Further Investigations on Transcription and Translation in *Limnaea* Embryos

R. L. Brahmachary

Indian Statistical Institute, Calcutta 35, India

AND

S. R. Palchoudhury

Biology Department, Carleton University, Ottawa, Canada

Received November 2, 1970

Brahmachary, R. L., and Palchoudhury, S. R. Further investigations on transcription and translation in *Limnaea* embryos. Can. J. Biochem. 49, 926–932 (1971).

^{14}C-amino acid incorporation in uncleaved *Limnaea* eggs is slight but it slowly increases up to the trochophore stage followed by a rapid rise during the veliger stage when ^{32}P incorporation is falling. There is a peak in the 10 S region of the sucrose density gradient profile of morula RNA. Presumably this morula RNA (messenger RNA) is necessary for final development and hatching of the veliger. The trochophore 10 S peak is the largest, stable and strongly depressed by actinomycin; on the contrary, the veliger 10 S RNA peak is insensitive to actinomycin and seems to be meant for immediate translation.

Actinomycin treatment of the uncleaved eggs prevents cleavage in a certain percentage of eggs but in the rest the treatment is not effective until the trochophore stage.

Brahmachary, R. L., et Palchoudhury, S. R. Further investigations on transcription and translation in *Limnaea* embryos. Can. J. Biochem. 49, 926–932 (1971).

L'incorporation d'un acide aminé-^{14}C dans les oeufs non clivés de *Limnaea* est faible mais elle augmente lentement jusqu'au stade trocophore et elle subit une élévation rapide durant le stade véligère alors que l'incorporation du ^{32}P diminue. Un pic apparaît dans la région 10 S du profil obtenu en centrifugeant l'ARN morulaire dans un gradient de densité de sucrose. Cet ARN morulaire (ARN messager) est probablement nécessaire pour le développement final et l'éclosion de la véligère. Le pic 10 S du stade trocophore est le plus important, il est stable et l'actinomycine le diminue fortement; au contraire, le pic 10 S de l'ARN du stade véligère est insensible à l'actinomycine et cet ARN semble désigné pour traduction immédiate.

Le traitement à l'actinomycine des oeufs non clivés empêche la segmentation d'un certain pourcentage d'oeufs mais ce traitement n'affecte pas les autres oeufs avant le stade trocophore.

Introduction

Studies of "embryonic information transfer" have been summarized by several authors (1–3). It seems that in sea-urchin eggs, a good deal of RNA synthesis takes place during oogenesis while just after fertilization there is a stimulation of protein synthesis by preexisting mRNA molecules; it is followed by a second peak of RNA and protein synthesis at bastula–gastrula stage. No such sudden marked change has been noted after fertilization in amphibian eggs but here too there is a good deal of RNA and protein synthesis in the maturing egg and a burst of RNA synthesis has been detected at gastrulation.

In *Limnaea* the transcription for most of organogenesis largely takes place during the peak of trochophore stage while translation occurs gradually during the veliger stage (4, 5). This report describes more results on the incorporation of ^{32}PO$_4$$^{3-}$ and ^{14}C-amino acids in normal and actinomycin-treated embryos. By comparing these results it has been possible to further confirm the thesis stated above and to reveal the presence of an early messenger RNA in the morula. The effects of actinomycin on uncleaved eggs were also studied.

Materials and Methods

Measurement of RNA and Protein Synthesis

The egg masses were pierced with a hooked wire and thus placed in the 100 μCi/ml of Na$_2$H^{32}PO$_4$ solution (specific activity 500 μCi/mmole) in a vial within a lead box. It was further established that most of the uncleaved eggs and morula can develop normally after 1 h treatment in 100 μCi/ml of ^{32}P solution if the pH is maintained at about 7.4. Uncleaved or morula egg masses were treated with 100 μg/ml of actinomycin for 1 h, washed twice with water, and then allowed to grow in 100 μCi/ml of ^{32}PO$_4$$^{3-}$ solution. After 4 h, the egg masses were diluted with an equal volume of 10% ice-cold TCA (trichloroacetic acid) along with 2 mg/ml of *E. coli* RNA as carrier and 0.025 M sodium azide. After 30 min in ice

the precipitate was collected by centrifugation, washed twice more with 5% cold TCA, and digested overnight with 0.4 N KOH at 37 °C. The digest was neutralized with 1 N HCl, diluted with an equal volume of 5% cold TCA, and the radioactivity of the supernatant fraction was determined. This mild alkali digestion has differential action (depolymerizing) on RNA and eliminates a possible error due to incorporation into phosphoproteins.

Eggs at different stages of development were also allowed to grow for 1 h in 20 μCi/ml of ^{14}C-amino acid mixture. Protein was isolated by hot TCA precipitation in the presence of 0.2 ml of 2% bovine serum albumin (6). The final precipitate was dissolved in liquid ammonia, dried in planchetts, and the radioactivity was measured by an ordinary Geiger Counter (counting efficiency is 20% for ^{32}P and 4% for ^{14}C radioactivity). For all isotopic experiments it was first determined that eggs treated with isotope solution, washed twice with water, and then put back to water were capable of normal development. $Na_2H^{32}PO_4$ (specific activity 500 μCi/mmole) was purchased from AEET, Bombay, and ^{14}C-amino acid mixtures (specific activity 50 mCi/mmole) from Amersham, England.

Actinomycin was a product of Merck, Sharpe and Dohme.

Density Gradient Analysis of RNA

^{32}P-containing egg masses were added to *E. coli* RNA (to be used as 23 S and 16 S markers) in 0.001 M Tris (pH 7.4) buffer containing 0.005 M Mg^{2+}. To 0.6 ml of the above mixture were added 0.1 ml sodium dodecyl sulfate and 0.6 ml hot phenol (60 °C), and the mixture was shaken for 5 min. The subsequent steps were performed at 4 °C in the following way. After phenol treatment the sample was centrifuged at 15 000 r.p.m. for 1 min and the aqueous phase was pipetted out. To remove the residual phenol, the aqueous layer was spun again at 2000 r.p.m. for 30 min, and the upper phase was precipitated with cold ethanol. The precipitate (RNA) was resuspended in the same Tris–magnesium (pH 7.4) buffer and 0.3 ml of this RNA was layered on 5–20% linear sucrose gradients which were centrifuged at 37 000 r.p.m. in an SW39 rotor for 4 h at 3 °C. After centrifugation 2–3 drop fractions were collected from the bottom of the tube for the measurement of optical density at 260 mμ (mainly for carrier *E. coli* RNA) and radioactivity (which indicates newly synthesized *Limnaea* RNA).

Plating of Limnaea Egg Masses

A mass of 50 eggs was plated without dilution on nutrient agar by agar overlay technique to assay the number of bacteria present as contamination. One hundred eighty four bacterial colonies were found in the egg masses which were negligible. Furthermore, carbol-fuchsin stained bacteria showed only 200 bacteria in a mass of 50 eggs even after 18 h immersion in nonsterile water.

Results

Actinomycin Treatment at the Uncleaved Stage
Effects on Development

In view of the earlier results (4, 5) that actinomycin treatment at morula stage (which does suppress RNA synthesis up to 75%) leads to abnormality only at the very late veliger stage, 41 experiments were carried out at the uncleaved stage of the Indian *Limnaea*. A control batch of uncleaved eggs was observed every 10 min while another batch from the same egg mass was treated with actinomycin (100 μg/ml). It was noted from the first few experiments that eggs left in the solution were arrested at the two- or four-cell stage or did not cleave. As the freshly laid egg may undergo cleavage as late as 2–3 h later (depending on both temperature and the genetic property of a particular batch of eggs), we then attempted to determine the sensitive period, if any, by treating batches of uncleaved eggs for successive periods of 1 h. Eggs treated during the uncleaved stage only revealed a remarkable effect at the early trochophore stage. These eggs remained as abnormal trochophores and sometimes as mere ciliated, moving fragments. Often the abnormal trochophores died. Inhibition of mitosis was not correlated with the length of treatment. Taking account of the variable results the significance of which has been strongly emphasized by Timourian (7, 8) in case of sea-urchin eggs, we can classify the results with *Limnaea* under two categories; namely, complete arrest of cleavage, or no arrest but a subsequent effect at the early trochophore (Table 1). Arrest of cleavage was variable ranging from uncleaved to four-cell eggs; incomplete first cleavage was also noted. In one egg mass, stages of arrest varied from two to eight cells.

Effect on RNA Synthesis

Unlike all other stages of development, uncleaved eggs showed stimulation of ^{32}P incorporation into the cold TCA-insoluble, alkali-labile fraction (RNA) after 1 h actinomycin treatment (Table 2).

Sucrose Density Gradients (Normal and Actinomycin-Treated Embryos)

Sucrose density gradient of the RNA from the morula (Fig. 1) shows a profile with a peak at the 10 S region which is comparable in size with the 23 S ribosomal RNA of *E. coli* used as marker. The 16 S peak, identified by a marker (*E. coli* 16 S rRNA), is significantly depressed. Again the 10 S peak of the veliger stage is also comparable with the ribosomal peak (Fig. 2) while only in this trochophore stage is this peak much larger than the ribosomal peak (4). This 10 S peak is also estimated to be small in the abnormal trocho-

TABLE 1. The results of actinomycin treatment at uncleaved stage[a]

Number of experiments with uncleaved egg masses in actinomycin permanently 41	Arrested at or after first or second cleavage 12	Not arrested 29
Number of experiments after 1 h treatment with actinomycin for watching subsequent action 18	Effect at early trochophore stage 16	No effect at early trochophore stage 2

[a]The concentration of actinomycin was 100 μg/ml. Immediately after 1 h treatment with actinomycin the uncleaved egg masses were washed twice with water and then put back in water for watching subsequent action.
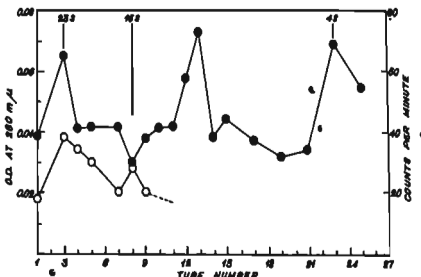

FIG. 1. Sucrose density gradient profile of the RNA from morula. 10 S RNA peak is comparable in size with the 23 S peak. (●) Radioactivity, (○) O.D. at 260 mμ.

phores produced by the actinomycin treatment (Fig. 3).

[14]C-Amino Acid Incorporation into Normal Embryos

For both the Indian Limnaea and Limnaea natalensis the count from live trochophores was 8–10 times higher than that for a comparable number of heat-killed trochophores (Table 3) when the two batches were fed with ^{14}C-amino acid for 1 h at different stages of development. The rate of incorporation increases rapidly during the trochophore and veliger stages (Fig. 4). To detect the effect of actinomycin on protein synthesis, the treated and untreated egg masses were left in the isotope for 1 h in veliger. The results show the suppression due to actinomycin treatment (Table 4).

Discussion

The strong effect exerted by actinomycin on the uncleaved eggs is interesting though the

TABLE 2. Effect on ^{32}P incorporation into cold TCA-insoluble, alkali-labile fraction (RNA) after 1 h actinomycin treatment of uncleaved egg masses[a]

	Number of eggs	Counts per minute
Actinomycin sample No. 1		
Control	52	2050
Actinomycin-treated	50	6000
Control	16	701
Actinomycin-treated	16	788
Actinomycin sample No. 2		
Control	16	95
Actinomycin-treated	16	150
Control	18	454
Actinomycin-treated	19	834

[a]Concentration of actinomycin was 100 μg/ml. Counts were taken for 10 min and then averaged for 1 min.

variable results are difficult to interpret. However, Timourian (7, 8) has shown that such variation reflects a difference at the level of molecular mechanisms and is indeed a natural phenomenon. It seems that certain batches of Limnaea eggs
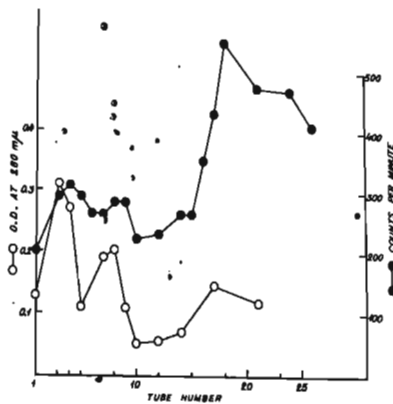
Fig. 2. Sucrose density gradient profile of the RNA from normal veliger stage. Unlike the normal trochophore there is no marked peak of labeled RNA between 16 S and 4 S. (●) Radioactivity, (○) O.D. at 260 mμ.

TABLE 3. The increase in ^{14}C-amino acid incorporation with development of embryos[a]

	Number of eggs	Stage of development	Counts per minute
Limnaea natalensis	15	Early trochophore	642
	14	Early veliger	907
	9	Morula	92
	9,	Very early trochophore	256
	9	Veliger	415
Indian *Limnaea*	16	Uncleaved	7
	15	Two to four cell	24
	15	Advanced trochophore	191
	15	Mid veliger	2069
	17	Very early veliger	565
	18	Advanced veliger	1102
	15	Very early veliger	279
	15	Advanced veliger	366
	23	Early to midtrochophore	80
	23	Very early veliger	265

[a]Batches of the comparable number of eggs from same egg mass were grown in ^{14}C-amino acid (35 μCi/ml) for 1 h at different stages of development. Counts were taken for 10 min and then averaged for 1 min.

transcribe RNA necessary for the first cleavage during the period preceding it and so actinomycin treatment at that time prevents cleavage. It is also to be noted that in all other egg masses, actinomycin treatment during the uncleaved stage exerts a marked action, viz. the trochophores become abnormal and remain so. However, as actinomycin causes an increase in incorporation into RNA rather than a decrease, no correlation can be made between the effects on "uncleaved" RNA and the morphological effects.

The profile of the density gradient of the late morula shows, besides the presence of a 10 S peak, that at this stage one ribosomal RNA subunit (23 S) significantly preponderates over the other (16 S). This has also been completely borne out by electrophoretic separation of *Limnaea* RNA on the ion agar (our unpublished data).
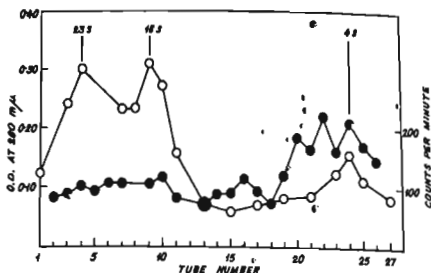
FIG. 3. Sucrose density gradient profile of the RNA from actinomycin-arrested trochophore. 10 S peak is much reduced compared with that of normal trochophore. (●) Radioactivity, (○) O.D. at 260 mμ.
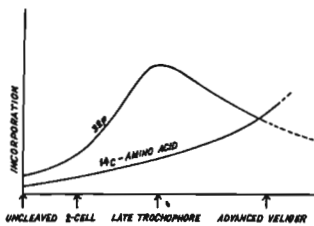

FIG. 4. A schematic diagram of the general nature of incorporation of ^{32}P and ^{14}C-amino acid at different stages of growth.

TABLE 4. The suppression of ^{14}C-amino acid incorporation in veligers for 1 h after actinomycin treatment

	Number of eggs	Treatment	Counts per minute
Limnaea natalensis	13	Untreated	3406
	14	Treated	1334
	20	Untreated	1067
	19	Treated	642
Indian Limnaea	16	Untreated	466
	16	Treated	254
	26	Untreated	260
	26	Treated	61
	18	Untreated	982
	18	Treated	217

NOTE: Parts of the same egg mass were used for comparing the results. One part was kept as control and watched for ^{14}C-amino acid incorporation during 1 h and also for further development after washing out the isotope. The other part was treated with 100 μg/ml of freshly prepared actinomycin for 1 h, washed with water, and then allowed to grow for 1 h in 20 μCi/ml of ^{14}C-amino acid mixture. Samples were withdrawn and examined for hot TCA-insoluble material as described in the Methods section.

Similar preponderance of the sea-urchin 18 S rRNA over 28 S rRNA has been reported by Giudice and Mutolo (9). We have observed that the Limnaea rRNA have nearly the same 'S' value as that of bacterial rRNA. The carrier E. coli RNA has been added at the beginning of the extraction procedure and still the O.D. peaks occur at 23 and 16 S, that indicates the extensive degradation of RNA has probably not taken place. Click and Tint (10) found animal rRNA of 31.7 S while Harris and Forrest (11) reported 24 S and 16 S in the milkweed bug. That the results with Limnaea are not due to bacterial contamination has been amply verified by plating (Materials and Methods). The definite rise and fall of incorporation into RNA and characteristic profiles of sucrose density gradient depending on developmental stages are so clear-cut that the results cannot be due to contamination.

The RNA peak at the 10 S region of the morula is presumably an early messenger. As was found earlier (4) actinomycin treatment at this stage can suppress about 75% of the ^{32}P incorporation and the corresponding morphological effect is an abnormality at the late veliger stage and inability to hatch. Baltus et al. (12) suggested that the mRNA of sea-urchin produced during cleavage must be stable for a few hours and Infante and Nemer (13) suggest that some of the early mRNA remains stored by being bound to 300 S polysomes. On the contrary, in another biological system Stewart and Papaconstantinou (14) showed that stable mRNA is synthesized when mitosis slows down.

The ^{14}C-amino acid incorporation pattern shows that unlike the plateau and peak following fertilization and. at gastrulation, respectively, in the sea-urchin egg, in the case of *Limnaea* there is a slow rise up to the trochophore stage followed by a very steep rise in the veliger. (In *Limnaea natalensis*, where the general rate of development is slower, the rise was less steep in the veliger.) This is indeed quite natural because body building, increase in size, and differentiation are rapidly taking place at this stage (which, however, incorporation of ^{32}P is falling steeply from its trochophore value). As it is less likely that ^{32}P is unable to penetrate when amino acid mixtures can do so, the observed phenomena probably indicate a real decline in transcription that goes hand in hand with increasing translation. That transcription precedes translation is interesting and is in line with the idea of masked mRNA or informosome. The immediate reduction in ^{14}C-amino acid incorporation due to actinomycin (Table 4), if correlated with the corresponding reduction in ^{32}P incorporation (4), would suggest that the 10 S peak of the normal veliger (Fig. 2) is meant for immediate translation. We have concluded that RNA synthesis increases to a maximal value at trochophore and that the major part of the trochophore RNA is an approximately 10 S fraction. The first part of the conclusion involves the question of permeability, for the enhanced incorporation at trochophore stage may be due to increased permeability of the embryo which would lead to a greater intracellular pool of the isotope.

In order to solve the problem, equal numbers of eggs (from the same egg mass) at morula and at trochophore stage were homogenated with a glass homogenizer in 0.01 M Tris buffer (pH 7.4) containing 0.1 M KCl and 0.01 M Mg^{2+} and incubated with 1 μCi of ^{32}P for 30 min at 25 °C. Table 5 shows the greatly enhanced degree of incorporation in the trochophore homogenate. This indicates a real increase in the rate of RNA synthesis (and not merely increased incorporation due to greater permeability) in the trochophore and that the factors responsible reside in the cytoplasm or at least do not require the normal nuclear-cytoplasmic interrelationships of the intact egg.

The very low rate of protein synthesis at the earliest stages (uncleaved, morula, etc.) may be due to low permeability or low RNA content or

TABLE 5. Incorporation of ^{32}P into acid-insoluble fraction

	c.p.m.
Morula	559
Early trochophore	5244

TABLE 6. Uncleaved homogenate incubated with ^{14}C-amino acids

	c.p.m.
Uncleaved homogenate plus morula RNA	1334
Uncleaved homogenate plus trochophore RNA	2771

undeveloped protein-synthesizing system. In order to shed more light, Mg–KCl–Tris homogenates of two batches of equal numbers of uncleaved eggs were mixed with morula and trochophore RNA (phenol extracted), respectively. These two batches were incubated with 10 μCi ^{14}C-amino acid mixtures for 1 h at 25 °C. Table 6 shows considerably higher incorporation into the acid-insoluble fraction with trochophore RNA. This suggests that the low rate of protein synthesis in the early stage is partly due to the small amount of RNA. Even the uncleaved egg homogenate has a potential system for a much greater degree of protein synthesis than what actually takes place.

Comparing the present findings with the earlier ones (4, 5) we thus have the following suggestions.

(*1*) A 10 S RNA fraction in the morula is somehow necessary for final growth and hatching.

(*2*) The major peak of RNA synthesis is at late trochophore and the major part of this RNA is due to a broad 10 S peak.

(*3*) Protein synthesis rises rapidly in the veliger stage when RNA synthesis is decreasing.

(*4*) The comparatively small 10 S peak of the veliger is responsible for immediate translation, but the suppression of this peak cannot prevent major organogenesis.

Thus the mRNA for major organogenesis and differentiation is probably transcribed earlier. Presumably this mRNA is part of the trochophore 10 S peak which is almost absent in the actinomycin-arrested abnormal trochophores.

The key to the crucial problem of differentiation is, in this case of *Limnaea*, to be sought for at the translation level, *i.e.* in the cytoplasm.

1. NEMER, M.: In Progress in nucleic acid research and molecular biology. Vol. 7. Edited by Davidson, J. N., and Cohn, W. E. Academic Press, Inc., New York. 1967. p. 243.

2. GROSS, P. R.: In Current topics in developmental biology. Vol. 2. Edited by Monroy, A., and Moscona, A. A. Academic Press, Inc., New York. 1967. p. 1.

3. SPIRIN, A. S.: In Current topics in developmental biology. Vol. 1. Edited by Monroy, A., and Moscona, A. A. Academic Press, Inc., New York. 1966. p. 1.

4. BRAHMACHARY, R. L., BANERJEE, K. P., and BASU, T. K.: Exp. Cell Res. 51, 177 (1968).

5. BRAHMACHARY, R. L., and BANERJEE, K. P.: Nature, 214, 172 (1967).

6. MANS, R. J., and NOVELLI, G. D.: Biochim. Biophys. Res. Commun. 3, 540 (1960).

7. TIMOURIAN, H.: Science, 154, 1055 (1966).

8. TIMOURIAN, H.: Exp. Cell Res. 48, 173 (1967).

9. GIUDICE, G., and MUTOLO, V.: Biochim. Biophys. Acta, 138, 276 (1967).

10. CLICK, R. E., and TINT, B. L.: J. Mol. Biol. 25, 111 (1967).

11. HARRIS, S. E., and FORREST, H. S.: Science, 156, 1613 (1967).

12. BALTUS, E QUERTIER, J., FICQ, A., and BRACHET, J.: Biochim. Biophys. Acta, 95, 408 (1965).

13. INFANTE, A. A., and NEMER, M.: Proc. Nat. Acad. Sci. U.S. 58, 681 (1967).

14. STEWART, J. A., and PAPACONSTANTINOU, J.: J. Mol. Biol. 29, 357 (1967).