarity with those obtained from free cytoplasmic mRNA, (c) the extensive region of adenosine residues (170-210) comprising the poly(A) tail at the 3'-end of the molecule, a finding which agrees well with the observation that most eukaryotic cytoplasmic mRNAs contain poly(A) segments of around 150 adenosine residues (11,14), while mRNA coded by mammalian and insect mitochondria contain much shorter poly(A) segments (14), and (d) the low percentage of mRNA capable of hybridizing to mitochondrial DNA. The non-complementary fraction presumably represents cytoplasmic mRNA bound to the membrane fraction. Our results suggest that $mRNA_I$ and $mRNA_{II}$ represent cytoplasmic mRNAs of two well-defined populations having molecular weights of 5.2×10^5, 3.6×10^5. This conclusion rests on the electrophoretic resolution of the membrane-bound mRNA in the presence or absence of formamide. Moreover, the removal of the poly(A) tail did not alter the homogeneous length distribution of the mRNA chains. It appears that within the cell, these mRNA molecules are probably associated with the membrane-bound "tight" polysomes (15,16). Indeed, membrane-associ-

ated fractions isolated in Mg^{++} containing buffer were found to contain massive quantities of cytoplasmic ribosomes, as determined by electrophoretic analysis of their RNA. Following the EDTA-buffer wash, the membrane preparations were found to be completely free of bound ribosomes. This washing procedure probably disrupted the polysomes, leaving behind naked mRNA molecules bound directly to the membrane. These results are in agreement with a model attributing direct association of the "tight" polysomal mRNA to the membrane, possibly via its poly(A) region, in addition to its indirect attachment via ribosomes and nascent products (17,18). A large quantity of membrane-associated mRNA had also been observed in bacterial spores, which may suggest that this association serves to stabilize the mRNA in dormant forms of different organisms (19). One would therefore look for a system in which differences between membrane-bound mRNA populations may be studied in dormant and developing forms. The Artemia system, we believe, is unique in having such characteristic differences between free and membrane-bound mRNA and may thus serve as a convenient system for the study of mRNA-membrane interactions.

ACKNOWLEDGEMENTS

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