*THE NATURE OF MESSENGER RNA IN THE EARLY STAGES OF SEA URCHIN DEVELOPMENT**

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The interrelated patterns of ribonucleic acid (RNA) and protein syntheses in developing organisms is becoming clear. In one of the most studied systems, sea urchins, protein synthesis begins immediately upon fertilization and accelerates.[1, 2] RNA synthesis follows a more complicated path. No deoxyribonucleic acid (DNA)-dependent synthesis of RNA is observable during the first two cleavages; however, labeled nucleotides are incorporated by 3′ terminal addition of cytosine and adenine (pCpCpA) to the soluble RNA (sRNA).[3, 4] After a few cleavages, newly synthesized DNA-like RNA, presumably messenger RNA (mRNA), begins to make its appearance. The synthesis of new ribosomal RNA (rRNA) is delayed until after the gastrula stage. The lag in the initiation and the subsequent low level of mRNA synthesis is consistent with the finding[5, 6] that neither the rate nor the type of proteins synthesized between fertilization and the blastula stage are inhibited by actinomycin D at concentrations that normally inhibit RNA synthesis. These observations imply that some mRNA is present in the unfertilized egg, and it is this that is utilized for the protein synthesis needed for the initial cell divisions and other basic biochemical functions.

It is against this background that one can ask how the newly synthesized mRNA relates to the maternal mRNA and whether mRNA produced at later stages of development is different or similar. In particular, one can find the production of some kinds of mRNA being stopped and that of others being started at intermediate stages of development.

We have examined this problem using the DNA-RNA hybridization technique of Gillespie and Spiegelman[7] to measure the extent of inhibition of hybridization of DNA and radioactive-labeled blastula RNA that is produced by various amounts of RNA isolated at different stages of sea urchin development. This work turns out to have been complementary to a somewhat similar study that has just been presented by Whiteley, McCarthy, and Whiteley[8] using the agar gel technique. While the basic conditions of the assay are extremely different, there is agreement in finding that the mRNA synthesized up to the blastula stage is essentially similar to the pre-existing maternal mRNA. By combining the results of the two studies, one can show that some gene transcription is turned off at the blastula stage and other transcription is turned on.

Methods and Materials.—Much of the methodology applied here has been presented previously.[3] All experiments were carried out on *Strongylocentrotus purpuratus* supplied by Dr. R. Fay, Pacific BioMarine Supply Co., Venice, Calif. The eggs as well as the sperm were liberated by injection of 1 *M* KCl. Only batches of eggs that were fertilizable to more than 95% were used.

The radioactive label was introduced by pulsing hatching blastula embryos with P^{32} phosphate having an activity of 1 μc/ml.

Isolation of RNA: Unlabeled RNA was isolated at five stages of development: unfertilized eggs, hatching-blastula, posthatching embryos (2 hr after hatching), gastrula, and prism. The method of extraction was the same as that previously described, except that in the room tempera-

ture extraction of the interphase the concentration of sodium dodecyl sulfate was increased to 2 gm/dl.

The labeled hatching blastula RNA was layered on a 5–20% sucrose gradient and centrifuged. Fractions corresponding to sedimentation constants greater than 7*S* were pooled. After dialysis and precipitation with ethanol, the 4*S*-free RNA was used for the hybridization experiments.

The radioactivity profile of the hatching blastula RNA in the sucrose gradient was the same as that previously described with a high peak at 4*S* and a broad irregular tail extending up to at least 35*S*. The optical density profiles of the unlabeled RNA preparations all showed the characteristic 28, 18, and 4*S* pattern.

Isolation of DNA: The DNA used in all the binding experiments was isolated from sea urchin sperm. About 0.5 gm of washed sperm was suspended in 0.01 SSC (SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7) and homogenized in a glass homogenizer. Sodium dodecyl sulfate was then added to a final concentration of 0.5 gm/dl. Gentle homogenization was continued at room temperature. The preparation was then extracted twice with phenol-water (50:50) and the final aqueous layer precipitated with 2 vol of ethanol. The DNA was dissolved in 0.01 SSC, dialyzed with repeated changes, and adjusted to 0.035 optical density units at 260 mμ. One ml of this solution was used for each filter prepared in the usual manner.[7]

Results.—Binding of labeled RNA to DNA: Various amounts of the labeled blastula RNA were incubated with sea urchin sperm DNA-impregnated filters for 12 hr at 60°C. The amount of radioactivity retained after washing is shown in Figure 1 as a function of the amount of labeled RNA in 1 ml of the incubation solution. It is seen that a well-defined plateau is attained above 100 μg RNA; consequently, this value was chosen for subsequent experiments.

Since the DNA content of each filter is about 2 μg of DNA, it is clear that a 50–100-fold excess of labeled RNA is required for saturation. This reflects the fact that the major portion of the RNA is ribosomal RNA and is both unlabeled and unbound. Indeed it is likely that the mRNA makes up less than 1 per cent of the sample, but it is rather completely bound at 100 μg/ml concentrations.

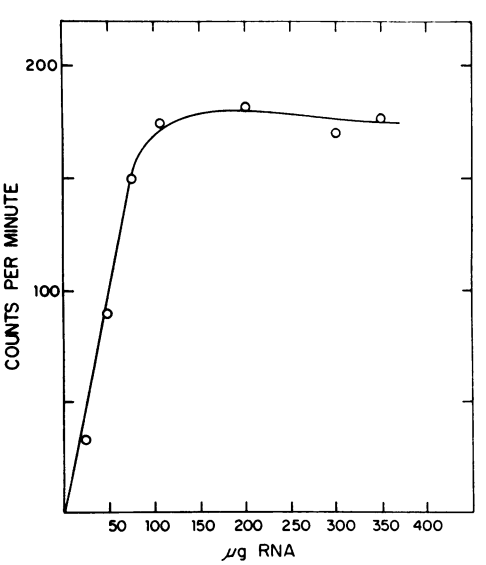

FIG. 1.—Binding of hatching-blastula mRNA to DNA. Various amounts of the labeled blastula RNA were incubated with 2 μg of sea urchin sperm DNA-impregnated filters for 12 hr at 60°C in a volume of 1.5 ml in 4 × SSC.

The RNA-DNA ratio employed here is in striking contrast to that employed by Whiteley *et al.*,[8] who incubated 4 μg of labeled RNA with 110 μg of DNA imbedded in the agar gel. Our ratio is 1400 times greater than theirs. As a consequence, their conditions are not near saturation, and most of the DNA is not being utilized for hybridization. While there are advantages and disadvantages to each extreme, and these will be important in further refinements of this kind of work, the main results at the present stage do not seem to be affected.

Comparison of RNA's from different stages of development: Unlabeled RNA samples prepared from five different stages of development (see *Methods and Materials*) were compared by determining their inhibition of binding 100 μg

of the labeled blastula RNA. The results for a range of RNA concentrations are shown in Figure 2. It is seen that each of the RNA preparations does compete. This means that each of the five preparations contains RNA molecules with sequences like those in the hatching blastula RNA. An examination of the competition profiles permits some conclusions to be drawn with respect to the composition of the various RNA preparations relative to the reference RNA, that is, the labeled blastula RNA.

First, the inhibition with unlabeled hatching blastula RNA is seen to be quite high; indeed, it follows within experimental error the theoretical curve expected. Second, the inhibition curves for unfertilized egg and postblastula RNA are essentially the same as for the hatching-blastula reference RNA. Hence, one concludes that the population of mRNA molecules, that is, the specific base sequences and their frequency of occurrence, is essentially the same in two blastula stages as in the unfertilized egg.

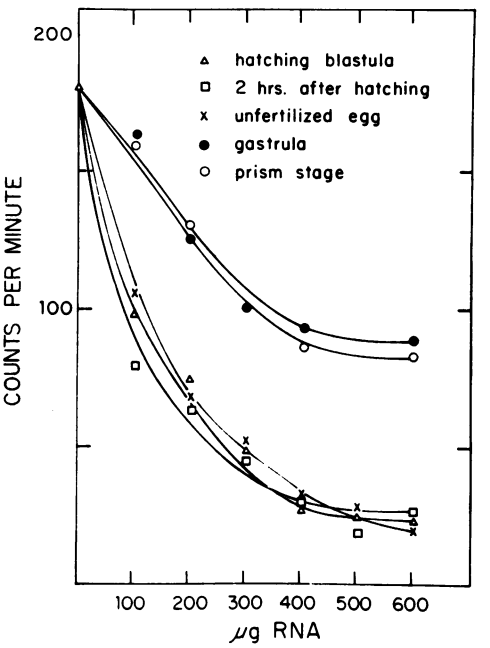

FIG. 2.—Comparison of RNA's from different stages of development. Unlabeled RNA samples prepared from different stages of development were compared by determining their inhibition of binding 100 μg of the labeled hatching-blastula RNA.

Third, the RNA from the gastrula and prism stages give rise to similar inhibition curves displaying a diminished inhibition corresponding to about 60 per cent homology in the RNA populations; that is, only about 60 per cent of the RNA present in the blastula stage continues to be generated in the two later stages.

Discussion.—At the level of discrimination provided by present techniques, it is seen that the mRNA population in developing sea urchins undergoes a shift between the blastula and gastrula stages. Our results show that about 40 per cent of the unfertilized egg or blastula-like mRNA is withdrawn from production in the later stages. To see if new types of mRNA are produced, we must turn to the experiments of Whiteley *et al.*,[8] who find indeed that unfertilized egg and blastula-type mRNA are deficient in approximately 40 per cent of the mRNA population characteristic of the gastrula and prism stages. Hence, between the blastula and gastrula stage there has been both a turning off of some gene transcription and a turning on of other gene transcription. Coincident with this is the continued and uninterrupted production of about 60 per cent of the mRNA which is common to all stages.

These results have an interesting bearing on the type and rate of protein synthesis that occurs in the early stages of development in view of the controversy over whether or not a significant qualitative change takes place in the new proteins

formed during early development. Spiegel *et al.*[9] have reported that in soluble proteins such a change does not take place even up to prism stage, which in *S. purpuratus* is reached only 72 hr after fertilization. However, a subsequent report by Terman and Gross[6] showed significant systematic changes in the pattern of proteins synthesized. These authors stated, "the differences in the kinds of proteins being synthesized or changing rates of the same set of proteins can be observed. The changes are obvious at gastrulation." Our results are consistent with the latter authors, and support the contention of Terman and Gross[6] that the "improved resolution of radioactive bands offered by autoradiographic methods" allowed the detection of changes of protein patterns not earlier observed.

It seems evident that a majority of the genes active in production of mRNA, at least in the very late stage of oögenesis, become activated soon after fertilization. We also know that the mRNA synthesized in the late stage of oögenesis is stored in the microsomes in the cytoplasm since the nonnucleate fragments which respond to activating stimuli can synthesize proteins.[10, 11] Obviously, the dormant stage of a mature unfertilized egg must be controlled at both the transcription and the translation level. Initially, at fertilization, an activation of the maternal mRNA takes place followed by an activation of the same sets of genes which were responsible for the synthesis of the maternal mRNA. This immediately raises the question of the importance and the role of the preblastula mRNA, since it has been shown[5] that in the presence of actinomycin D sufficient to inhibit the synthesis of mRNA, the embryos can develop up to the hatching-blastula stage. The inhibition of mRNA synthesis in the preblastula period neither affects the rate nor the type of protein synthesis, nor does it seem to affect significantly the rate of cell division. This last observation apparently holds only for *Arbacia punctulata*, since the same concentration of actinomycin D has a very severe effect on the rate of cell division in the case of *S. purpuratus*.[9] When the control embryos have reached the 16-cell stage, the actinomycin D-treated embryos were only in the eight-cell stage. The retardation in the advanced embryos was even more pronounced. It will be of great interest to know whether the proteins are synthesized at the same rate in the latter species, too.

Since the population of mRNA remains essentially constant through the hatching-blastula stage and an embryo can develop rather normally to that stage, there is no obvious role in this period for the mRNA being newly synthesized. However, this can be accommodated, along with the observation of Spiegel *et al.*[9] that the development of sea urchin embryos under conditions of total inhibition of mRNA synthesis is very much delayed, while there is no change in the protein pattern up to blastula stage[6] by assuming that the diminished supply of maternal mRNA per cell leads directly to a slower rate of cell division. This explanation would require that this type of messenger RNA be relatively stable.

The mRNA molecules which might be responsible for the production, for example, of the hatching enzymes or the development of gastrula (G factor) cannot be present in the unfertilized egg, since in the presence of actinomycin D the development is arrested at the blastula stage. Of course, there is a possibility that a very few new genes are activated between the postfertilization and hatching periods but these lie below the sensitivity of present methods.

Although our results and those of Whiteley *et al.*[8] show a considerable similarity

or identity between the maternal and blastula mRNA, possible existence of masked or stored mRNA that becomes functional only at a later stage cannot be ruled out. There are numerous cases reported in the literature where the simplest interpretation of the results assumes the existence of a masked, nonfunctional mRNA.[12]

Summary.—mRNA synthesized in the developing sea urchin through the blastula stage is essentially the same as that in the unfertilized egg. Between the blastula stage and the gastrula stage, about 40 per cent of this population disappears and is replaced by different mRNA that is characteristic of the later stages of development. However, this major shift in mRNA population arising from a combination of gene activation and deactivation leaves unchanged the maintenance of functions common to all stages programmed by an invariant portion of the mRNA population.

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