Release of maternal RNA from some particles as a mechanism of activation of protein synthesis by fertilization in sea urchin eggs*,**

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Since the discovery of activation of protein synthesis by fertilization, a number of mechanisms have been proposed. Recent investigations have been concentrated on the mode of utilization of preformed m-RNA, i.e. "maternal RNA" as proposed by Spirin and Nemer (1). Monroy et al. (2 - 4) concluded from their model experiment with trypsin that the removal of an inhibitory protein from ribosomes was a principal mechanism in the activation of protein synthesis by fertilization. The present paper shows that the mt-RNA bearing particles are different entities from ribosomes and that the release of mt-RNA from those particles may be a principal mechanism for the activation.

Matured eggs of the sea urchins, Hemicentrotus pulcherrimus and Pseudocentrotus depressus were used. No significant difference was observed between the two species. The subcellular distribution of RNA in fertilized and unfertilized eggs, fractionated by a modification of Hultin's method (5) in the presence of Ca^{++}, was similar to that seen in other animal tissues, and remained rather constant throughout fertilization while that of protein changed considerably. From these fractions RNA was extracted and purified, and the template RNA activity (6) was examined as shown in Table I. The stimulating activity of the RNA

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** Abbreviations: mt-RNA, maternal RNA; m-RNA, messenger RNA (this is used only for that synthesized after fertilization); t-RNA, transfer RNA.

Table I

Change of distribution of template RNA activity in subcellular fractions resulting from fertilization and tryptic digestion

	With RNA (0.5 mg) derived from				Without RNA
	H	S	M	P	cpm incorporated
Unfertilized	256	158	80	334	38
Fertilized	307	252	328	96	202
Unfertilized, trypsinized	321	218	317	136	73

(H, unfertilized whole homogenate; S, 105,000 x g supernatant; M, microsomes; P, 12,000 x g precipitate in the following fractionation.)

H. pulcherrimus eggs were fractionated by a modification of the method of Hultin (5) with addition of 2 mM $CaCl_2$ at the homogenization and centrifugation of the first precipitate. Fertilized eggs used were those developed for 30 min. after fertilization at 20°. The conditions for trypsinization were as follows: A 25% homogenate of unfertilized eggs in a medium containing 0.15 M sucrose, 0.1 M KCl, 0.01 M $MgCl_2$ and 0.05 M Tris buffer of pH 7.8 was treated with crystalline trypsin in a ratio of 1:2,000 of the enzyme to the protein as substrate for 10 min. at 28° at pH 7.8. The reaction was stopped by addition of an amount of crystalline soybean trypsin inhibitor double that of the trypsin added. RNA was prepared by the method of Kirby (7) with addition of DNase (20 µg per ml) and trypsin (50 µg per ml) in a medium of 0.01 M $MgCl_2$ and 0.05 M Tris buffer of pH 7.8 at 37° for 30 min. followed by the addition of trypsin inhibitor as above before the final dialysis. The incubation mixture for the assay of the effect of RNA contained 0.5 mg of RNA, 3.0 mg protein of ribosomes freshly prepared from unfertilized eggs (5), 3.0 mg protein of pH 5 enzyme (8) prepared from unfertilized eggs, 0.5 mg, 28,000 cpm of C^{14}-aminoacyl t-RNA which was prepared from E. coli t-RNA and C^{14}-algal acid protein hydrolysate (6.88 mc per mC) by the method of von Ehrenstein and Lipmann (9), 5 µM PEP, 1 µM ATP, 0.2 µM GTP, 0.2 unit of crystalline pyruvate kinase, 180 µM sucrose, 10 µM $MgCl_2$, 120 µM KCl, and 60 µM Tris buffer of pH 7.8 in a total volume of 0.5 ml. Incubation was at 28° for 30 min. The reaction was stopped by addition of an equal volume of 10% TCA containing 0.2% casamino acid. Preparation of the sample was followed by the method of Hultin (5) after dissolving in 5% NH_3. The table includes in the last line the values of ribosomal activity without addition of RNA for comparison.

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resided mainly in the 12,000 x g precipitate fraction in unfertilized eggs, but in the microsomal and the supernatant fractions after fertilization. Such a change in distribution of mt-RNA was also observed after brief treatment of the unfertilized homogenate with trypsin, or when unfertilized eggs were homogenized in the absence of Ca^{++}. But the same treatment with chymotrypsin gave no such effect. Trypsin treatment, as short as five minutes, was sufficient to cause the change in the

Table II

Stimulation of protein synthesis with the trypsin treated unfertilized ribosomes prepared under various conditions

Ribosomes prepared from the supernatant previously centrifuged at	Trypsinization	cpm incorporated
8,000 x g, 10 min.	−	36
	+	127
12,000 x g, 10 min.	−	33
	+	68
17,000 x g, 30 min	−	41
	+	46

Ribosomes were prepared from unfertilized H. pulcherrimus eggs as in Table I from the supernatant specified above. The ribosomes were subjected to tryptic digestion under the following conditions: the ribosomal preparation was trypsinized in a ratio of 1:100 of the enzyme to the protein as substrate with the same buffer as shown in the trypsinization in Table I for 10 min. at 37°. After the reaction, soybean trypsin inhibitor was added as in Table I and the mixture together with non-treated ribosomes in suspension were layered separately on a medium containing 0.3 M sucrose, 0.6 M KCl, 0.01 M $MgCl_2$, 0.035 M Tris buffer of pH 7.8, 0.006 M β-mercaptoethanol and centrifuged at 105,000 x g for 120 min. The ribosomal pellet was suspended in a medium of 0.15 M sucrose, 0.1 M KCl, 0.01 M $MgCl_2$ and 0.05 M Tris buffer of pH 7.8. The condition of the assay system was the same as in Table I using the ribosomal preparation without addition of RNA.

pattern of RNA template activity. This time is comparable to that required for the activation at fertilization. It can hardly be considered that the treatment induces a substantial effect on the binding of the RNA and ribosomes, since no effect was observed by the treatment of unfertilized ribosomes on additional stimulating activity with added RNA. These facts suggest that the mt-RNA resides in some particles other than ribosomes. The ribosomal preparations from various supernatants shown in Table II had different stimulating activities, the higher g supernatant from which ribosomes were prepared providing less activity. This result shows the mt-RNA is contained in particles which sediment faster than ribosomes. This suggests that the activation by the trypsin treatment may be due not to the removal of some inhibitory proteins from ribosomes but to the release of the mt-RNA from other particles besides ribosomes. The RNA of the particles seems to be liable in release by

Table III

Effect of combination of the particulate fractions on the stimulation of protein synthesis by trypsinization

	P	M	P + M
			cpm incorporated
Non-treated	10	22	38
Trypsinized	74	51	113

Preparation of the particles and the conditions of trypsinizations, and thus abbreviations are the same as in Table I. The samples as indicated were incubated with or without trypsin for 10 min. at 28° in advance. The protein and RNA contents of the samples were 27.6 mg and 0.45 mg for P, and 8.4 mg and 1.42 mg for M, respectively. The volume in the trypsinization and control incubation was 0.15 ml, and after the reaction, it was changed to 0.20 ml. The assay for the stimulation of protein synthesis was carried out in the same way as in Table I with the use of the samples in place of RNA directly. The addition of unfertilized ribosomes (2.8 mg in protein) was changed in the system of M and P + M to that of 1.4 mg in protein, which were regarded as an equal amount of ribosomes as a whole on calculating the content of RNA.

trypsinization compared with that of ribosomes, i.e. the selective release of RNA from the particles was observed by the treatment.

Sucrose density gradient analysis of RNA prepared from P^{32}-labeled ovaries showed the transfer of RNA of about 10S from the 12,000 x g precipitate to the microsomal fraction after a brief trypsinization. In a similar way, it was observed that the RNA of the precipitate from short term labeled ovaries combined with ribosomes to form polyribosomes without any energy supply *in vitro*. Thus there may be no particular necessity to assume that the mt-RNA binds to ribosomes by a further complicated mechanism in the formation of polyribosomes, as was suggested by Hultin (10, 11).

The above experiments suggest that the mt-RNA is contained in particles sedimenting faster than ribosomes and is released by the action of trypsin. The released RNA may combine with ribosomes to form polyribosomes spontaneously. This hypothesis was tested directly by comparing the effect of trypsinization on the combined 12,000 x g precipitate and microsomal fractions with the effect on the separate frac-

tions. The result showed that the combined fraction had a much higher ability to synthesize protein than the individual fractions (Table III).

The last possible mechanism which have been considered to explain the activation of protein synthesis following fertilization is the activation of t-RNA by the addition of the CCA-terminal nucleotides (12) as was suggested by instant incorporation of nucleosides into t-RNA after fertilization (12 - 14). We compared the stimulating activity of t-RNA purified from fertilized and unfertilized eggs and found no difference between them. Thus the activation of t-RNA seems not to be involved in the activation. Recent studies are failed to show any changes in amino acid activating enzymes (15), transfer factors (5, 16), and the participation of m-RNA (13, 17 - 21) following fertilization.

The combination of m-RNA and particular particles other than ribosomes in animal cells is now generally accepted (1, 22 - 24). The localization of the mt-RNA in fairly heavy particles is supported on the basis the experiment of Baltus et al. (13). The "heavy polyribosomes" in the work of Spirin and Nemer (1) were found after fertilization, and may be quite different from the particles implicated here (25). In the authors' opinion, the result obtained by Monroy et al. (2, 3) may be attributable to a possible contamination of the latter ribosomal preparation with the particles. According to the definition of Spirin et al. (22), the particles are thought to be a kind of "informosomes". The attachment of the mt-RNA to the particles may be favorable for the protection of the RNA against possible decomposition for a long survival awaiting fertilization.

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