Termination of Quiescence in Crustacea

THE ROLE OF TRANSFER RNA AMINOACYLATION IN THE BRINE SHRIMP ARTEMIA

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In quiescent embryos of the brine shrimp Artemia, the level of aminoacylation of transfer RNAs is low. During resumption of development the charging level of transfer RNAs increases, concomitant with the activation of protein synthesis. The total level of charging rises dramatically from an average of 4% to 50% within a period of 24 h of development. The restriction of in vitro translation of the quiescent embryo extract can be partially released by the addition of charged aminoacyl-tRNA, which apparently starts the flow of ribosomes into polyribosome structures. Complete reactivation of translation by aminoacyl-tRNA occurs when mRNA from preformed mRNA-ribosome complexes, like the polyribosomes extracted from developing embryos or poly(U)-programmed ribosomes, are offered to quiescent embryo extracts. With respect to the mechanism of in vitro recharging of tRNAs, we observed that the level of several aminoacyl-tRNA synthetases increase during development. Methionyl-tRNA synthetase rises more than 10-fold. In the case of valyl-tRNA synthetase, the activation is lower and shown to be due to the de novo synthesis of its mRNA and the corresponding protein product as well. We conclude that protein synthesis and thereby the gradual animation of cryptobiotic Artemia embryos is determined to a large extent by the rate by which aminoacyl-tRNAs are replenished during development at both the initiation and elongation level.

Multicellular organisms like the crustacean Artemia can survive long periods of environmental stress by entering a cryptobiotic stage (see Refs. 1–4 for reviews). Tissue culture cells can protect themselves against adverse growing conditions by entering the Go phase of the cell cycle. In both cases, the rate of protein synthesis is reduced to a low level and its reactivation is a prerequisite for the cells to re-enter the cell cycle (5, 6). For a better understanding of the processes that determine cell growth and division, it is important to know how protein synthesis can be regulated.

Upon re-immersion of the quiescent dehydrated Artemia embryo in sea water, development quickly resumes. After a defined period of pre-emergence development, a free-swimming nauplius with distinct morphological features emerges from its shell. At the start of this period, protein synthesis and transcription resume quickly, both occurring in the absence of DNA synthesis or cell division (1, 7).

After 30 years of research on the re-activation of translation in Artemia, it is clear that quiescent and developing embryos contain approximately equal amounts of ribosomes (8), mRNA (9), initiation factors (10), elongation factors (11), and termination factors (12), which are all equally active in in vitro translation assays. At the onset of development, a slow but definite shift from 80 S ribosomes to polysomes can be observed (5, 13). However, extracts from quiescent embryos are inactive in translation of their endogenous mRNAs (14). This paradox has led several investigators to search for specific inhibitors and activators of translation in extracts from non-developing and developing embryos, respectively (15). Until now, none of these approaches has provided a definite answer.

Since ribosomes, factors, and mRNAs appear to be normal in the quiescent embryo, we have explored the level of charged tRNA during development. Although the amount of tRNA as such does not change significantly during development (16), the degree of tRNA aminoacylation has until now received little attention. The results of such studies are presented here.

EXPERIMENTAL PROCEDURES

Culturing Conditions—Dried Artemia embryos (San Francisco Bay Brand Inc., San Francisco, CA) were washed with 2% NaOCl as described (17) and either used directly (quiescent embryos) or cultured for the time indicated in aerated artificial sea water at 27 °C (developing embryos). Under these conditions, approximately 80% of the embryos emerge from their shells within 20 h.

In Vivo tRNA Charging Levels—Dried embryos (25 g) were cultured for the time indicated and washed with distilled water and 50 mM NaAc, pH 4.5, 150 mM NaCl. The volume was adjusted to 75 ml with the same buffer, 50 ml of phenol (saturated with the same buffer) was added, and this mixture was homogenized three times for 45 s with a Polytron homogenizer. Upon centrifugation for 12 min at 3000 × g in a Beckman JA 10 rotor, the aqueous (upper) phase was precipitated with ethanol (18). The dried pellet was dissolved in 15 ml of 10 mM NaAc, pH 4.5, and lithium chloride was added to a concentration of 0.8 M, followed by centrifugation for 20 min at 9000 × g in a Sorvall SS 34 rotor. The tRNA present in the supernatant was precipitated with ethanol and then dissolved in 5 mM NaAc, pH 4.5, 0.5 M NaCl, and 10 mM MgCl2. Complete separation of tRNA from mRNA was achieved by gel filtration on a Superose 12 FPLC column in the same buffer. The tRNA eluting at the position of tRNA from brewers' yeast (Boehringer Mannheim) was pooled and precipitated with ethanol. The dried pellets were dissolved in 10 mM ammonium acetate, pH 4.5, and A260 was measured. Samples containing 200 μg (3.7 A260 units) of tRNA were adjusted to pH 8 by addition of ammonium carbonate, pH 8, to 100 mM and incubated for 1 h at 37 °C to achieve the complete deacylation of aminoacyl-tRNA (19). Released amino acids were separated from tRNA by ultrafiltration (Microcon-3, Amicon), modified by dimethylaminobenzene sulfonyl chloride treatment, separated by reverse phase chromatography on a C18 high performance liquid chromatography column (100RP18e, Merck) and quantified at 346 nm by comparison to known amounts of amino acids (20). When the tRNA samples were incubated at pH 4.5 instead of 8, no significant amount of amino acid was found in the ultrafiltrate, indicating that our tRNA preparations are essentially devoid of contaminating free amino acids.

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Preparation of Extracts—Cell-free extracts from quiescent and developing Artemia embryos were prepared precisely as described in Ref. 14. These extracts, referred to as "embryo lysate," were used as such only for the in vitro translation experiments of Fig. 1. In the other experiments, the endogenous amino acid and nucleotide pools were removed from S30 extracts by passage of 2 ml of embryo lysate through a G-25 column (20 ml), previously equilibrated in 20 mM Hepes, pH 7.6, 100 mM potassium acetate, 1 mM magnesium acetate, 6 mM DTE, and 10% glycerol. Void-volume fractions, termed S30 extract, were pooled and stored in aliquots at -80 °C. The 100,000 × g supernatant (S100) and 0.5 M KCl-washed (poly)ribosomal fraction as used in Fig. 4 were obtained from S30 extracts as described.

Preparation of [3H]Val-labeled Aminoacyl-tRNA—Aminoacyl-tRNA from developing embryos was first deacylated as described above and separated from released amino acids by gel filtration on Superose 12. Next, 1 mg of the deacylated tRNA was recharged by an incubation for 30 min at 27 °C with 10 mM Tris-Cl, pH 7.4, 5 mM MgCl2, 150 mM KCl, 0.5 mM DTE, 10 mM ATP, 10 mM phosphocreatine, 25 units of creatine phosphokinase, 0.1 mM each amino acid, and in the presence of 400 μg of S100 protein from developing embryos and 200 μCi of [3H]Val. The aminoacyl-tRNAs were re-extracted with phenol as described (17) and purified by gel filtration on Superose 12. Judged from the [3H]Val radioactivity measured in a liquid scintillation spectrometer. Incorporation of 1.09 nmol of Val/20.8 nmol of tRNA, each tRNA species was assumed to be fully charged with its cognate amino acid.

Aminoacyl-tRNA Synthetase and Elongation Factor-1 Assays—

Poly(Phe) Synthesis—Poly(U)-directed poly(Phe) synthesis was adapted from (21). To remove the endogenous mRNA pool completely, S30 extracts (30 μl) were first treated for 15 min at 20 °C with 1 unit of micrococcal nuclease-treated extracts (see below), and the complete reaction mixture (40 μl) was processed to determine the incorporation of [3H]Val into protein. Incorporation was found to be time-dependent and globin mRNA concentration-dependent with an optimum at 2.5 μg of globin mRNA.

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RESULTS

General Characteristics of in Vitro Translation during Development of Artemia Embryos—The rate of in vitro translation of endogenous mRNAs is low in quiescent embryo extracts, but increases strongly throughout pre-emergence development (Fig. 1A). In Fig. 1B, the time courses of [3H]Val incorporation into protein by extracts prepared from quiescent and 20-h developing embryos are compared. The latter exhibits a fairly steep incorporation throughout the whole incubation period, which is in excellent agreement with the results of De Haro's group, where [35S]Met was used as a label (14). The incorporation of [3H]Phe and [3H]Leu into protein proceeds at a comparable rate.

Concerning the nature of the process of reactivation of protein synthesis in Artemia, translational repressors and activators have been postulated to be present in quiescent and developing embryos, respectively (15). However, when we mixed extracts prepared from quiescent and 20-h developing embryos in different ratios, we did not observe any effect of the extract from quiescent embryos on the translational activity of that of the developing embryos, nor the reverse. In fact, the extract prepared from quiescent embryos behaves as a buffer (Fig. 1C). Thus, our results do not indicate the presence of a strong translational repressor in the quiescent nor an activator in the developing embryo, but rather suggest a shortage of one or several active translational components, which gradually get replenished during development of the quiescent embryo.

Change in in Vitro tRNA Charging Levels during Development—We used direct amino acid analysis on purified aminoacyl-tRNA to determine the degree of in vitro aminoacylation. The purification procedure includes homogenization of embryos in phenol at pH 4.5 to prevent deacylation, isolation of total RNA, followed by precipitation with lithium chloride to remove the major part of rRNA (26), and final purification of

1 The abbreviations used are: DTE, dithioerythritol; ValRS, valyl-tRNA synthetase; poly(U), poly(adenylic acid).
tRNA by gel filtration (see also Ref. 19). Compared with the method of periodate oxidation, the major advantage of this procedure is that the charging degree of all 20 different tRNA species can be assessed in a single experiment.

Neither the total yield of tRNA nor the ratio of tRNA to rRNA changed significantly during development. The average yield of 4.4 ± 0.5 mg (n = 6) tRNA/25 g of dried embryos is in the expected order when considering the EF-1a content of 22 mg/25 g of embryos (27) and assuming equal molar amounts of EF-1a and tRNA present in Artemia (28). Moreover, the amino acid acceptance of tRNA prepared from quiescent and 20-h developing embryos was the same for valine, leucine, lysine, phenylalanine, and glycine, when using nauplius S100 as a source of aminocyl-tRNA synthetases. In the case of valine, both tRNA pools could be charged to a maximum of 2.7% of the total tRNA pool by purified ValRS from Artemia (22). Under the assumption that tRNA

\[ \text{Val} \] represents 5% of the total tRNA pool, this indicates that at least half of the valine-accepting ends are intact, both in quiescent and developing embryos. We conclude that neither the total amount of tRNA nor its processing is significantly elevated during development.

As seen from Fig. 2, there is a dramatic increase in the charging degree of each of the 20 tRNA-species during the first 20 h of development, from an average of 4 ± 4% (n = 3) in quiescent to 52 ± 3% (n = 2) in 20-h developing embryos. The mutual relationship between the charging degree of tRNA and the rate of translation is obvious, especially when taking into account that more than half of the 20 different tRNA species are not charged at all in the quiescent embryo (detection limit 2% charging). However, not all tRNAs appear to be recharged in concert. After 1 h, tRNA\(^{\text{Val}}\) already shows 40% charging, whereas tRNA\(^{\text{Phe}}\) after this time hardly carries any amino acid (Fig. 2). Therefore, some aminocyl-tRNA species may clearly contribute more to the repression of overall protein synthesis in quiescent embryo lysates than others. On the whole, the results of Figs. 1 and 2 clearly demonstrate a positive correlation between the capacity for protein synthesis and the degree of tRNA aminoacylation, and therefore support a model in which the arrest of translation in quiescent Artemia embryos is based on a shortage of its natural substrate, aminocyl-tRNA.

The Role of tRNA Aminoacylation in Poly(phenylalanine) Synthesis—Whether the observed paucity of aminocyl-tRNA actually restricts elongation can be determined experimentally by using the poly(U)-directed poly(phenylalanine) synthesis assay. The flow of \(^{3}H\)phenylalanine into poly (\(^{3}H\)phenylala-

nine) may be represented by two subsequent reactions (Reaction 1).

\[
\begin{align*}
\text{Phe-tRNA synthetase} & \rightarrow \text{[3H]Phe-tRNA} \\
& \rightarrow \text{[3H]Phe} + \text{tRNA}
\end{align*}
\]

\text{REACTION 1}

When the substrates \(^{3}H\)Phe and uncharged tRNA are added separately, the rate of poly(Phe) synthesis is expected to depend on the amounts of elongation factors and phenylalanyl-tRNA synthetase present in the extracts. In this case, the activity of the quiescent embryo extract is found to be about 2.5-fold lower than that of the developing embryo extract (Fig. 3A). However, when charged \(^{3}H\)Phe-tRNA is used instead of \(^{3}H\)Phe and tRNA, the difference in poly(Phe) synthesis between quiescent and developing embryo extracts disappears completely (Fig. 3B). The results of Fig. 3B indicate that elongation factors 1 and 2 are equally active in quiescent and developing embryos. We conclude that a shortage of charged phenylalanyl-tRNA limits the elongation phase of protein synthesis in extracts of quiescent embryos, at least under the direction of a synthetic messenger.

The Role of tRNA Aminoacylation in the Reactivation of Protein Synthesis—The extent to which total cell-free protein synthesis of endogenous mRNA is limited by the shortage of aminocyl-tRNAs was assessed as follows. In the presence of the two separate substrates, amino acids and total tRNA, the rate of protein synthesis in the quiescent embryo extract is about 16 times lower as that observed in the developing embryo, i.e. the relative rate is 6.4 ± 1.3% (mean ± S.D.; n = 3) (Fig. 4A). Addition of the full complement of aminocyl-tRNAs, however, markedly enhanced \textit{in vitro} protein synthesis of the quiescent embryo from a relative rate of 6.4 ± 1.3% to a relative rate of 23.6 ± 1.2% (Fig. 4B). This proves that a paucity of charged aminocyl-tRNA significantly limits protein synthesis in extracts of the quiescent embryo.

Since protein synthesis could not be further stimulated by up to 20 \(\mu\)m aminoacyl-tRNAs, a second restriction in translation of available mRNAs appears to be present in the quiescent embryo. Note that the level of mRNAs is comparable in both types of embryo extracts (9). As a first attempt, we fractionated the quiescent and developing embryo extracts into the cytosolic (S100), polyribosomal, and ribosomal wash fraction. Interestingly, the purified polyribosomal fraction of developing em-

**FIG. 2. Aminoacylation levels of transfer RNA during development.** Charging is expressed as the amount of each individual amino acid attached to 2 nmol of total tRNA from Artemia embryos at different times of development (0, 1, and 20 h). Assuming that each of the 20 tRNA species represents 5% of the total tRNA population, 100 pmol of a specific amino acid/2 nmol of total tRNA equals a 100% charging level for this type of specific tRNA. The result of a single representative experiment is shown.
bryos was quite effective in stimulating protein synthesis of the quiescent embryo extract (S30), while the other fractions, including the ribosomes from quiescent embryos, were not. In fact, the polyribosomes from developing embryos activate protein synthesis of the quiescent embryo S100 to a relative rate 23.4 ± 1.6% of that of the developing embryo S100 (Fig. 4C).

Ultimately, the further addition of fully charged aminoacyl-tRNAs completely restored protein synthesis in the extract of the quiescent embryo to the level of the developed embryo (Fig. 4D). Moreover, the ribosomal wash fractions, which contained large amounts of initiation factors (10), were without effect, thus indicating that they were not rate-limiting. As shown previously by others, initiation factor eIF-2 was found to remain constant in amount and activity during development (10).

A substantial effect of charged tRNAs was also seen on the translation of globin mRNA by the quiescent embryo extract. Addition of the full complement of aminoacyl-tRNAs to RNase-treated extracts of the quiescent embryo raises the synthesis of globin from a relative rate of 34 ± 8% to a relative rate of 76 ± 10%. Absolute values, however, were an order of magnitude less than those observed with polyribosomes in the two types of extract. This was also the case with translation of poly(A)"mRNAs extracted from the developing embryo, indicating that "naked" mRNA is a poor substrate for translation by the quiescent and the developing embryo extract as well. We conclude, therefore, that during the development of *Artemia* embryos, protein synthesis is activated by the recharging of aminoacyl-tRNAs, but due to the virtual absence of polyribosomes in the quiescent embryo (1, 5, 13), the rate of protein synthesis remains lower than in the developing embryo.

**Levels of Aminoacyl-tRNA Synthetases during Development**—Upon rehydration of the quiescent embryos, tRNA may be recharged by its cognate aminoacyl-tRNA synthetase as present at this stage. However, the low degree of charging of most of the tRNAs even after 1 h of development (Fig. 2) indicates a severe limitation in the supply of aminoacyl-tRNAs. Apparently, the aminoacylation reaction is limited by one or more of its reacting components.

![Aminoacyl-tRNA Synthetases during Development](image-url)
The poly(A) fraction from developing embryos contains about 3 times more ribosomes (5, 9, 13). In fact, we have found that the polyribosomal fraction of ribosomes, present as polysomes in quiescent embryos, does not necessarily prevail to reactivate protein synthesis after a period of quiescence like in the brine shrimp Artemia. For instance, efforts to prove deficiencies in the initiation factors of quiescent embryos have been rather unconvincing (31), and levels of the initiation factor eIF-2 are found to be the same before and after development (10). Most characteristic of quiescent embryos, however, is the inactive form of its mRNAs and the slow reappearance of polysomes during development (9, 13). However, this by itself does not explain that the encapsulated embryo exhibits no protein synthesis unless resumption of development ensues. In this context, we have proven the absence of an inhibitor, although adequate levels of mRNAs are present in such extracts (9). How protein synthesis is reactivated during development of Artemia has therefore remained a long-standing question (8–15). We demonstrate that during the development of quiescent embryos into metabolically active embryos there is a dramatic increase in the level of tRNA aminoacylation. We also establish that the difference in protein synthesis capacity between extracts from quiescent and developing embryos can be completely abolished by solely adding aminoacyl-tRNA synthetase activities in extracts of quiescent and developing embryos

| Aminoacyl-tRNA synthetase specific for | Specific activity a | Increase |
|-------------------------------------|--------------------|---------|
| | Quiescent | Developing | |
| Valine | 43 | 67 | 1.6 |
| Methionine | 3.7 | 51 | 14 |
| Lysine | 180 | 400 | 2.2 |

a Specific activities were calculated from the linear region of concentration-dependent plots obtained with extracts of quiescent and 20-h developing embryo, incubated for 10 min at 25 °C in the presence of 3.5 mg/ml yeast tRNA and [3H]-labeled amino acid. One unit of activity corresponds to the formation of 1 pmol of aminoacyl-tRNA/min.

DisCUSSION

It is generally agreed that the rate-limiting steps in protein synthesis lies at the level of initiation (30). The same situation does not necessarily prevail to reactivate protein synthesis after a period of quiescence like in the brine shrimp Artemia. For instance, efforts to prove deficiencies in the initiation factors of quiescent embryos have been rather unconvincing (31), and levels of the initiation factor eIF-2 are found to be the same before and after development (10). Most characteristic of quiescent embryos, however, is the inactive form of its mRNAs and the slow reappearance of polysomes during development (9, 13). However, this by itself does not explain that the encapsulated embryo exhibits no protein synthesis unless resumption of development ensues. In this context, we have proven the absence of an inhibitor, although adequate levels of mRNAs are present in such extracts (9). How protein synthesis is reactivated during development of Artemia has therefore remained a long-standing question (8–15). We demonstrate that during the development of quiescent embryos into metabolically active embryos there is a dramatic increase in the level of tRNA aminoacylation. We also establish that the difference in protein synthesis capacity between extracts from quiescent and developing embryos can be completely abolished by solely adding aminoacyl-tRNA synthetase activities in extracts of quiescent and developing embryos.

We have demonstrated here that protein synthesis itself is controlled at the level of tRNA charging during the natural development of a eukaryote. Interestingly, in bacterial spores, the level of tRNA charging is also very low and increases rapidly upon germination (34). Moreover, tRNAs specific for valine, arginine, and histidine are among the most significantly charged tRNAs in these spores, as also seems to be the case in Artemia embryos. Furthermore, studies on E. coli and mouse ascites tumor cells show that protein synthesis decreases when the charging degree of tRNAs is lowered on purpose (38, 39).

Aminoacylation of tRNAs therefore appears to function as a sensor that regulates the biosynthesis of amino acids via transcription factor GCN4 in yeast (35) and also the transcription of aminoacyl-tRNA synthetase genes in Gram-positive bacteria (36, 37). We have demonstrated here that protein synthesis itself is controlled at the level of tRNA charging during the natural development of a eukaryote. Interestingly, in bacterial spores, the level of tRNA charging is also very low and increases rapidly upon germination (34). Moreover, tRNAs specific for valine, arginine, and histidine are among the most significantly charged tRNAs in these spores, as also seems to be the case in Artemia embryos. Furthermore, studies on E. coli and mouse ascites tumor cells show that protein synthesis decreases when the charging degree of tRNAs is lowered on purpose (38, 39).

Aminoacylation of tRNAs therefore appears to play a general regulatory role in escaping routes from dormancy and may also be important for instance in germination of plant seeds and perhaps also in the revival of G0-arrested vertebrate cells. Since the present day adaptor molecule, tRNA, is believed to have been of prime importance in the genesis of protein synthesis and life itself (40), the regulation of protein synthesis may even have started at the level of tRNA aminoacylation, while fine tuning of mRNA expression at the level of ribosome-induced initiation and elongation events occurred later on in evolution.

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