Centrifugation does not alter spatial distribution of 'BEP4' mRNA in paracentrotus lividus EGG

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Abstract ***Paracentrotus lividus*** **unfertilized eggs were centrifuged in a sucrose gradient, so to split each into two parts: a nucleated light fragment and an anucleated heavy fragment. Northern blot analyses utilizing a bep4 probe as animal marker and H2A histone gene and 12S-mit RNA as controls indicate that the eggs are elongated along the animal-vegetal axis during centrifugation and thereafter split into an animal and a vegetal half. Treatment of the eggs with colchicine before centrifugation abolishes the animal localization of bep4 mRNA.**

Key words: Centrifugation; A/V axis; Localized mRNA; Cortex

1. Introduction

Animal-vegetal axis is already fixed in a sea urchin unfertilized egg [1]. Maternal cytoplasmic factors localized in particular regions of the same egg are responsible for specification during early embryogenesis. It is not easy to isolate these factors which might help to study cell fate. Centrifugation has been a useful tool in sea urchin developmental research [2]. When sea urchin eggs are loaded onto a discontinuous sucrose density gradient, a cytoplasmic stratification occurs. They, indeed, assume first a dumbbell shape and then are divided in a centripetal half containing the nucleus and the cortical cytoplasm, and in a centrifugal half containing mitochondria, yolk and pigments.

This study has been carried out to begin to understand whether some cytoplasmic components, such as factors that are relevant for animal-vegetal axis determination, are maintained in their position after centrifugation. For this purpose we utilized as a molecular marker a mRNA, called bep4, coding for a cell surface protein that we have already demonstrated to be localized in the animal half of the *P. lividus* egg and that is relevant for the development of the part of the embryo deriving from this side of the egg [3–7]. Moreover, we have also demonstrated that the cytoskeleton is involved in bep4 localization, and probably this association is important to establish the animal-vegetal axis of the egg and embryo [8].

Here we provide evidence that centrifugation does not alter bep4 localization, and that this represents a good method to separate animal and vegetal morphogens in bulk amounts.

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Abbreviations: *P. lividus*, *Paracentrotus lividus*; sw, sea water

2. Materials and methods

2.1. Centrifugal bisection

Sea urchin unfertilized eggs were loaded onto a two layer discontinuous sucrose gradient. The sucrose gradient consists of the following solutions: at the bottom 1 M sucrose in sea water (sw) and at the top 0.5 M sucrose in sea water. The eggs were centrifuged in a SW28 rotor at 16000 rpm for 15 min at 4°C. After centrifugation, the light nucleated fragments were found in the upper layer and the heavy anucleated fragments in the lower layer. The fragments were collected and rinsed two times with sw.

In the experiments in which the microtubule inhibitor was employed, the eggs were incubated for 1 h in sea water containing 1 μg/ml colchicine, and then centrifuged.

2.2. Northern blot hybridization

RNAs extracted from nucleated and anucleated fragments were separated on denaturing gel and the Northern blots hybridized with the labeled *Sal-Stu* fragment of bep4, the *Drosophila* 12S mitochondrial RNA (mtRNA) (gift of S. Alziari) and with the H2A histone gene (gift of G. Spinelli), utilizing standard procedures [9].

2.3. In situ identification of BEP RNA

Whole mount in situ hybridization and in vitro transcription of the utilized probe were carried out according to Montana et al. (1996) [10].

3. Results

3.1. Northern blots of RNAs from nucleated and anucleated halves

P. lividus eggs were centrifuged in the discontinuous gradient and were separated in nucleated and anucleated fragments. The nucleated and anucleated fragments were obtained as a light centripetal and a heavy centrifugal fraction respectively. Usually, under the utilized conditions, the nucleated fragments were larger than the anucleated ones.

In order to ascertain whether the distribution of the RNAs changed after centrifugation, we extracted total RNAs from nucleated and anucleated fragments. Northern blots, containing these RNAs were hybridized with a labeled specific fragment of a bep4 clone which is known to be localized in the animal half of the *P. lividus* egg [6,7]. The same filter was also hybridized both with labeled fragments of 12S mtRNA that should reside in the anucleated fragment and with the labeled H2A histone gene, which is known to be localized in the pronucleus of the mature egg [11]. The results are shown in Fig. 1. The bep4 mRNA is present only in the nucleated fragment, as it is the H2A, whereas the 12S mtRNA is present in the RNA extracted from the anucleate fragments where the mitochondria are present. These data shown that centrifugation does not alter the position of *bep4* mRNA.

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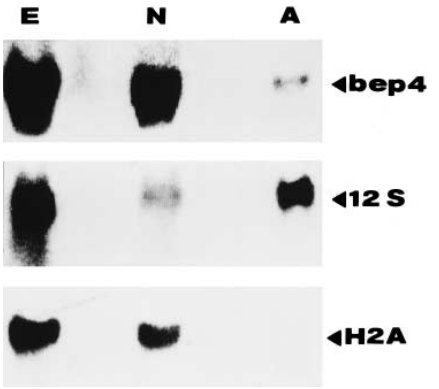

Fig. 1. Distribution of RNAs in of *P. lividus* egg halves obtained after centrifugation. Total RNA was extracted from entire eggs (E), nucleated fragments (N) and anucleated fragments (A) and the Northern blot was hybridized either with bep4 or with 12S or with H2A histone probes. The arrowheads indicate the hybridization band of each probe used.

3.2. *Localization of bep4 mRNA in nucleated and anucleated fragments by in situ hybridization*

In order to further confirm the data obtained by Northern blot hybridization, we investigated about the localization of *bep4* mRNA by whole mount in situ hybridization of both nucleated and anucleated fragments. Fig. 2 shows the results obtained utilizing as probe an antisense fragment of *bep4*. As expected, only the nucleated fragment is stained with the *bep4* probe, whereas no hybridization is detected in the anucleated fragment.

3.3. *Effect of microtubule inhibitor on localization of bep4 as revealed by Northern blot analysis*

Our previous studies have demonstrated that bep4 mRNA is associated to the cytoskeleton [8]. In order to investigate whether this association permits the maintenance of this mRNA in the nucleated part of *P. lividus* eggs during centrifugation, we incubated the eggs with colchicine before centrifugation. Colchicine is known to bind to tubulin and to cause depolymerization of microtubules. After this treatment we extracted total RNA from nucleated and anucleated fragments. Northern blot hybridizations with bep4 probe show that the message is now present in both fragments (Fig. 3). The H2A mRNA, instead, that in the previous experiment shared the fate of bep4, is not affected by the colchicine treatment and remains present only in the RNA extracted from the nucleated fragment in agreement with the fact that it is located inside the pronucleus [11]. Moreover, the 12S probe always hybridizes with the RNA extracted from the anucleated fragment, as expected because it resides inside the mitochondria, localized in the centrifugal part independently of the cytoskeleton. This result indicates that bep4 is associated with the network of microtubules present in the cortex.

3.4. *Detection by in situ hybridization of bep4 in egg fragments after treatment with colchicine*

In order to visualize the results obtained above, the fragments obtained after incubation of the eggs with colchicine were fixed for whole mount in situ hybridization that was carried out by utilizing bep4 antisense fragments. The result, shown in Fig. 4, indicates that the bep4 message is now present in both nucleated and anucleated fragments. Thus, colchicine treatment has caused releasing of this mRNA from its normal localization, and allowed free diffusion in the cytoplasm.

4. Discussion

In this report we provide evidence that bep4, a localized mRNA, remains in its position after high speed centrifugation able to split the eggs into nucleated and non-nucleated halves. Moreover, two other maternal localized mRNAs, belonging to the same gene family, bep1 and bep3, maintain the same position after centrifugation at the same speed (data not shown). This result indicates that the animal-vegetal axis of the egg approximately coincides with the centrifugal axis under these experimental conditions. Bep4 mRNA, the marker utilized in these experiments, is localized in the animal part of the egg and this localization is due to association with the cytoskeleton. This association is the factor that permits this RNA to remain immobile under a centrifugal force which causes egg splitting. Treatment with colchicine, indeed, destroys the cytoskeleton network, and the bep4 mRNA, normally present only in the nucleated fragment, diffuses also into the anucleated one. This is in agreement with Morgan's hypothesis [12] that the cortex, a centrifugally immovable component, might play a crucial role in the determination

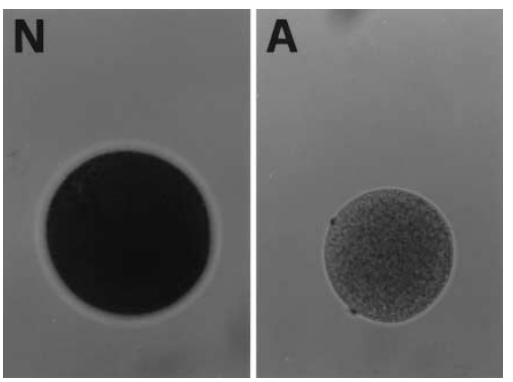

Fig. 2. Localization of bep4 mRNA in centrifugally bisected *P. lividus* eggs. Three dimensional images of whole mount in situ hybridization with antisense bep4 of nucleated (N) and anucleated (A) fragments.

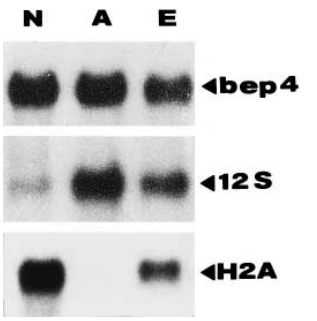

Fig. 3. Effect of colchicine on bep4 mRNA representation in nucleated and anucleated fragments. Northern blot of RNA extracted from *P. lividus* eggs (E) incubated with colchicine, divided in nucleated (N) and anucleated (A) fragments and hybridized with bep4, 12S and H2A probes. The arrowheads indicate the hybridization band for each probe used.

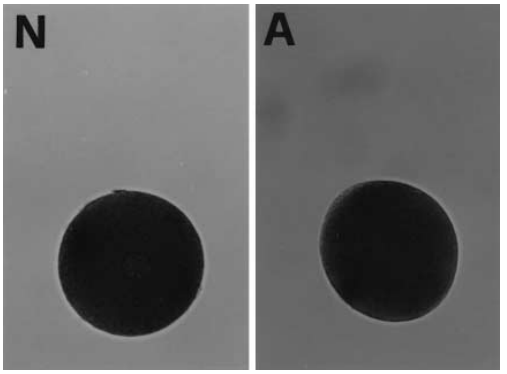

Fig. 4. Delocalization of bep4 mRNA in *P. lividus* bisected eggs treated with colchicine. Whole mount in situ hybridization of nucleated (N) and anucleated (A) fragments incubated with antisense bep4 mRNA.

of the animal-vegetal axis. At the moment it is not known if all the mRNAs that are relevant for the development of the animal parts are associated with the cytoskeleton. Probably, there are cytoplasmic components that are bound to the cytoskeleton and that remain immobile when submitted to centrifugal force and other ones that are mobile and are divided between the light and the heavy fragments. Experiments done by classical embryologists, indeed, have not revealed clearcut differences in developmental capacity between light and heavy fragments of the egg. The centripetal halves, if fertilized, tendentially develop into permanent blastulae but sometimes develop into plutei, whereas centrifugal halves always become plutei. This indicates that the nucleated half behaves tendentially like an animal half and the anucleate half like a vegetal half. This difference in development is probably due to the point at which the egg is divided during centrifugation. Indeed, microsurgery experiments in which *P. lividus* egg has been cut along the equator with a glass needle in two halves have recently demonstrated that the animal half corresponds to the side in which the pronucleus is located [6,7]. Unfortunately at the moment no molecular marker of the vegetal part of the unfertilized egg has been cloned and we are not able to hypothesize what permits localization in the vegetal part of the egg and if these components are moved by centrifugation. However centrifugation may represent a simple method to enrich animal or vegetal components that might be then separated and analyzed to study cell lineage. Recently, indeed, this technique has been employed to enrich three different basic proteins with heavy fragments of starfish oocytes [13].

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