# MESSENGER RNA IN UNDEVELOPED AND DEVELOPING ARTEMIA SALINA EMBRYOS

Jose Manuel Sierra[1], Witold Filipowicz[2], and Severo Ochoa

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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SUMMARY

Messenger activity of RNA species retained [poly (A)+] and not retained [poly (A)-] by oligo(dT) cellulose was assayed in cytoplasmic fractions of Artemia salina embryos using a wheat germ system. In undeveloped embryos, mRNA activity was found in a fraction sedimenting at 15,000 x g (p15) and about half as much in the cytosol (s105). The ribosome (R) fraction, consisting mainly of 80S monomers, had no activity. Only p15 contained [poly (A)+] mRNA amounting to about one tenth of the total mRNA activity of undeveloped embryos. After development for 15-16 hrs, the total mRNA activity of the embryos increased 3-fold. All three cell fractions contained mRNA activity in both [poly (A)-] and [poly (A)+] RNA. Over half of the total was in the polysome-containing R fraction and about one fifth in [poly (A)+] RNA. The most pronounced change with development was in the [poly (A)+] mRNA activity which increased over 6-fold. The patterns of polypeptides synthesized by translation of [poly (A)+] mRNA fractions from undeveloped and developing embryos were similar but not identical.

In 1968, Hultin and Morris (1) found no endogenous protein synthetic activity in preparations from undeveloped A. salina embryos but observed that activity appeared upon resumption of development by hydration of the cysts and increased as development progressed. Similar observations were made by Clegg and Golub (2). These results were confirmed by Sierra et al. (3) and Filipowicz et al. (4) who found further that undeveloped embryo extracts failed to translate added natural mRNAs because of an initiation factor deficiency that was corrected with resumption of development. The absence of endogenous protein synthesis could among other things be due to mRNA being either absent or present in a form unavailable for translation. Recently, Nilsson and Hultin (5) found mRNA activity, as assayed in an E. coli system, in cytoplasmic fractions from undeveloped embryos including a fraction sedimenting at 15,000 x g composed mainly of endoplasmic vesicles

[1]Postdoctoral Fellow of the Institute of Molecular Biology, Madrid, Spain.

[2]Permanent address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland.

and mitochondria. The mRNA in this fraction contained [poly (A)+] species (6). The presence of mRNA activity in undeveloped embryos was also observed in this laboratory (7,8). In this paper, we report that mRNA activity is present in cytoplasmic fractions of dormant A. salina embryos and increases markedly with development. After 15-16 hrs, the total mRNA activity is 3 times higher than in the resting embryos and more than half of this activity is in the ribosome/polysome fraction. The amount of [poly (A)-] mRNA is in all cases much higher than that of the [poly (A)+] species. However, development is accompanied by a relatively greater increase of the polyadenylated species. While this paper was in preparation, Grosfeld and Littauer (9) also reported the presence of mRNA activity in undeveloped A. salina embryos.

## MATERIALS AND METHODS

Cell fractions and RNAs. Postribosomal supernatant (s105) fraction and unwashed ribosomes/polysomes were prepared from 200 g batches of undeveloped or developed A. salina embryos as described earlier (3). The s105 supernatant was immediately precipitated with 2 volumes of ethanol at -20°. For preparation of the fraction sedimenting at 15,000 x g the initial extract was centrifuged for 10 min at 1,400 x g to remove the nuclei and the supernatant recentrifuged for another 10 min at 15,000 x g.

RNA was prepared by a modification of the method of Brawerman (10). The various fractions were suspended in 0.1 M Tris-HCl buffer, pH 9.0, containing 5 mM EDTA and 0.5% SDS; the RNA was isolated by the phenol procedure and precipitated with ethanol as described previously (8). RNA from s105 fractions was further fractionated by chromatography on a Sephadex G-100 column (2.5 x 55 cm) in 10 mM Tris-HCl, pH 7.5. Fig. 1 illustrates this fractionation for RNA derived from developed cysts. The excluded material (peak 1) had messenger activity (Fig. 1, inset) and was devoid of amino acid acceptor activity. This fraction was used for mRNA assays. Peak 2 had amino acid acceptor activity and probably consisted mostly of tRNA. Peak 3 consisted mainly of nucleoside mono and polyphosphates. s105 RNA from undeveloped cysts gave an identical pattern.

Oligo (dT)-cellulose chromatography. For separation of [poly (A)+] and [poly (A)-] RNA, the RNA obtained from the various cell fractions as outlined above was submitted to affinity chromatography on oligo (dT)-cellulose (Collaborative Research, Inc.) essentially as described by Aviv and Leder (11). The RNA fractions were dissolved in application buffer (0.01 M Tris-HCl, pH 7.5, 0.5 M KCl) at concentrations of 20, 50, and 2 $A_{260}$ units/ml for p15, R, and s105 fractions, respectively. Non-retained material, [poly (A)-] RNA, was eluted by washing with application buffer and precipitated with 2 volumes of ethanol at -20°. The retained material [poly (A)+] RNA, was eluted with 0.01 M Tris-HCl, pH 7.5, and frozen directly. 4-5% of the mRNA in the non-retained fraction was retained after a second passage through the column, but this was not routinely performed. Chromatography of [poly (A)-]

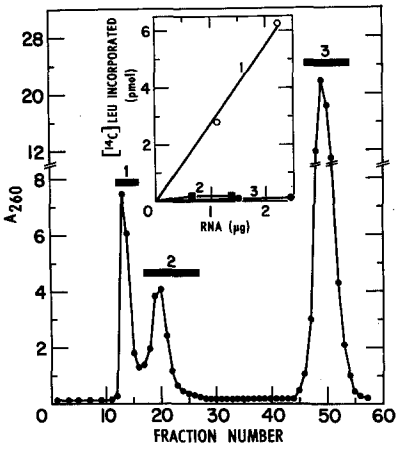


FIG. 1. Sephadex G-100 chromatography of RNA from s105 fraction of developed embryos. Inset. Assay of pooled fractions 1,2,3, for mRNA activity in wheat germ system.

RNA fractions on poly (U)-sepharose did not result in additional RNA retention. This suggests (12) that these fractions did not contain significant proportions of RNA having short poly A (< 25 residues) segments.

Translation in wheat germ extracts. s30 extract of raw wheat germ (Niblack, Rochester, N.Y.) was prepared as described (13). Standard protein synthesis assays contained in 50 µl, 20 mM N-2-hydroxyethylpiperazine- N'2-ethanesulfonic acid (Hepes) buffer adjusted to pH 7.6 with KOH, 70 mM KCl, 3.5 mM Mg $(AcO)_2$, 1 mM dithiothreitol, 1.3 mM ATP, 0.33 mM GTP, 10 mM creatine phosphate, 5µg creatine phosphokinase, 19 amino acids, each 0.02 mM, 0.3 µCi of L-[$^{14}$C] leucine (324 mCi/mmol), 1.5-2.0 $A_{260}$ units of preincubated wheat germ s30 extract, and A. salina RNA fraction as indicated. After incubation for 60 min at 25°, the radioactivity incorporated into hot trichloroacetic acid-insoluble material was determined as described (3).

Gel electrophoresis of translation products. The incubations were carried out as above except for the substitution of L-[$^{35}$S] methionine (40 µCi, 340 Ci/mmol) for L-[$^{14}$C] leucine and of 2.5 mM Mg $(AcO)_2$ and 0.1 mM spermine for 3.5 mM Mg $(AcO)_2$. After incubation, Tris-HCl, pH 6.8, SDS glycerol, and 2-mercaptoethanol were added to each sample at final concentrations of 62.5 mM, 2%, 10%, and 5%, respectively. The samples were heated at 100° for 3 min, cooled, and 15-25 µl aliquots subjected to electrophoresis on 12.5% polyacrylamide gel slabs followed by autoradiography according to Laemmli (14).

## RESULTS

The effect of RNAs from various cell fractions on the incorporation of [$^{14}$C] leucine into acid-insoluble material in the wheat germ system is shown in Fig. 2 as a function of the RNA concentration. As seen in panel B, the [poly (A)-] RNA preparation from the ribosome (R) fraction of undeveloped

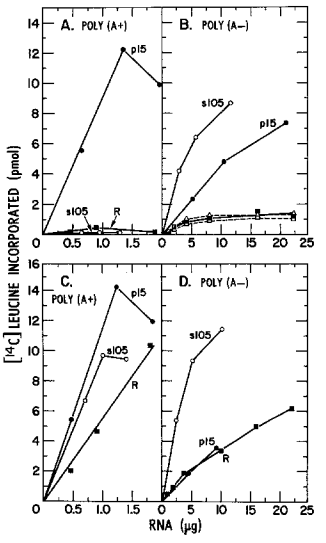


FIG. 2. Assay of cytoplasmic fractions from undeveloped and developed embryos for mRNA activity in the wheat germ system. Activity as a function of the RNA concentration. A. Undeveloped, [poly (A)+]. B. Undeveloped, [poly (A)-]. C. Developed, [poly (A)+]. D. Developed, [poly (A)-]. -●-●-, p15; -o-o-, s105; -■-■- , R; -▲-▲-, RNA from 60S subunits; -□-□-, RNA from 40S subunits.

embryos contained only slight stimulatory activity, no different from that seen in preparations of RNA from 40S and 60S A. salina ribosomal subunits. This stimulation was thus considered to be non-specific and was used to correct the values obtained with [poly (A)-] RNAs from polysomes of developing embryos. These RNA preparations are always contaminated with transfer and/or ribosomal RNA and estimation of their total mRNA activity is only approximate.

The specific activity of the various RNA fractions was determined from the ascending linear portion of the curves of Fig. 2 and was used to calculate the total amount of mRNA activity in the various fractions. These data are given in Table 1. It is apparent from this table, and even more readily from the histogram and numerical data of Fig. 3, that: a. Undeveloped embryos contain both [poly (A)+] and [poly (A)-] mRNA activity in the membrane-rich p15 fraction,

Table 1. Distribution of mRNA activity in cytoplasmic fractions from undeveloped and developed Artemia salina embryos

| Development | Fraction | Poly (A)+ | | | Poly (A)- | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | mg | S.A. ($x10^{-3}$) | Units ($x10^{-3}$) | mg | S.A. ($x10^{-3}$) | Units ($x10^{-3}$) | Units ($x10^{-3}$) |
| Undeveloped | p15 | 0.23 | 8.8 | 2.02 | 28.6 | 0.46 | 13.15 | 15.17 |
| | s105 | 0.07 | 0 | 0 | 4.13 | 1.46 | 6.03 | 6.03 |
| | R | 0.16 | 0 | 0 | 173.2 | 0 | 0 | 0 |
| Developed | p15 | 0.37 | 11.6 | 4.29 | 37.3 | 0.39 | 14.55 | 18.84 |
| | s105 | 0.22 | 9.7 | 2.13 | 3.5 | 2.12 | 7.42 | 9.55 |
| | R | 1.14 | 5.8 | 6.61 | 161.4 | 0.19 | 30.66 | 37.27 |

1 unit of mRNA activity is the amount of RNA promoting the incorporation of 1 pmol of [$^{14}$C] leucine into hot trichloroacetic acid-precipitable material in the wheat germ assay system. Specific activity (S.A.) = units/mg RNA. S.A. was calculated from the ascending linear portion of the curves of Fig. 2. Values for s105 [poly (A)-] fractions wer corrected for unspecific stimulation of [$^{14}$C] leucine incorporation by non-messenger RNAs (e.g., rRNA). Total units were obtained by multiplying S.A. by the total mg RNA in the corresponding fraction. Fractions were prepared from 200 g dry weight cysts.

only [poly (A)-] activity in the s105 fraction (cytosol)[1], and no activity in the ribosome (R) fraction, which consists mainly of 80S monomers. b. There is a marked increase (about 3-fold) of total mRNA activity upon resumption of development. All three, p15, s105, and R (now consisting of monosomes and polysomes) fractions contain both [poly (A)+] and [poly (A)-] mRNA activity, but the amount of [poly (A)-] greatly exceeds that of [poly (A)+] mRNA in all three fractions. More than half of the total activity is in the R fraction. The most pronounced change with resumption of development is in the [poly (A)+] RNA the total activity in this fraction increasing over 6-fold.

As seen in Fig. 4, cell-free translation of [poly (A)+] RNAs from (a) p15

[1]When A. salina cysts are broken up in a French press, rather than by grinding in a mortar, some [poly (A)+] RNA is found in the s105 fraction (7,8). This may be due to disruption of membrane structures with concomitant release of mRNA.

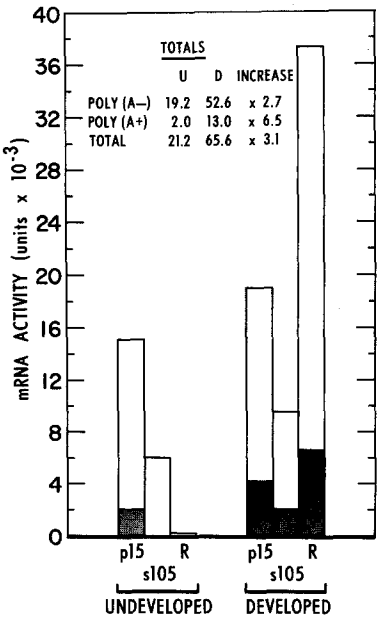


FIG. 3. Histogram showing changes of mRNA activity upon resumption of development of A. salina embryos. [Poly (A)−] RNA. [poly (A)+] RNA. U, undeveloped; D, developed embryos.

fraction of undeveloped embryos (channel 2), (b) p15 fraction of developed embryos (channel 3), and (c) R fraction of developed embryos (channel 4), which is derived from actively translating polysomes, yields a large number of polypeptides (over 40) and, with a few exceptions, the translation patterns are very similar. Note, however, that band No. 27 in the middle region of the electropherogram, which is very heavy upon translation of undeveloped p15 mRNA (channel 2), is considerably lighter upon translation of developed p15 and R mRNA (channels 3 and 4). Note also that band No. 26 is missing from the translation products of mRNAs from developed embryos (channels 3 and 4).

## DISCUSSION

Our results (7,8, this paper) and those of others (5,6,9) clearly establish the existence of preformed mRNA in dry A. salina cysts, a small fraction of which is polyadenylated. This RNA, particularly the one in the p15 fraction, may represent a storage form of cytoplasmic and mitochondrial mRNA during cryptobiosis.

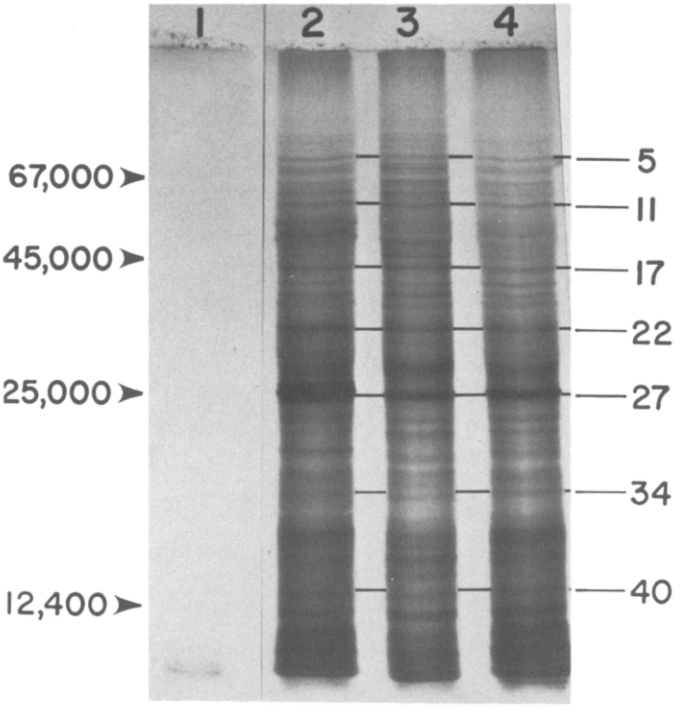


FIG. 4. [$^{35}$S]Methionine-labelled products of wheat germ system translation of mRNA from undeveloped and developed embryos as analyzed by SDS gel electrophoresis and autoradiography. Channel 1, no mRNA. Channel 2, p15 [poly (A)+] from undeveloped embryos ([$^{35}$S] radioactivity applied, 390 x $10^3$ cpm). Channel 3, p15[poly (A)+] RNA from developed embryos ([$^{35}$S] radioactivity applied, 399 x $10^3$ cpm). Channel 4, R[poly (A)+] RNA from developed embryos ([$^{35}$S] radioactivity applied, 391 x $10^3$ cpm). Mol. wt. markers: Bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), cytochrome c (12,400).

Since mRNAs from both undeveloped and developed embryos have 5'-terminal $m^7G$ (8), failure of the messenger in dormant cysts to associate to ribosomes cannot be attributed to absence of this cap in the stored mRNA. This failure is most likely due to the low levels of initiation factors in undeveloped embryos (3,4). Upon hydration, translation of preformed and newly transcribed messengers, slow at first because of the initiation factor deficiency, gradually restores normal initiation factor levels and protein synthesis eventually reaches optimal rates. Removal or neutralization of translation inhibitors (15) upon rehydration of the cysts might play an additional role in the transition from cryptobiosis to active development. The similarity of translation patterns of mRNA from dormant embryos

or from developing ones, whether from the p15 or the actively translating polysome fraction, suggests that most messengers utilized at the time of development examined here are already present during cryptobiosis.

The bulk of the mRNA in the cytoplasm of A. salina embryos, whether undeveloped or developed, is not polyadenylated (Fig. 3). Coexistence of non-histone [poly (A)-] with [poly (A)+] mRNA has been reported in sea urchin embryos (16) and HeLa cells (17) but the [poly (A)-] species is not as preponderant in these cells as in A. salina embryo cells. There appeared to be some differences in the pattern of polypeptides synthesized by translation of [poly (A)+] and [poly (A)-] mRNA fractions but this requires further study.

The pronounced increase in mRNA activity observed upon resumption of development is probably the result of new transcription of embryo DNA, but the possibility that the increased activity may be due to some modification of preformed, inactive mRNA cannot be ruled out. An increase in activity of several RNA polymerases has been observed during this period (18). Since there is a relatively greater increase of the polyadenylated than the non-polyadenylated mRNA species when development resumes it is possible that some of the preformed mRNA is polyadenylated at this time. Polyadenylation of preexisting RNA molecules has been found to occur in sea urchin eggs after fertilization (19,20).

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REFERENCES

1. Hultin, T. and Morris, J.E. (1968). Develop. Biol. 17, 143-164.
2. Clegg, J.S. and Golub, A.L. (1969). Develop. Biol. 19, 178-200.
3. Sierra, J.M., Meier, D. and Ochoa, S. (1974). Proc. Nat. Acad. Sci. USA 71, 2693-2697.
4. Filipowicz, W., Sierra, J.M., Nombela, C., Ochoa, S., Merrick, W.C. and Anderson, W.F. (1976). Proc. Nat. Acad. Sci. USA 73, (in press).
5. Nilsson, M.O. and Hultin, T. (1974). Develop. Biol. 38, 138-149.
6. Nilsson, M.O. and Hultin, T. (1975). FEBS Letters 52, 269-272.
7. Filipowicz, W. and Sierra, J.M. (1975). Federation Proc. 34, 675.
8. Muthukrishnan, S., Filipowicz, W., Sierra, J.M., Both, G.W., Shatkin, A.J. and Ochoa, S. (1976). J. Biol. Chem. (in press).

9. Grosfeld, H. and Littauer, U.Z. (1975). Biochem. Biophys. Res. Commun. 67, 176-181.
10. Brawerman, G. (1974). In Methods in Enzymology (K. Moldave and L. Grossman, eds.) Vol. 30, part F, pp. 605-612.
11. Aviv, H. and Leder, P. (1972). Proc. Nat. Acad. Sci. USA 69, 1408-1412.
12. Dubroff, L.M. and Nemer, M. (1975). J. Mol. Biol. 95, 455-476.
13. Roberts, B.E. and Paterson, B.M. (1973). Proc. Nat. Acad. Sci. USA 70, 2330-2334.
14. Laemmli, V.K. (1970). Nature 227, 680-685.
15. Huang, F.L. and Warner, A.H. (1974). Arch. Biochem. Biophys. 163, 716-727.
16. Nemer, M., Graham, M. and Dubroff, L.M. (1974). J. Mol. Biol. 89, 435-454.
17. Milcarek, C., Price, R. and Penman, S. (1974). Cell 3, 1-10.
18. Renart, J., Osuna, C. and Sebastian, J. (1975). Abstracts of VIth Congress of Spanish Biochem. Soc., p. 97.
19. Wilt, F.H. (1973). Proc. Nat. Acad. Sci. USA 70, 2345-2349.
20. Slater, I. and Slater, D.W. (1974). Proc. Nat. Acad. Sci. USA 71, 1103-1107.