Characterization of cell-free protein-synthesis systems from undeveloped and developing *Artemia* embryos*

Aurora MORENO, Raul MENDEZ and Cesar DE HARO†

Centro de Biología Molecular, Consejo Superior de Investigaciones Científicas–Universidad Autónoma de Madrid, Canto Blanco, 28049 Madrid, Spain

We have developed and characterized translationally active cell-free systems from *Artemia* embryos at different developmental times. The optimized lysates from 16 h-developed embryos incorporated radiolabelled amino acids into polypeptides for up to 120 min. The polypeptides synthesized ranged in M_r from 150 000 to 10 000, suggesting that the endogenous mRNA was capable of directing the synthesis of complete polypeptides. Similar results were obtained by using lysates from early developmental stages; even the cell-free system prepared from 1 h-developed embryos was partially active in protein synthesis. Furthermore, all these lysates were capable of re-initiation, as demonstrated by inhibition of initiation with the inhibitors edeine and 7-methylguanosine 5'-triphosphate. Because we found no endogenous protein-synthetic activity in the corresponding lysates from undeveloped embryos, we have used cell-free translation systems from 0 h- and 16 h-developed *Artemia* embryos to analyse the mechanisms limiting protein synthesis at very early developmental stages. Undeveloped-embryo lysates supplemented with nuclease-treated reticulocyte lysate were capable of translating endogenous mRNAs to give products with a wide spectrum of M_r values, but lysates of 16 h-developed embryos supplemented in this way were not further stimulated. The nuclease-treated lysate appeared to be unnecessary 5 h after resumption of development. These results suggested that a component(s) limiting translation in the undeveloped-embryo lysate was provided by the nuclease-treated reticulocyte lysate, and that this component(s) no longer limited protein synthesis after development. In view of these results, partially fractionated reticulocyte lysates were tested for restoration of protein-synthetic activity in the undeveloped embryo lysate. A high-salt ribosomal wash devoid of ribosomal subunits, which is considered a crude polypeptide-initiation-factor preparation, also restored translation activity in the undeveloped embryo lysate and made it capable of directing the synthesis of both endogenous mRNAs and exogenous (globin) mRNA.

INTRODUCTION

The development of the embryos of the brine shrimp *Artemia* may be arrested at the gastrula stage; the embryos enter a state of dormancy, characterized by an almost complete cessation of metabolic activity. The period of dormancy may last several years, but the cysts promptly resume development upon rehydration in a solution of adequate salinity (Finamore & Clegg, 1969). During pre-emergence development (0–8 h) of encysted embryos of *Artemia*, differentiation and morphogenesis occur in the complete absence of cell division (Nakanishi *et al.*, 1962) and DNA synthesis (Golub & Clegg, 1968). The inability of *Artemia* to synthesize purines *de novo* (Clegg *et al.*, 1967) and the observation that *Artemia* embryos are almost completely impervious to nucleic acid precursors (Warner & Finamore, 1967) present difficulties in studying RNA metabolism during this period of intense morphogenetic activity. By using [32 P]P_i as precursor of RNA synthesis, Susheela & Jayaraman (1976) had showed that synthesis *de novo* of heavy RNA species occurs only after the emergence of the embryos. Furthermore, [32 P]P_i- and [3 H]adenosine-incorporation studies indicate that RNA synthesis during development of *Artemia* is most intense immediately after hatching (McClellan & Warner, 1971). More recently, Möller *et al.* (1987) have found that levels of mRNA for eukaryotic elongation factor EF-1 α and ribosomal proteins eL12 and eL12' increase co-ordinately already after 4 h of development of the cyst. However, this behaviour does not reflect a more general resumption of transcriptional activity in the developing cyst, since equivalent amounts of total RNA and poly(A)-containing

mRNA have been obtained from dormant and 12 h-developed *Artemia* embryos (Wahba & Woodley, 1984). Moreover, the expression of histone (Bagshaw, 1982) and globin (Manning *et al.*, 1989) genes takes place only in the nauplius stage, after 24 h of development. Thus protein synthesis should play a major role in executing the early developmental programme, since any changes in biochemical parameters reflect differentiation of pre-existing cells (Hentschel & Tata, 1976). Indeed, protein synthesis has been shown to accompany cyst development (Golub & Clegg, 1968).

Brine-shrimp cysts are not at the same developmental stage as unfertilized eggs of sea urchins (Davidson *et al.*, 1982) or immature oocytes of the frog *Xenopus laevis* (Woodland, 1974; Wasserman *et al.*, 1982), but the biochemical events associated with resumption of protein synthesis are similar (Finamore & Clegg, 1969; Rinaldi & Monroy, 1969). Several mechanisms for regulating maternal mRNA expression during development have been proposed. Thus, protein synthesis may be regulated by the physiological state of the ribosomes, by the availability of functional mRNAs, by highly specific limitation of initiation factors, or by a combination of all three parameters. Investigation of the mechanisms promoting the stimulation of translation during *Artemia* development has been severely handicapped by its impermeability, which makes difficult studies *in vivo*, and by the lack of suitable cell-free protein-synthesizing systems.

Encysted embryos of the brine shrimp *Artemia* contain competent mRNA (Nilsson & Hultin, 1974; Grosfeld & Littauer, 1976; Sierra *et al.*, 1976), large quantities of 80 S ribosomes (Golub & Clegg, 1968; Hultin & Morris, 1968), elongation

Abbreviations used: m⁷GTP, 7-methylguanosine 5'-triphosphate; HSW, ribosomal high-salt wash; HSS, post-ribosomal high-speed supernatant.

* Dedicated to Severo Ochoa on the occasion of his 85th birthday.

† To whom reprint requests and other correspondence should be sent.

factors (Slobin & Möller, 1976), peptide-chain-termination factors (Reddington *et al.*, 1978) and aminoacyl-tRNA synthetases (Bagshaw *et al.*, 1970). Polysomes are observed within 15 min when hydrated cysts resume development (Hultin & Morris, 1968; Golub & Clegg, 1968). It is during this period that embryos must overcome factors that repress protein synthesis and select transcripts to be translated from the store of mRNAs. However, cell-free extracts of developed embryos were capable of elongation on pre-formed polysomes, but were unable to initiate protein synthesis on endogenous mRNA (Sierra *et al.*, 1974; Groner *et al.*, 1976). All these difficulties may explain why our current understanding of translational control during early embryonic development of the brine shrimp *Artemia* is so limited.

Here we prepare and characterize translationally active *Artemia* cell-free systems from embryos at different stages of development. They are capable of initiating protein synthesis on both endogenous and exogenous mRNAs and produce full-length polypeptide products. In addition, we present initial results suggesting that availability of initiation factor(s) is the primary control of translation in undeveloped embryos of *Artemia*.

MATERIALS AND METHODS

All reagents were from Sigma Chemical Co., except as follows. RNasin, the RNAase inhibitor from human placenta, was from Promega Corp., Madison, WI, U.S.A. Soybean trypsin inhibitor, bovine lung aprotinin and *Staphylococcus aureus* nuclease were from Boehringer Mannheim. Protein was determined by the method of Lowry *et al.* (1951).

Preparation of undeveloped *Artemia* embryos

All operations were conducted at 0–4 °C, and amounts given were per 100 g (dry wt.) of encysted *Artemia* sp. embryos obtained from San Francisco Bay Brand (Newark, CA, U.S.A.). pH values were at 25 °C. The cysts were stirred for 20 min with 900 ml of ice-cold 5% NaClO, the embryos were allowed to settle and the turbid supernatant was removed by suction. This treatment removes the granular parts of the shell without damaging the embryo, and produces a preparation that can be gently disrupted. The cysts were washed 6–10 times with 15 litres (total) of ice-cold distilled water until the NaClO was completely removed, and then once with 10 mM-Hepes buffer, pH 7.6, and collected by filtration through a Millipore stainless-steel support screen. If the temperature is maintained at 0–4 °C during these operations, the embryos remain dormant.

Development of cysts

We used a modification of the method described by Sierra *et al.* (1974). The cysts after the last washing with distilled water were suspended in 10 litres of artificial sea water (Warner *et al.*, 1979) and incubated at 28–29 °C for 16 h, with vigorous aeration to ensure adequate oxygen supply. Little or no hatching occurred during this time. In some cases, embryos of early developmental stages were collected (10 g dry wt.) as specified in the legend of the corresponding Figure. The mixtures were chilled in ice, and the embryos were washed 5–6 times with 20 litres (total) of ice-cold distilled water until the washes were virtually salt-free, then once with 10 mM-Hepes buffer, pH 7.6, and collected by filtration as above.

Preparation of extracts from undeveloped and developing embryos

The sedimented embryos were suspended in a minimal volume of stock solutions of Hepes, pH 7.6, dithiothreitol, EGTA and

soybean trypsin inhibitor, to give final concentrations of 10 mM, 3 mM, 6.5 mM and 0.13 mg/ml respectively, except for the undeveloped embryos, which were resuspended in 0.5 vol. of the same buffer. Each cell suspension was transferred to a chilled mortar and thoroughly ground. The broken embryos were homogenized in a tight-fitting Dounce homogenizer (15 strokes), and then $\frac{1}{2}$ vol. of buffer containing 100 mM-Hepes, pH 7.6, 1 M-potassium acetate, 10 mM-magnesium acetate and 30 mM-dithiothreitol was added. The homogenate was centrifuged for 20 min at 20000 g; the top 75% of the clear supernatant was removed and adjusted to 64 units/ml in creatine kinase (EC 2.7.3.2) and 10% in glycerol. This fraction, which will be referred to as the embryo lysate, was kept frozen at –70 °C until use.

Amino acid incorporation assays with *Artemia* extracts

For endogenous mRNA translation in *Artemia* systems, the reaction mixture (20 μ l) contained 30 mM-Hepes buffer (adjusted to pH 7.6 with KOH), 80 mM-potassium acetate, 1 mM-magnesium acetate, 0.1 mM-spermidine, 15 mM-phosphocreatine, 0.51 unit of creatine kinase, 15 μ Ci of L-[³⁵S]methionine (sp. radioactivity 1020–1475 Ci/mmol; Amersham International), 25 μ M each of the remaining 19 unlabelled L-amino acids, 10 units of RNasin and 8 μ l (about 1.2 A_{280} units) of *Artemia* extract. Incubations were for 60 min at 28 °C. Samples of the reaction mixture (5 μ l) were measured into 1.5 ml of a solution containing 0.33 M-NaOH, 0.16 M-H₂O₂ and 2.2 mM-methionine. After 15 min at 40 °C, protein was precipitated with 0.5 ml of 50% trichloroacetic acid. The samples were then placed on ice for 15 min and collected on GF/C filters (Whatman). The filters were washed with 15 ml of cold 5% trichloroacetic acid and dried. Radioactivity was measured in a liquid-scintillation spectrometer.

Preparation of reticulocyte components

The preparation of reticulocyte lysates from phenylhydrazine-treated rabbits has been described (Palomo *et al.*, 1985). To obtain post-ribosomal supernatant and polysomes, the lysate was centrifuged in a T865 rotor (Sorvall) for 150 min at 55000 rev./min (219000 g). The preparation of ribosomal high-salt wash (HSW) from rabbit reticulocyte polysomes has been described (Maroto & Sierra, 1989). Globin mRNA was prepared by phenol/chloroform/3-methylbutan-1-ol (50:49:1, by vol.) extraction of pelleted reticulocyte polysomes. The RNA was ethanol-precipitated, and the reticulocyte poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose Type 7 (Pharmacia) as described by Aviv & Leder (1972). The preparation of micrococcal-nuclease-treated reticulocyte lysate has been described (Jackson & Hunt, 1983). For preparation of the ribosomal high-salt wash (HSW) and post-ribosomal supernatant (HSS), reticulocytes were washed and lysed with solutions containing 1 mM-EGTA, 50 μ M-haemin, 0.5 mM-phenylmethanesulphonyl fluoride, 0.1 mg of soybean trypsin inhibitor/ml and 8 μ g of aprotinin/ml.

Analysis of translation products by PAGE

Protein-synthesis assays (20 μ l) were carried out as described above. After incubation (1 h), portions (usually 8 μ l) of the reaction mixtures were made 62.5 mM in Tris/HCl, pH 6.8, 2% in SDS, 10% in glycerol and 5% in 2-mercaptoethanol, and the [³⁵S]methionine-labelled polypeptides were analysed by SDS/PAGE (12.5% acrylamide, 0.34% bisacrylamide) as described by Laemmli (1970), followed by fluorography. Autoradiographs were prepared by exposure of Kodak X-Omat S film to the gels for a time specified in the legend of each Figure. For gel

calibration the following M_r markers were used: phosphorylase b (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20000) and α -lactalbumin (14400).

RESULTS

Preparation of translationally active lysates from developing *Artemia*

To obtain cell-free extracts from *Artemia* embryos at various developmental times, we first used the conditions outlined by Maroto & Sierra (1988) for preparation of lysates from *Drosophila melanogaster* embryos. It was necessary, however, to introduce some modifications to obtain translationally active lysates from early developmental stages, as described in the Materials and methods section.

Several conditions appeared essential for preparation of the *Artemia* lysates. (a) The homogenization procedure. This was the same for all developmental stages, from 0 to 16 h. Grinding in a chilled mortar before homogenization was crucial, because a stronger initial rupture yielded inactivate lysates from developed embryos, whereas lighter grinding methods were ineffective for the cyst. (b) Because of high levels of endogenous proteinases (Warner, 1987), a mixture of proteinase inhibitors was included in the homogenizing medium. This mixture enhanced initiation on endogenous mRNA. (c) The final concentration of the lysates (about 130 A_{280} units/ml; $A_{280}/A_{260} \sim 0.7$ –0.8) is also critical.

Fig. 1. *Artemia* lysates initiate new polypeptide-chain synthesis

(a) Time course of incorporation of [³⁵S]methionine into protein by *Artemia* extracts prepared as described in the Materials and methods section from undeveloped (○) or 16 h-developed (●) embryos. The effect of adding the initiation inhibitors edeine (b) and m⁷GTP (c) to *Artemia* extracts prepared from undeveloped (○) or 16 h-developed (●) embryos was studied. Protein synthesis was measured by [³⁵S]methionine incorporation for 60 min; each translation was carried out at a different edeine (b) or m⁷GTP (c) concentration. (d) Translation of endogenous mRNAs in *Artemia* cell-free translations systems prepared from embryos of different developmental times. RNA content in each extract was estimated from that obtained when RNA from corresponding extracts was isolated by phenol extraction. Each of the lysates was incubated in the absence (●) or presence (○) of 2 μM-edeine in the translation medium. Protein synthesis was measured by [³⁵S]methionine incorporation for 60 min.

This explains why we had to dilute the undeveloped embryos with 0.5 vol. of buffer. (d) Owing to the endogenous RNAases, the RNAase inhibitor from human placenta, RNasin, was included in the standard cell-free translation assay.

Optimization of the lysates

Studies were carried out with 16 h-developed *Artemia* extracts to determine the optimal conditions for translation. In most of the experiments the incubation was for 60 min, although the incorporation of [³⁵S]methionine into protein may continue for up to 120 min (Fig. 1a). The final ion concentrations selected for preparation of the lysates were 100 mM-K⁺ and 1 mM-Mg²⁺ (see above). In the assay, addition of extra K⁺ and Mg²⁺ ions over those already contributed by the extract was needed, and the optimal concentrations were found to be 80–100 mM and 0.5–1.0 mM respectively (results not shown). Incorporation decreased rapidly at concentrations above or below the optima. Addition of a mixture containing 19 amino acids to the system did not significantly affect translation. Nevertheless, amino acids were routinely added at a final concentration of 25 μM each. Spermidine, which slightly enhanced the rate of [³⁵S]methionine incorporation, was added at a final concentration of 0.15 mM. In the presence of 250 μg of creatine kinase/ml the incorporation of radiolabel increased linearly with the addition of phosphocreatine up to an optimum of 15 mM. Addition of exogenous ATP with equimolar Mg²⁺ concentrations did not affect translation; thus, the endogenous ATP pool appeared to be sufficient. The optimum temperature was 28 °C; incorporation decreased significantly at temperatures above 35 °C and below 20 °C. The incorporation was proportional to the amount of extract added; optimal activity was obtained with about 1 mg of protein, which corresponded to 40 % of the assay volume, in our case. Finally, RNasin was added at various concentrations between 0 and 2 units/μl, and the optimum was found to be approx. 0.5–1 unit/μl. In contrast with the cell-free systems described by Lopo *et al.* (1989) for the sea urchin *Strongylocentrotus purpuratus*, where RNasin had to be added before homogenization, we found that it was possible to prepare lysates active in translation from any developing stage of *Artemia* if RNasin was just included in the cell-free-translation assay. This optimized assay was used for endogenous translation with *Artemia* lysates from each developmental stage.

It should be emphasized that, as in most of the cell-free systems so far described, to obtain fully active lysates consistently it is also necessary to work in a scrupulously clean environment, with use of baked glassware, sterile plasticware and sterile solutions.

Characterization of the lysates

Translation with development. Under the conditions used for the 16 h-developed *Artemia* extracts, we obtained completely inactive lysates from undeveloped embryos (Fig. 1a, ○), but there was small, though highly reproducible, activity with lysates from 1 h-developed lysates. The rate of incorporation increased significantly at later stages of development (Fig. 1d); with lysates from 5 h-developing embryos, the incorporation of [³⁵S]methionine reached a maximum.

The lysates initiate protein synthesis. A cell-free translation system is most useful if it is able to initiate translation. Previously, Sierra *et al.* (1974), using a fractionated system with ribosomes from developed embryos and cytosol from undeveloped or developed embryos, and Groner *et al.* (1976), using an *Artemia* cell-free translation system, observed that the endogenous protein synthesis was mostly due to the elongation of preformed polypeptide chains. To see whether initiation of protein synthesis on endogenous mRNA occurred *in vitro*, translation assays were

Fig. 2. Translation products of the *Artemia* lysates

(a) SDS/PAGE of proteins extracted from 0 h (undeveloped embryos) and 1 h-, 3 h-, 5 h-, 7 h-, 10 h-, 13 h- and 16 h-developed embryos (lanes 1–8 respectively) and marker proteins (lane 9): phosphorylase *b* (M_r 94000); BSA (M_r 67000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 30000); soybean trypsin inhibitor (M_r 20000); α -lactalbumin (M_r 14400). Samples (in a range of 120–140 μ g of protein) were analysed by SDS/PAGE (12.5% gel). The gel was then stained with Coomassie Brilliant Blue. (b) and (c) The lysates from different developmental times of *Artemia* embryos were incubated for 60 min as described in the Materials and methods section. Samples containing either the same concentrations of extracts (15 μ g/ml) (b) or the same amount of radioactivity (5.6×10^4 c.p.m. of incorporated [35 S]methionine) (c) were analysed by SDS/PAGE. The gels were dried and autoradiograms prepared by a 7-day (b) or 4-day (c) exposure. A photograph of the autoradiogram is shown. The positions of migration of M_r markers are shown on the right. Arrows show the position of the M_r -28000 and 27000 polypeptides. (b) Lane 1, undeveloped-embryo lysate (4.8×10^3 c.p.m.); lanes 2–8, 1 h- (25×10^3 c.p.m.), 3 h- (5×10^4 c.p.m.), 5 h- (15×10^4 c.p.m.), 7 h- (20×10^4 c.p.m.), 10 h- (30×10^4 c.p.m.), 13 h- (25×10^4 c.p.m.) and 16 h- (20×10^4 c.p.m.) developed-embryo lysates respectively. (c) Lanes 1–5: 1 h-, 3 h-, 5 h-, 10 h- and 16 h-developed-embryo lysates respectively.

performed in the presence of specific inhibitors of initiation such as edeine (Fig. 1b) or 7-methylguanosine 5'-triphosphate (m⁷GTP) (Fig. 1c). Edeine inhibits initiation by blocking the joining of the 60 S subunit with the 48 S initiation complex; m⁷GTP inhibits cap-dependent initiation. With both inhibitors there was a partial but significant decrease in the total amount of incorporation in 16 h-developed lysates (Figs. 1b and 1c). The residual incorporation in the initiation-inhibited lysates may be due to continued elongation on preformed polysomes in the lysates. About 45–60% of the incorporation after 60 min incubation with the 16 h-developed lysate is due to new initiation. The same degree of initiation was obtained with lysates of different stages of development (Fig. 1d), except for the undeveloped embryos, where the minimal residual incorporation was not prevented by initiation inhibitors.

Translation products. The optimized lysates from different stages of development of *Artemia* embryos incorporate radio-labelled amino acids into polypeptides in the range of M_r 10000 to > 150000 , as measured by SDS/PAGE (Figs. 2b and 2c). This is particularly evident with 5 h-developed embryos, when the translational machinery becomes fully active. Thus the mRNA in the lysates appears to be intact and capable of directing the synthesis of complete proteins. With inclusion of proteinase inhibitors, proteolytic degradation is not a problem in our optimized lysates. Lysates from earlier-developing embryos had a much lower translational activity (Fig. 2b, lanes 2 and 3) whereas lysates from undeveloped embryos were completely inactive (Fig. 2b, lane 1). It should be noted that the dormant embryos have both poly(A)-containing and poly(A)-deficient mRNAs, both of which can be actively translated in cell-free

systems (Nilsson & Hultin, 1974; Grosfeld & Littauer, 1976; Sierra *et al.*, 1976; A. Moreno & C. de Haro, unpublished work).

Previously, Grosfeld & Littauer (1976) had obtained evidence that the pattern of soluble proteins extracted from dormant cysts and 22 h-developed *Artemia* are similar, but not identical. The same results have been obtained in the present study, with lysates of dormant and any developing stage of *Artemia* embryos (Fig. 2a).

The spectrum of products synthesized by lysates at all developmental stages is similar (Fig. 2b), but shows some qualitative differences. Some of these differences accurately reproduce certain molecular aspects of development of *Artemia* described in the literature. Thus the dormant cysts contain two predominant proteins in the M_r -20000–30000 range, which disappear almost completely after hatching. One of these low- M_r cyst-specific *Artemia* proteins has been studied extensively by De Herdt *et al.* (1980) and by Slobin (1980), who has named it 'artemin'. Artemin is an RNA-binding protein which aggregates to form the abundant 19 S complexes which are found in the cyst cytoplasm. It has been assigned a tentative role in the maintenance of the integrity of the dry cyst, possibly as a cytoskeletal component (De Herdt *et al.*, 1980) or in the control of recommended metabolic activity following rehydration (Grosfeld & Littauer, 1976). In our lysates, these proteins (M_r 27000 and 28000, indicated by arrows in the Figures) are evident at 5 h of development and earlier if the lysates are supplemented, as shown below. It has also been described that the relative amount of a protein of M_r 42000, co-migrating with purified chick actin marker on SDS/PAGE, increases 3-fold during cyst development (Grosfeld & Littauer, 1976). Here too, translation products

Fig. 3. Response of the *Artemia* lysates to nuclease-treated rabbit reticulocyte lysates

Undeveloped-embryo (a) or 16 h-developed-embryo (b) lysates were partially replaced with various amounts of nuclease-treated reticulocyte lysate (RL) (as shown), and incorporation of [35 S]methionine at 60 min was determined as described in the Materials and methods section. Samples (8 μ l) were analysed by SDS/PAGE. The gels were dried and autoradiograms prepared by a 7-day (a) or 2-day (b) exposure. The positions of migration of M_r markers are shown on the right.

Fig. 4. The translational machinery appears to be limiting during early developmental stages of *Artemia*

Nuclease-treated reticulocyte lysate (RL_{Nuc}) or HSW from reticulocyte lysate were added to lysates of undeveloped embryo (a) or of 1 h-, 3 h- and 5 h-developed embryo (b), and analysed as described in the legend to Fig. 3. The autoradiograms were prepared by a 14-day (a) or 12-day (b) exposure. Arrows show the position of the M_r -28000 and -27000 polypeptides. The incorporation of [35 S]methionine [in c.p.m. ($\times 10^{-3}$) per sample] is indicated below in parentheses for each lane. (a) Undeveloped-embryo lysates. In addition, the samples contained the following: lane 1, none (3.5); lane 2, RL_{Nuc} , 3 A_{260} (37.2); lane 3, RL_{Nuc} , 4 A_{260} units (27.2); lane 4, HSW, 0.3 A_{260} unit (27.1). (b) 1 h- (lanes 1-3), 3 h- (lanes 4-6) and 5 h- (lanes 7-9) developed-embryo lysates. In addition, the samples contained the following: lanes 1, 4 and 7, none; lanes 2, 5 and 8, RL_{Nuc} , 3 A_{260} units; lanes 3, 6 and 9, HSW.

directed by *Artemia* mRNA *in vitro* show the same distinct developmental specificity.

On the other hand, we found that one of the cyst-specific proteins is the major product synthesized at the beginning of development (Figs. 2b and 2c), but the lower- M_r protein is only

actively synthesized in lysates at 5 h of development, when the translational machinery is fully active, although both mRNAs are present in a functionally intact form in the dormant cyst lysates (Fig. 3a, and results not shown). This result is in good agreement with the notion that part of the mRNA exists in the

Fig. 5. Effect of adding the initiation and elongation inhibitors (eideine and sparsomycin respectively) to *Artemia* lysates

Undevolved embryos supplemented with nuclease-treated reticulocyte lysate (RL_{Nuc}) (a), or 16 h-developed-embryo lysates (b), were incubated as described in the Materials and methods section, except that the initiation inhibitor eideine or the elongation inhibitor sparsomycin were included in the translation medium, when present. Samples (8 μ l) were analysed by SDS/PAGE. The gels were dried and autoradiograms prepared by a 12-day (a) or 2-day (b) exposure. The incorporation of [35 S]methionine [in c.p.m. ($\times 10^{-3}$) per sample] is indicated below in parentheses for each lane. (a) Undevolved-embryo lysate. In addition, the samples containing the following: lane 1, none (3.9); lane 2, RL_{Nuc}, 3 A_{260} units (36.3); lane 3, RL_{Nuc} and 2 μ M-eideine (3.5); lane 4, RL_{Nuc} and 4 μ M-sparsomycin (2.6). (b) 16 h-developed-embryo lysate. In addition, the samples contained the following: lane 1, none (188.0); lane 2, eideine (92.1); lane 3, sparsomycin (8.2).

dehydrated cysts in a 'masked' form of mRNA-protein (Grosfeld & Littauer, 1975). The synthesis of both cyst-specific proteins decreased markedly in 13–16 h-embryo lysates, probably as a result of a parallel decline in the content of cyst-specific mRNAs observed when total RNA from corresponding extracts was phenol-extracted and translated in a messenger-dependent reticulocyte lysate (results not shown).

The translational machinery is limiting during early development

Our results (the present paper; A. Moreno & C. de Haro, unpublished work) and previously those of others (Nilsson & Hultin, 1974, 1975; Grosfeld & Littauer, 1975; Sierra *et al.*, 1976) clearly established the existence of preformed mRNAs in dry *Artemia* cysts which are competent as templates for polypeptide synthesis. Then, if a translational component (or components) other than mRNA is limiting in the undeveloped *Artemia* lysates, supplementing these lysates with whole or partial fractions of an active translation system may provide sufficient amounts of the missing component to stimulate the lysates. The same approach had been applied with success for the *S. purpuratus* lysates (Lopo *et al.*, 1988). To test this possibility, the undeveloped *Artemia* lysates were supplemented with nuclease-treated rabbit reticulocyte lysate, which contains no mRNA but is otherwise intact with respect to the translational machinery.

When the undeveloped-embryo lysates were supplemented with nuclease-treated reticulocyte lysate, active translation of the

preformed mRNAs in dry *Artemia* cysts was observed (Fig. 3a). Interestingly, higher translational efficiency was obtained with a small amount (20%) of undeveloped-embryo lysates and 60% reticulocyte lysate. Under these conditions the two 'cyst-specific' proteins were synthesized in large amounts. When the amount of undeveloped lysates in the mixture was increased (40%), keeping constant the amount of reticulocyte lysate (60%), the incorporation of radiolabel into protein decreased, in agreement with the idea that the dormant cysts contain a protein inhibitor of protein synthesis which disappears during development (Huang & Warner, 1974). On the other hand, when increasing amounts of reticulocyte lysate (40% and 60%) was added to a saturating amount of 0 h lysate (40%), the expression of endogenous *Artemia* mRNAs was strongly increased (Fig. 3a, compare 40% Und. plus 40% RL with 40% Und. plus 60% RL). The synthesis of the higher- M_r cyst protein (28000; p 28) seems to be saturated with the lower amount of reticulocyte lysate (Fig. 3b, lanes 6 and 10), although, when the amount of reticulocyte lysate present is too low (20%), even the expression of p 28 mRNA is markedly diminished (Fig. 3b, lane 7). The presence of the polypeptides in the M_r -20000–30000 range in Fig. 3a (lanes 4 and 9) may be due to the expression of some minor and nuclease-resistant mRNAs from reticulocyte lysates. When the *Artemia* extracts are present, the competition between mRNAs from both sources could negatively affect the expression of the reticulocyte mRNA species.

In this kind of experiment it is not possible to discard the possibility that the reticulocyte translational machinery, which is more efficient, is supplanting that of *Artemia*. Thus, with a small amount (20–40%) of 16 h-developed lysate, stimulation is also observed (Fig. 3b), but no effect was found when the reticulocyte lysate was added to an optimized cell-free translation system from 5 h- (see Fig. 4b) or 16 h- (results not shown) developed *Artemia*. These results suggest that the nuclease-treated lysate provides a component (or components) that limits translation in the undeveloped lysate, but no longer limits protein synthesis after development.

Limiting translation component may be an initiation factor(s)

In order to find out whether the limiting translation component(s) in undeveloped lysates were the ribosomal subunits or soluble elements which are present in the reticulocyte lysate, we prepared high-speed supernatant (HSS), ribosomal high-salt wash (HSW) and washed polysomes from reticulocyte lysates. Each of these fractions was then used to supplement the undeveloped-embryo lysates. The addition of HSW, which mainly contains initiation factors, promoted activation of endogenous mRNA translation similar to that seen with the nuclease-treated lysate (Fig. 4a, lane 4).

Addition of HSS from reticulocytes stimulated the undeveloped-embryo lysates only marginally, whereas the addition of washed reticulocyte polysomes did not affect the endogenous *Artemia* mRNA translation, although we found newly synthesized proteins mainly owing to continued elongation of pre-formed polysomes (results not shown). This result may suggest that the undeveloped *Artemia* lysates contain sufficient amounts of tRNAs, aminoacyl-tRNA synthetases, and elongation and termination factors.

The translational machinery appears to be limiting only during the early developmental stages of *Artemia*. Thus the addition of either nuclease-treated reticulocyte lysate or HSW markedly stimulated translation of the *Artemia* mRNAs in 1 h or 3 h lysates (Fig. 4b), but with 5 h-developing lysates, which appear to be fully active in translation (Figs. 1d and 2b), the overall amino acid incorporation was not affected (Fig. 4b, lanes 7–9).

Protein synthesis in the undeveloped-embryo lysates supplemented with nuclease-treated reticulocyte lysates was essentially

Fig. 6. Lysates from undeveloped *Artemia* supplemented with HSW utilize exogenous mRNA

Purified globin mRNA ($1 \mu\text{g}/20 \mu\text{l}$ assay) was added to undeveloped embryo lysate supplemented with HSW from reticulocyte lysate, and the mixtures were incubated for protein synthesis as described in the Materials and methods section. Samples ($8 \mu\text{l}$) were taken for SDS/PAGE, and the autoradiogram was prepared by a 18-day exposure. Lanes 1–4, without globin mRNA; lanes 5–8, with globin mRNA. Lanes 1 and 5, undeveloped-embryo lysate alone; lanes 2 and 6, with $25 \mu\text{g}$ of HSW; lanes 3 and 7, with $50 \mu\text{g}$ of HSW; lanes 4 and 8, with $100 \mu\text{g}$ of HSW.

abolished by edeine (Fig. 5a). This indicates that most of the translation was due to initiation events *in vitro*, and that there were few pre-formed polysomes capable of run-off, as expected for the undeveloped embryos (Hultin & Morris, 1968; Clegg & Golub, 1969). However, in the 16 h-embryo lysates, there is appreciable incorporation in the initiation-inhibited lysates (Fig. 5b). This incorporation is likely to be due to continued elongation of preformed polysomes present when the development is resumed (Golub & Clegg, 1968). As expected, protein synthesis was blocked in both lysates by sparsomycin, an inhibitor of polypeptide-chain elongation (Figs. 5a and 5b). These results suggest that, in the dormant-cyst lysates, but not in the developed-embryo lysates, the translational machinery is limiting at the level of initiation. The potentiating effect of the reticulocyte HSW preparation on the translation of endogenous *Artemia* cyst mRNA in the undeveloped lysates raises the question of the identity of the factor(s) that are present in the reticulocyte extract but are absent from or limiting in the *Artemia* cell-free system. Further work on the roles of individual initiation factors or various combinations of them is required.

Finally, it is widely thought that part of the preformed mRNA is preserved in the cysts in a latent form, unavailable for translation, possibly by the presence of associated inhibitory or 'masking' proteins (Nilsson & Hultin, 1974; Grosfeld & Littauer, 1975). Masked mRNA has been demonstrated not only in *Xenopus* oocytes (Richter, 1987) but also in the seeds of several plants (Marcus & Feeley, 1966; Chen *et al.*, 1968), which shows striking analogies with *Artemia* cysts. A carefully regulated removal of this masking factor during early development allows the mRNA to be translated while no transcription of new poly(A)-containing mRNA occurs in nuclei (Felicetti *et al.*,

1975; Susheela & Jayaraman, 1976). If mRNA is a limiting component, supplementing the undeveloped lysates with exogenous mRNA should result in an increase in the total amino acid incorporation. In these studies, we used total reticulocyte poly(A)-containing RNA as a source of globin mRNA. Protein synthesis in the undeveloped-embryo lysate is only slightly stimulated by the exogenous mRNA (Fig. 6), suggesting that, in fact, message availability is not the primary control of translation in the undeveloped embryo. When the lysate was supplemented with increasing amounts of reticulocyte HSW, it was capable of translating globin mRNA as well as its own mRNAs (Fig. 6).

DISCUSSION

In this paper we characterize cell-free translation systems from undeveloped embryos and embryos at several developmental stages of *Artemia*. The mechanisms by which gene expression is regulated at the levels of transcription and translation during early embryo development of eukaryotic systems is a major question in molecular biology. Indeed, at the level of translational control, it is not clear how specific messages from the mRNA and mRNP pool are recruited. In addition, it is not known how initiation-factor activities are modulated.

Several laboratories have reported results from work with cell-free translation systems derived from sea-urchin eggs and zygotes (Winkler *et al.*, 1985; Lopo & Hershey, 1985; Colin *et al.*, 1987; Hansen *et al.*, 1987; Lopo *et al.*, 1988). Patrick *et al.* (1989) have prepared extract from *Xenopus* oocytes and eggs with translational activity comparable with that of intact cells (Richter *et al.*, 1982). These extracts have provided valuable information on the molecular mechanisms of stimulation of protein synthesis in early development. Two important aspects have been considered; first, the role of individual initiation factors in mediating the response to fertilization, and second, the mechanisms regulating the recruitment of the cellular mRNA for translation. Nevertheless, oocytes, which have not yet started to differentiate, are primed protein-synthesizing systems and may not require specific factors to select different classes of mRNA. On the other hand, developing *Artemia* embryos contain large amounts of stored mRNA and must select and translate mRNA appropriate for each particular stage of development. There are important differences between oocytes and dormant *Artemia* embryos. Thus it is accepted that in *Artemia* cellular differentiation does indeed occur in the absence of any cell division or DNA synthesis throughout the pre-emergence development stage (0–8 h) (see review by Clegg & Conte, 1980). It is not known when RNA synthesis resumes after cyst activation. It is a general idea that the period of most intense RNA synthesis occurs immediately after hatching of the nauplii, although the synthesis of a few and probably specific mRNAs starts earlier during development of *Artemia*. Thus *ras* and ubiquitin mRNAs are stored in large amounts in the cyst, and their levels increase at 5 h of development (Sastre *et al.*, 1989). In any case, it appears reasonable to postulate that the initial development (0–4 h) must proceed in the absence of RNA synthesis. Therefore a differentiating system of this kind is ideal for the study of translation regulation of mRNA activity and the influence this may have on development.

Before emergence, *Artemia* embryos are impermeable to RNA and protein precursors (Clegg, 1966; de Chaffoy *et al.*, 1978), and experiments *in vivo* with developing *Artemia* cysts, before hatching, cannot be performed. On the other hand, up to now, all the cell-free extracts obtained from this organism were rather inefficient at initiation of new polypeptide chains (Sierra *et al.*, 1974; Groner *et al.*, 1976). These limitations may explain in part the difficulties so far encountered in trying to understand translational control in the early development of *Artemia*.

The *Artemia* lysates that we describe in this paper meet important criteria of usefulness: (1) they mimic the intact cell, in some molecular aspects of development; (2) they produce full-length polypeptides, i.e. they do not display premature termination; (3) they are capable of initiation of new polypeptide chains.

We are successfully using these cell-free translation systems, and they should provide a means for identifying and analysing individual reactions of protein synthesis and for studying in depth some molecular mechanisms by which gene expression is regulated at the translational level during early embryonic development of *Artemia*.

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