BASE COMPOSITION OF RNA SYNTHESIZED DURING CLEAVAGE OF THE SEA URCHIN EMBRYO

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Received January 15, 1965

During the period from fertilization to hatching of the blastula, the sea urchin embryo cleaves to about 1,000 cells. It shows no net gain in dry mass, protein, or RNA. Incorporation of labeled precursors into RNA begins very early, - probably during the first cell division in all species, and on the basis of sedimentation behavior, ribosomal RNA appears to be absent from the newly-synthesized material (Gross, Malkin, & Moyer, 1964; Nemer, 1963; Wilt, 1964; Comb & Brown, 1964; Glisin & Glisin, 1965). Cleavage RNA is heterogeneous in other embryos as well, e.g., amphibians (Brown & Littna, 1964) and teleost fish (Ajtkozhin, Belitsina, & Spirin, 1964). While the incorporation of precursors into "heavy" RNA is Actinomycin-sensitive (Gross, 1964), a significant fraction of the early incorporation is in low molecular weight material, and this labeling is largely insensitive to Actinomycin. RNA from Actinomycin-treated embryos exposed to labeled uridine shows a very large shift of radioactivity to CMP (Malkin, Gross & Romanoff, 1964), suggesting that most of the Actinomycin-insensitive incorporation represents terminal addition in the pCpCpA sequence of sRNA. The cytoplasm is the main site of incorporation (Gross, 1964). The results reported here indicate that end-labeling of sRNA is in fact an important part of the incorporation through the period of cleavage, and that the remainder of the new RNA is DNA-like.

Methods

1. Blastula RNA. Eggs from one ripe animal (Arbacia punctulata, supplied by Mr. Norris Hill, from the North Carolina coast) were extensively washed in Millipore-filtered sea water and freed of their jelly coats. They were suspended at a density of 10^3/ml in sea water containing 0.25 mg/ml streptomycin and $^{32}PO_4$ (2.25 µc/ml, carrierfree, neutralized form, from Iso-Serve, Inc., Cambridge, Mass.). The eggs were fertilized with a drop of concentrated sperm suspension and allowed to develop at 20°C on a rocker platform. Incubation was continued until hatching was complete (9 hours at this temperature). Embryos were collected by centrifugation and washed extensively in cold Ca++-Mg++-free Millipore-filtered sea water, then quick-frozen at -40°C. Our experience is that, combined with our routine methods for obtaining gametes (Gross and Cousineau, 1964), this procedure renders negligible the contamination of extracted RNA by radioactive RNA form microorganisms.

RNA was extracted from the pellets by a cold phenol technique slightly modified from that used by Brown and Littna (1964). The main changes were a 1-hour DNA'ase digestion of the purified RNA, followed by two re-extractions with phenol, and 18 hours of dialysis against a large volume of 0.01 M acetate buffer, ph 5.1, in the cold. 0.2 ml samples of the RNA were layered on 4.6 ml linear sucrose gradients (20-5% w/w) and centrifuged for 5 hours at 37,000 rpm. Two-drop fractions were collected and diluted with 1 ml distilled water. Absorbance at 260 mµ was determined for each fraction and then the radioactivity of an 0.1 ml aliquot from each was determined by liquid scintillation counting. The samples were pooled in four groups, representing four regions of the sedimentation profile, as shown in fig. 1. From each sample, RNA was precipitated in the presence of carrier yeast RNA by making the solutions 0.1 M in NaCl, 10^{-3}M in $MgCl_2$, and 66% (v/v) in ethanol.

The precipitates were washed with ethanol-ether and dried, then hydrolyzed in 0.3N KOH at 37°C for 20 hours. Hydrolyzates were neutralized with 70% PCA and centrifuged. The supernatant, containing the 2',3'-mononucleotides, was applied to Whatman 3MM paper, and the nucleotides separated by flat-plate electrophoresis at 40 V/cm for two hours (0.05M NH_4-formate at pH 3.5). Separated nucleotides were located on the paper by their ultraviolet absorption. Radioactivity was determined by scintillation or planchette counting of the spots, cut from the paper, with pieces of similar size cut from the regions between spots to give backgrounds, or by counting in a 4π strip-counter coupled to an analytical rate meter and recorder.

2. Ribosomal RNA. RNA was extracted from eggs containing ^{32}P incorporated during seven days of oögenesis. The extraction was with hot phenol under conditions that give 28S and 18S ribosomal RNA negligibly contaminated with non-ribosomal species. This was assessed from the constancy of specific activity across the sedimentation profiles*. The composition of the pool of acid-soluble nucleotides does not vary drastically from the unfertilized egg to the blastula (Nilsson, 1961), hence the finding of ribosomal RNA base ratios for 18S and 28S RNA made during maturation and non-ribosomal RNA base-ratios after fertilization would not be attributable simply to changes in the composition of the pools.

Results

The distribution of absorbancy at 260 mμ (i.e., pre-existing RNA) and counts (new RNA) in the RNA extracted from the blastulae is shown in fig. 1., and the base ratios for the new RNA from the four regions indicated on the figure (I through IV, centers of gravity at approx. 28,

*These preparations were made in connection with a study of RNA synthesis during oögenesis which will be reported elsewhere in detail (Gross, Malkin, and Hubbard, 1965).

18, 10, and 3 Svedberg units) are given in Table I. The table also contains base ratios for 18S and 28S ribosomal RNA made during oögenesis, and for comparison, analytical base ratios for bulk RNA and sperm DNA.

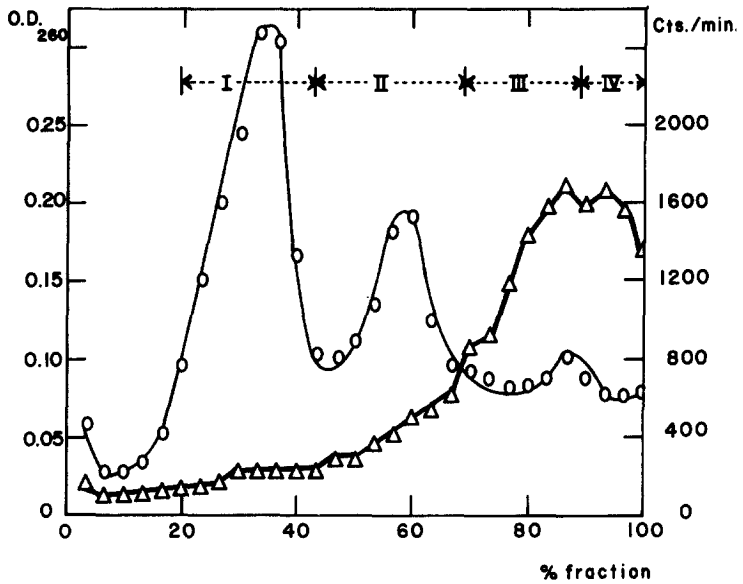

Figure 1. Sedimentation profile for absorbancy and radioactivity in RNA extracted from blastulae which had been exposed to $^{32}PO_4$ continuously from fertilization. Circles represent optical density, i.e., the stable, pre-existing material, and the three peaks, reading toward higher fraction numbers (centripetally) are 28S and 18S ribosomal RNA and 4S material, mainly sRNA. Triangles represent counts incorporated into RNA. This is the typical pattern, - heterogeneous material, distributed throughout the gradient, but rising very sharply in the 4S region. The Roman numerals represent the fractions pooled for base ratio determination.

The RNA made during oögenesis, and prepared under conditions that would tend to remove non-ribosomal species, has ribosomal base composition, at least to the extent that radioactivity base ratios agree with the bulk analytical values, as shown in the table, and with the values for G+C content characteristic of other animal cells. There is thus nothing inherent in the method that would tend to give distorted ratios if ribosomal RNA were being made during cleavage. None of the new RNA from the blastulae, however, has ribosomal composition, and the RNA of high molecular weight, i.e., Fractions I and II, is distinctly DNA-

Table I

BASE COMPOSITIONS (in mole %) OF SEA URCHIN RNA

Sample	A	U	G	C	G+C	Source
Fraction I	28.9	24.4	23.6	23.1	46.7	These experiments
Fraction II	28.1	27.4	21.8	22.8	44.6	" "
Fraction III	33.8	16.2	18.2	31.8	50.0	" "
Fraction IV	14.4	12.9	14.5	58.2	72.7	" "
28S rRNA*	22.4	18.8	32.8	26.0	58.8	" "
18S rRNA*	24.4	21.7	30.0	24.0	54.1	" "
Bulk RNA	22.3	20.7	29.6	27.4	57.0	Elson, Gustafson, & Chargaff (1954)
Sperm DNA	28.4	32.8(T)	19.5	19.3	38.8	Daly, et al. (1950)

like, both as to G+C content and on the basis that A/U and G/C are close to one. Fraction III has a higher G+C content than the heavy ones, but the origin of this effect is a sharp reduction in labeled UMP and GMP. Fraction IV has a very high G+C content, but again, this results from a dramatic elevation in labeled CMP, and a depression of labeling in the other nucleotides. This fraction represents the descending side of the 4S peak in bulk RNA, and its composition is close to that expected if most of the radioactivity had been incorporated through the action of an sRNA-pyrophosphorylase (Hecht, et al., 1958, Furth, et al., 1961, Lagerkvist and Berg, 1962, etc.) i.e., by end-labeling of sRNA in the pCpCpA triplet. The composition of fraction III suggests a mixture of dRNA and the RNA represented by fraction IV, although the AMP content is rather high. AMP is low, on the other hand, in fraction IV, if the base adjacent to the pCpCpA triplet is predominantly adenine, as it is in other species (see, e.g., Lagerkvist and Berg, 1962, Herbert and Wilson, 1962).

Discussion

These results indicate that most of the RNA made during the period of cleavage is DNA-like, and probably messenger RNA. Since there is no growth, in the strictest sense, during this period, and a reorganization of the pattern of protein synthesis is in progress (Gross and Baker, 1965), the situation is reminiscent, and perhaps not in a trivial way, of that following a "step-down" in microbial cultures. The intense end-labeling activity may reflect activation or synthesis of a pyrophosphorylase at fertilization, and it explains the actinomycin-resistant fraction of the RNA synthesis. It could be a cause of the activation of protein synthesis at the beginning of development. It should be noted, however, that evidence from the behavior of cell-free amino acid incorporation systems suggests that the levels and transfer capacity of sRNA do not differ significantly between the unfertilized and the newly-fertilized egg (Nemer and Bard, 1963, Hultin, 1961).

Acknowledgments

These experiments are supported by grants from the National Institutes of Health (GM 11534-01), the National Science Foundation (GB-2502), and the American Cancer Society (E-285A). We are grateful to Dr. V. R. Glisin, who allowed us to examine, in advance of its publication, a paper describing comprehensive studies on ^{32}P-labeled RNA from the cleavage stages of Lytechinus.

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