On the Protein Composition of Ribonucleoprotein Particles before and after Fertilization of Sea Urchin Eggs (*Echinus esculentus*)

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Ribonucleoprotein Particles

Experiments indicate that eggs and embryos of the North Sea sea urchin *Echinus esculentus* are excellent material to investigate the regulation of protein synthesis before and after fertilization. The dormant egg contains several proteins which are absent from embryos. Such additional proteins have been detected on the small ribosomal subunits, in the salt wash of whole ribosomes, and associated with cytoplasmic messenger ribonucleoprotein particles.

Introduction

Early development of sea urchin embryos depends on newly activated protein synthesis. The increase in rate of proteins ynthesis is not paralleled with an equally rapid onset of transcription [1]. Ribosomes and messenger RNA found in the system at the moment of insemination have been synthesized by the maturing oocyte in the maternal organism [2, 3]. The question why neither ribosomes nor messenger RNA assume full activity before fertilization has been explained in three ways by different groups. It has been suggested and in part experimentally supported,

1. that most of the ribosomes are inactive, *e. g.* blocked by an inhibitor [4, 5];
2. that messenger RNA is present in "masked" form [6, 7] as protein complexes, called informosomes [8];
3. that initiation and elongation factors are lacking or are barred away from the protein synthesizing complex [9].

At this moment neither suggestion can be excluded, most likely all three are realized.

That ribosomes of unfertilized sea urchin eggs are inactive *in vivo* and *in vitro* [10, 11] is obviously due to an inhibitory protein, for it can be removed by proteases [4]. Recently Metafora *et al.* [12] have isolated such a protein from Mediterranean sea urchin species, which is detached from egg ribosomes by buffered 1 M NH_4Cl.

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Abbreviations: SDS, sodium dodecylsulfate; RNP(s), ribonucleoprotein particle(s) *i. e.* ribosomes and/or informosomes.

Echinus esculentus of the North Sea proved to be suitable to reinvestigate the subject; because mid May "full" animals provide numerous clear eggs, which develop with a high degree of synchrony after artificial insemination. In this contribution we report on supplementary proteins in the egg, likely for causing its dormancy, which disappear after fertilization.

Material and Methods

Sea urchin eggs and embryos preparation

Sea urchins *Echinus esculentus* were collected inside and outside of the Helgoland harbor constructions. They were stored in tanks with running sea water (10 °C) until used (1 – 5 days). The gonads were carefully excised and transferred to millipore-filtered sea water. Through all further manipulations eggs were held at approximately 12 °C (temperature of the North Sea at this time). Spontaneously shed eggs were resuspended in sea water and allowed to settle twice. The jelly coat was removed with acid sea water (pH 5), and settled jellyless eggs were resuspended once in sea water and allowed to settle again. An aliquot of these cells was inseminated. Only preparations yielding less than 5% eggs without high fertilization membranes were used in the experiments. Some batches of fertilized eggs were raised to two-cell or eight-cell embryos in a beaker on a magnetic stirrer at 12 °C. Under these conditions plutei will develop within 4 – 5 days.

Ribosomes preparation

Eggs or embryos were sedimented by low speed centrifugation and resuspended in an equal volume of buffer A (10 mM Tris-HCl, pH 7.4; 10 mM KCl; 3 mM $MgCl_2$; 7 mM mercaptoethanol). They were homogenized in a Dounce homogenizer, and ribosomes were prepared and purified by ultracentri-

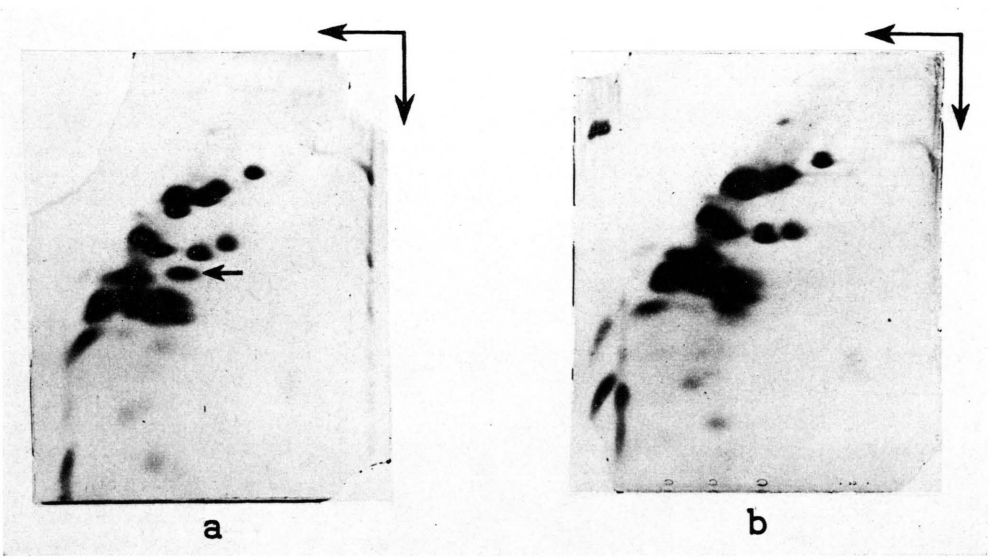

Fig. 2. Protein pattern of the small ribosomal subunits from eggs and emrbyos as revealed by two dimensional acrylamide gel electrophoresis according to the method of Kaltschmidt and Wittmann [14]. Electrophoresis in the first dimension was run in tubes on a Shandon-apparatus for the second dimension a Desaga-apparatus was used. a. Proteins of small ribosomal subunits from eggs; b. proteins of small ribosomal subunits from embryos.

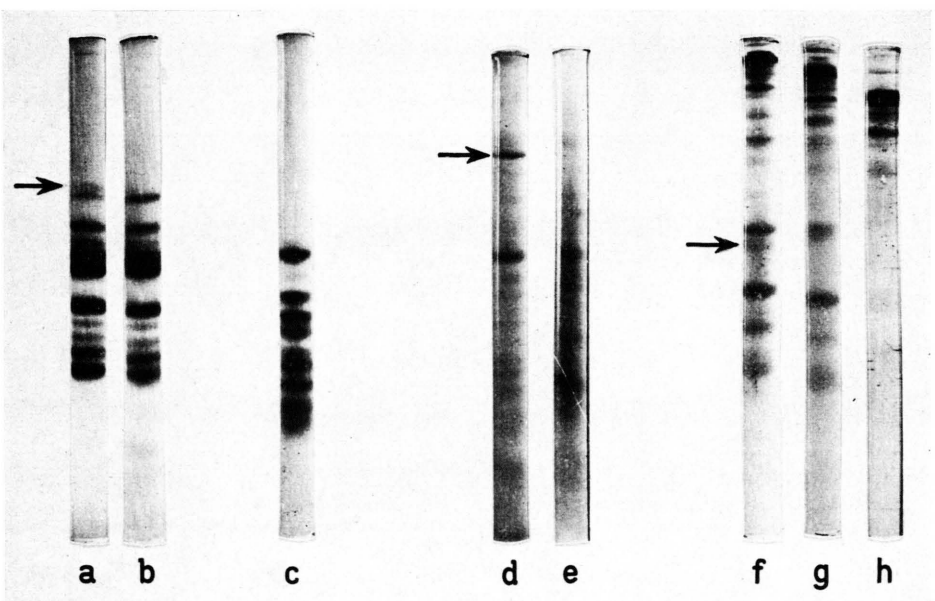

Fig. 3. Acrylamide gel electrophoresis of protein fraction from eggs and embryos; a. proteins of the small ribosomal subunit (45S) from eggs; the arrow (1) indicates a band not found in b; b. proteins of the small ribosomal subunit (45S) from embryos; c. proteins of the large ribosomal subunit (60S) of eggs; d. proteins sloughed off in buffered 500 mM KCl (*i. e.* salt wash, S.W.) from egg ribosomes; e. same as d. but from embryo ribosomes; f. informosomal proteins from egg cytoplasm; g. informosomal proteins from embryo cytoplasm; h. control egg cytoplasm not RNase treated.

fugation. Only fractions containing the 80S monoribosomal peak were pooled and sedimented by centrifugation (Spinco L 3, rotor 65, at 65000 rpm, 5 hours).

Ribosomal subunits were prepared by resuspending the ribosomal pellet in buffer B (10 mM Tris-HCl pH 7.4; 500 mM KCl; 3 mM $MgCl_2$; 7 mM mercaptoethanol) which was layered on a 15 – 30% sucrose gradient in buffer B. The subunits were adequately separated after 16 hours centrifugation (rotor SW 27, at 24000 rpm, 4 °C). The fractions containing either subunit were pooled; the particles were precipitated with 2 vol. of ethanol at – 12 °C overnight and collected by centrifugation. The pellets were heated to 60 °C and dissolved in buffer C (10 mM sodium phosphat buffer, pH 7.4 with 14 mM mercaptoethanol and 2% SDS), and analysed by gel electrophoresis according to Weber *et al.* [13], or Kaltschmidt *et al.* [14].

Preparation of free cytoplasmic particles containing mRNA (informosomes) [8]

Informosomes were obtained by centrifugation of a postmitochondrial supernatant at 60000 rpm (rotor 65) for 3 hours. The pellet was resuspended in buffer B, and centrifuged at 18000 rpm for 20 min. The supernatant was transferred to a tube containing a cushion of 30% sucrose in buffer B, and centrifuged at 30000 rpm for 17 hours. The pellet was then resuspended in buffer B and treated with pancreatic RNase (2 μg/ml) at 20 °C for 20 min. The digest was cleared from remaining aggregates by centrifugation (SW 65, at 35000 rpm, 5 hours). The upper third, the protein fraction, was analysed by gel electrophoresis.

Density analysis

CsCl density gradient centrifugations of glutaraldehyde fixed ribosomes, were performed as described elsewhere [16].

Results

Differences in density of egg and embryo ribosomes

We wondered whether *Echinus esculentus* possesses on its ribosomes an inhibitor, which is sloughed off after fertilization, or which is exchanged for or neutralized by an additional protein. Since the first mechanism leads to a different protein content of the embryo ribosomes, we have subjected ribosomes from eggs and embryos to CsCl density gradient centrifugation. As seen from Fig. 1 embryo ribosomes have a higher density than egg ribosomes. 11 experiments yielded a density of $\varrho = 1.5730$ for egg ribosomes. The densities of embryonic ribosomes were scattered over a broader range between 1.5750 and 1.5880. The mean density resulting from all experiments with egg ribosomes is $\varrho_1 = 1.5750 \pm 0.0031$. The mean density of embryo ribosomes is $\varrho_2 = 1.5827 \pm 0.0040$.

According to Student's equation the difference is significant: meaning that the 2 series of measurements were obtained from 2 different types of ribosomes. According to Spirin's formula % protein $= \frac{1.85 - \varrho}{0.006}$ egg ribosomes have 45.8% proteins and ribosomes from embryos 44.5% (considering for rRNAs 5S, 18S, 28S a total molecular weight of 2.2×10^6).

This difference in density can be explained, by a difference in the protein content of the ribosome. But, it may also be possible that denser ribosomes contain additional RNA (*e. g.* tRNA or mRNA fragments); conformational changes could also explain differences in density.

Ribosomal proteins

In order to assess the three possibilities ribosomes were further analysed for their protein composition by means of mono and bi-dimensional gel electrophoresis. They were dissociated in buffered 0.5 M KCl, yielding besides large and small subunits a supernatant fraction, the so-called "salt wash" (or "wash"). As seen from Fig. 2 * the small ribosomal subunits from eggs contain at least one additional protein.

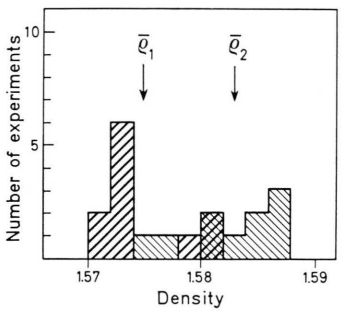

Fig. 1. Densities of egg and embryo ribosomes. 0.25 mg of ribosomes were suspended in buffer A fixed with glutaraldehyde and centrifuged in a CsCl gradient with ranged from 30 to 57% CsCl at equilibrium (39000 rpm, 20 hours at 20 °C). Fractions of 5 drops were collected, the refractory index and the absorbance at 260 nm were determined. The mean density for egg ribosomes is $\varrho_1 = 1.5750$, for embryo ribosomes $\varrho_2 = 1.5827$.

* Figs 2 and 3 see Plate on page 464 b.

Since these proteins tend to become insoluble in urea, routine experiments were performed by one-dimensional SDS-gel electrophoresis. Small subunits of egg ribosomes possess a protein fraction of 50000 daltons approximately, which is lacking in preparations of embryos Fig. 3 a and b). Large subunits of egg and early embryo ribosomes show no difference in protein composition (Fig. 3 c).

We have noticed that the "salt washes" from egg ribosomes contain higher quantities of protein than "salt washes" from embryonic ribosomes. There are also qualitative differences: "washes" from egg ribosomes show two more clearly defined bands of 90000 daltons and 50000 daltons, the former is absent from embryo "washes" (Fig. 3 d and e). Besides a host of minor bands is detected with our method.

Informosomal proteins

As indicated in the introduction cytoplasmic mRNA has been found associated with proteins first in unfertilized eggs by Spirin and Nemer [8]. It has been proposed that such mRNA-protein complexes (informosomes) are inactive and "masked" formes of mRNA [8]. Later, informosomes have also been found in embryos [3] and in other cells [18]. Consequently, the "masked" form of mRNA is not explained by the mere presence of proteins alone. It is, however, apparent that there is among them a specific protein, which renders egg "informosomes" inactive. We have, therefore, investigated the protein composition of mRNA containing particles with sedimentation rates between 20S – 100S, *i. e.* informosomes from egg and embryo cytoplasm. According to our experience with HeLa cells, a rapid analysis of the proteins moiety of informosomes is achieved by digesting the particles with RNase and separating the liberated proteins by SDS-gel electrophoresis [15]. Informosomes from eggs yields 5 bands, whereas the same preparations from embryos show 4 bands only (Fig. 3 f and g). There are, however, some higher molecular bands on the top of the gel. As seen from Fig. 3 h these were also present in controls which had not been treated with RNase. We consider these bands as contaminants carried over from the cytosol. The 4 or 5 bands evidently associated with RNA, are likely to be informosomal proteins.

Discussion

The research reported in this paper has been limited to the question: whether and where additional proteins are provided by the oocyte, likely to cause the dormancy of the egg. Our results are in good agreement with the ones of Gambino *et al.* [17], but diverge in some interesting points. Egg ribosomes, likely to carry more protein than embryo ribosomes, are found to be less dense in CsCl density gradient centrifugation experiments. Prior fixation by glutaraldehyde yields sharp homogeneous fractions; formaldehyde-fixed particles tend to be more heterogeneous. Formaldehyde-fixed egg and embryo ribosomes of the Mediterranean species did not exhibit such differences in density [17].

High salt buffers remove a host of proteins from both egg and embryo ribosomes. There is, at least, one fraction missing from embryo salt wash. Gambino *et al.* [17] use 1 M NH_4Cl and 18 mM Mg^{2+}. whereas we have applied 0.5 M KCl and 3 mM Mg^{2+} according to our routine with HeLa cells. We have demonstrated in a earlier paper [16] that the different alkali ions have a quantitatively different action on ribosomes (0.5 M KCl give results similar to 0.9 M NH_4Cl) and act as antagonist to Mg^{2+} ions; qualitative differences have not been demonstrated.

We also confirm the existence of informosomes, in the original sense of Spirin and Nemer [8] (see also ref. 3). These particles are more abundant before fertilization and seem to diminish gradually. They contain a host of proteins in the range of 20000 daltons to 50000 daltons, which corresponds well with data obtained from HeLa cell informosomes [15]. In sea urchin embryo informosomes again one protein is missing. This could indicate that inactive egg informosomes loose this protein to become potentially available to translation after fertilization.

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