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Spatial distribution of two maternal messengers in *Paracentrotus lividus* during oogenesis and embryogenesis

(sea urchin/*in situ* hybridization/mRNA localization/cell surface proteins/protein localization)

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ABSTRACT **We demonstrated that two mRNAs that are synthesized during the vitellogenic period of oogenesis and that code for cell surface proteins are asymmetrically distributed in the unfertilized egg of *Paracentrotus lividus*. At fertilization, these RNAs rapidly localize in the cortical zone at the animal pole of the egg. They are then detected in the mesomeres and the macromeres, but not in the micromeres, and thereafter are found in the ectoderm but not in the vegetal plate, mesenchyme cells, or early intestine. They disappear in late gastrula. The proteins synthesized by these mRNAs show the same territorial location during the period examined here, which included the unfertilized egg and the 16-blastomere stage. These conclusions were reached on the basis of *in situ* hybridization and immunostaining experiments, as well as Northern and Western blot analyses of isolated blastomeres. The possible significance of this asymmetric distribution of these two mRNAs and proteins in the establishment of the animal/vegetal axis is discussed.**

Determination of the embryonic axes has long been a central problem in developmental biology. Recent studies in *Drosophila* on the underlying molecular mechanisms have demonstrated that information that specifies the anteroposterior and the dorsoventral axes of the egg is already laid down during oogenesis (1) and that a cascade of events determines the position of the head and of the thorax. Certain maternal genes lead to localization of the product of the bicoid gene, a transcription factor, producing an anterior/posterior concentration gradient that regulates zygotic target genes (2, 3). A maternal RNA, nanos, the product of which prevents the translation of a transcription factor encoded by a ubiquitous maternal RNA is initially localized at the posterior pole (4). Another cascade of events occurs in the establishment of the dorsoventral axis in which the product of the maternal Toll gene, probably a membrane receptor, and the maternal dorsal gene are involved (5, 6). The possibility that determination of the embryonic axes is correlated with different locations of maternal RNAs in different egg territories has also been suggested for the eggs of *Xenopus*, where Melton (7, 8) found that the Vg1 RNA, whose product shares homologies with a type β transforming growth factor, is transferred to the vegetal pole of the oocyte at the time of its maturation.

In contrast to *Drosophila* and *Xenopus*, nothing is known to date about the molecular basis of axis determination in sea urchin embryos, where the problem of an underlying mechanism was first raised at the beginning of this century. Microsurgery experiments performed mostly by Horstadius (9) had demonstrated that if unfertilized eggs were equatorially cut into two halves and each half was fertilized, the animal halves always developed into ciliated hollow spheres ("animalized" embryos), whereas the vegetal halves produced "vegetalized" embryos with an excess of vegetal structures; while observation indicated the presence of some asymmetry along the animal/vegetal axis of the unfertilized sea urchin egg, no satisfactory explanation for this asymmetry was provided, except for the suggestion of a double gradient proposed in 1928 by Runnstrom (10, 11). There was no evidence of a differential RNA localization in echinoderm eggs, as has been demonstrated in *Xenopus* and *Drosophila* eggs.

We report here that two maternal mRNAs, *bep1* and *bep4*, both of 1.4 kb, are asymmetrically distributed in the unfertilized egg of *Paracentrotus lividus*. They belong to a multigene family and code for proteins that become localized in the cell surface during early embryogenesis and may be relevant for cell adhesion (12, 13). Their molecular mass is 33 kDa, and their messengers show a particular structural organization; two well-conserved domains surrounding a single specific domain (12, 13). We report here that both these mRNAs and the corresponding proteins are located exclusively in one-half of the unfertilized egg (probably the animal half) and become localized at the animal pole immediately after fertilization. Those parts of the embryo that are derived from the vegetal half of the egg are devoid of these two mRNAs. After the gastrula stage, these mRNAs are no longer found in the embryo.

MATERIALS AND METHODS

***In Vitro* Transcription Using pGEM Plasmid.** The *Sac* I/*Stu* I and *Ava* I/*Stu* I fragments of *bep1* and *bep4*, respectively, containing specific parts of both clones (12), were subcloned in the *Sac* I/*Hin*cII and *Ava* I/*Hin*cII sites of the transcription vector pGEM2. These constructs were used to synthesize antisense RNA probes with a Riboprobe kit (Promega), according to the manufacturer's instructions with uridine 5′-[α-[^{35}S]thio]triphosphate (1000 Ci/mmol; 1 Ci = 37 GBq; Amersham). Probes were synthesized to a specific activity of 2×10^8 cpm/mg. The final concentration of the probe in the hybridization solution was equivalent to 12.5×10^6 cpm/ml. The presence of transcripts was analyzed by 6% polyacrylamide/7 M urea gel.

***In Situ* Hybridization.** Paraffin sections of eggs and embryos were spread onto polylysine-coated slides, as described by Cox *et al.* (14). Hybridization and washing were performed according to Cox *et al.* (14); the only modification was inclusion of 10 mM 2-mercaptoethanol in all the washing steps. Slides were processed for autoradiography as described by Cox *et al.* (14). Exposure time was 2 days for the sections shown.

Oocyte Purification. Purification was carried out according to Giudice *et al.* (15). Sea urchin female gonads were suspended in 100 ml of Ca^{2+}-free seawater containing 100 mg of Pronase (Calbiochem; B-grade) and the released oocytes were freed from the gonads by filtration. The sedimented

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oocytes were layered on top of a 5–20% Ficoll gradient in seawater and centrifuged in a swing-out rotor at 700 × *g* for 20 sec.

Separation of Meso- and Macromeres and Micromeres. The cells were separated by a modification of the Harkey and Whiteley procedure (16). Sea urchin eggs were fertilized in 2 mM *p*-aminobenzoic acid. Sixteen-cell stage embryos were dissociated into single cells in Ca^{2+}- and Mg^{2+}-free seawater, layered on top of a 5–20% Ficoll gradient in seawater and centrifuged in a swing-out rotor at 1000 × *g* for 30 sec.

RNA Extraction and Northern Blot Hybridization. Total RNA was isolated by using an acid guanidinium isothiocyanate/phenol/chloroform method (17). The RNA (10–20 mg) was electrophoresed on 1% agarose/1.1 M formaldehyde gel and transferred onto nylon membranes (Amersham). Blots were hybridized with labeled fragments specific for bep1 and bep4 (12) and with a fragment containing a portion of an 18S rRNA gene (gift from Rainer Barbieri) at 65°C in 4× standard saline citrate/5× Denhardt's solution/0.5% SDS.

Gel Electrophoresis and Western Blot Analysis. Samples (50 mg) were resuspended in an equal volume of 2× sample buffer (0.0625 M Tris·HCl, pH 6.8/1% glycerol/0.2% SDS/0.7 M 2-mercaptoethanol/0.1% bromophenol blue) and subjected to electrophoresis on SDS/10% polyacrylamide gel (18). Proteins were then transferred onto nylon membranes (Amersham) using an LKB Multiphor blot apparatus. After washing in Tris-buffered saline (TBS), filters were incubated with primary antibodies, produced against a portion of bep1 and bep4 proteins, diluted 1:500 as described by Romancino *et al.* (13).

Immunostaining of Egg Sections. Paraffin sections of *P. lividus* eggs were prepared for staining according to Levi *et al.* (19). Sections (3 mm thick) were deparaffinized and incubated with the polyclonal antibodies anti-bep1 and anti-bep4 (13) diluted 1:100 in TBS according to a standard procedure (20). After washing in TBS, the sections were incubated with mouse anti-rabbit IgG peroxidase conjugate (Promega) diluted 1:50 in TBS. After washing in TBS, the peroxidase color reaction was developed by bathing the sections in a solution containing diaminobenzidine (6 mg/ml), 0.05 M Tris·HCl (pH 7.6), and 0.03% H_2O_2.

RESULTS

We have previously demonstrated that two maternal mRNAs, *bep1* and *bep4*, are transcribed during oogenesis and persist throughout early development up to the gastrula stage, when they disappear (12). To investigate possible changes in their territorial distribution within eggs and early embryos, we prepared two antisense RNAs, labeled as described in *Materials and Methods* and shown in Fig. 1, and used them for *in situ* hybridization.

Fig. 2*A* shows that both *bep1* (I) and *bep4* (II) mRNAs are localized in only a part of the unfertilized egg, which corresponds roughly to one-half of the egg. When the hybridization was carried out on sections of embryos at the 16-cell stage (Fig. 2*C*), no hybridization was observed in the micromeres; at later stages, there was no hybridization in the vegetal part of the blastulae, in the primary mesenchyme cells, or in the gut (Fig. 2 *D* and *E*), whereas hybridization was observed in the other regions of the embryos at all these stages. This strongly suggested that *bep1* and *bep4* were confined to the animal half of the unfertilized egg, unless one assumes that their localization became reversed in the interval between fertilization and the 16-blastomere stage. Of particular interest was the finding that after fertilization the two mRNAs became localized close to the egg pole which is identified as the animal pole, for the reasons discussed above (Fig. 2*B*). Consistent with earlier evidence from Northern blots (12), *in situ* hybridization was not observed in later stages (data not shown).

The above evidence that micromeres did not contain *bep1* and *bep4* RNAs was further confirmed by the following experiment: embryos at the 16-blastomere stage were dissociated into cells, and the micromeres were separated from the mixture of mesomeres and macromeres by the procedure of Harkey and Whiteley (16) modified as shown schematically in Fig. 3*A*; total RNA was separately extracted from these two cell populations and the presence of *bep1* and *bep4* was investigated by Northern blot analysis, using as probes specific fragments of both cDNAs. As shown in Fig. 3 *B* and *C*, in the micromeres there was no hybridization signal for these two mRNAs, whereas a clear signal for both mRNAs was obtained in the meso- and macromeres and in the 16-cell

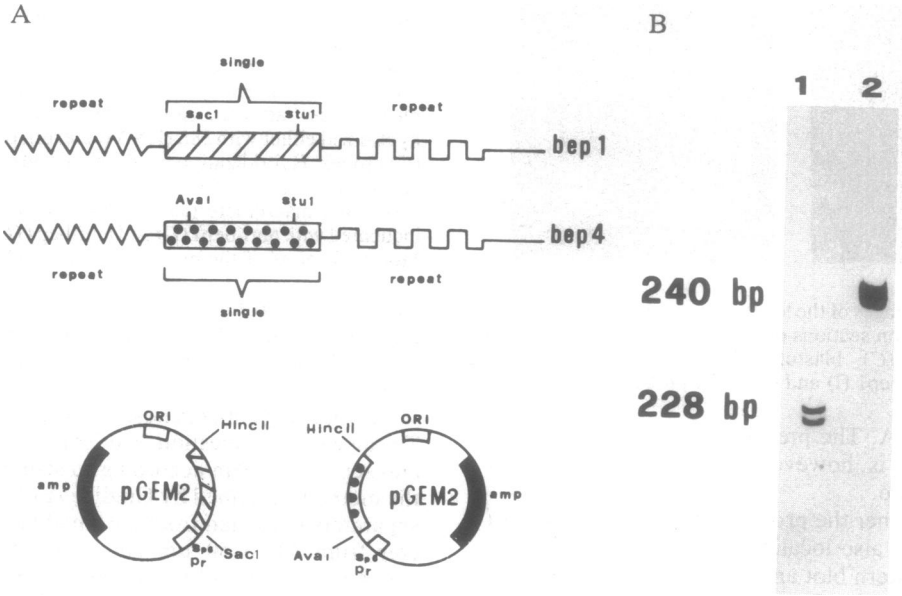

FIG. 1. (*A*) Construction of clones for *in vitro* transcription. Zigzag and open square lines indicate the regions common to bep1 and bep4. Hatched and dotted boxes indicate the specific region of *bep1* and *bep4*, respectively. *Sac* I, *Ava* I, *Stu* I, and *Hin*cII show the restriction sites from which the fragments inserted into pGEM2 have been prepared. ORI, origin of replication; amp, ampicillin resistance site; Sp6 PR, promoter site for Sp6 polymerase. (*B*) Autoradiograms of fragments obtained by *in vitro* transcription; numbers of base pairs synthesized are indicated on the left.

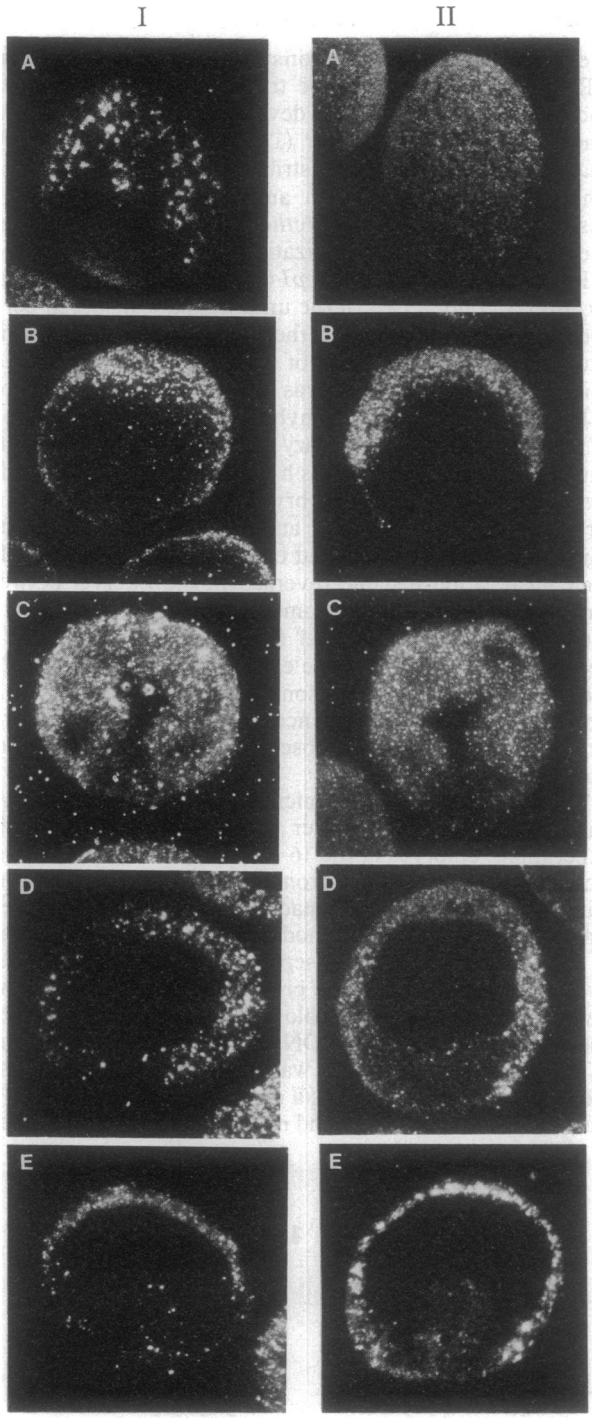

FIG. 2. Dark-field images of the location of *bep1* and *bep4* mRNA by *in situ* hybridization on sections of unfertilized egg (*A*), fertilized egg (*B*), 16-cell stage (*C*), blastula (*D*), and gastrula (*E*) with single-strand antisense bep1 (I) and bep4 (II) probes.

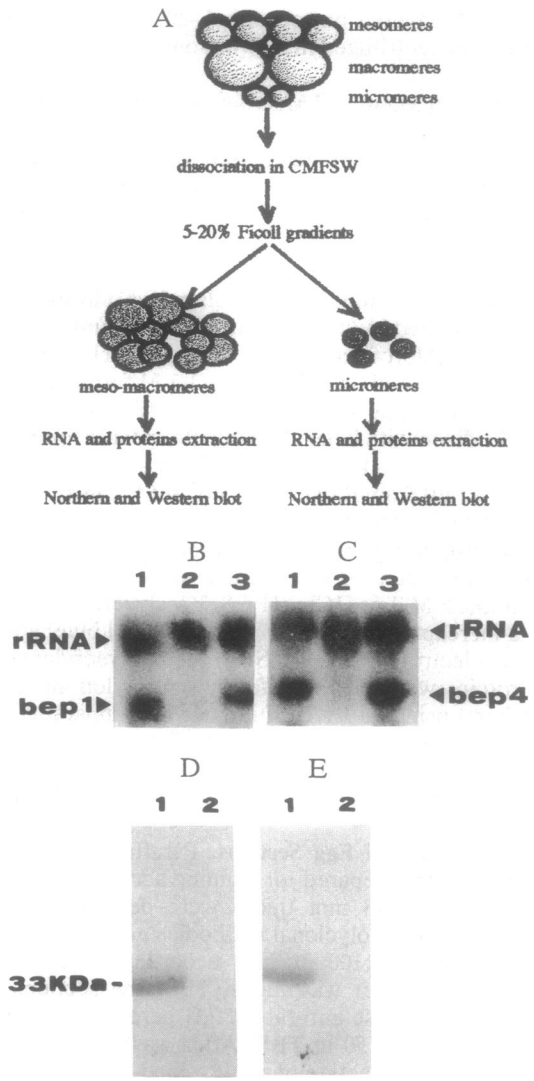

FIG. 3. Separation of meso- and macromeres and micromeres and Northern and Western blot analyses. (*A*) Schematic representation of meso- and macromeres and micromere separation from 16-cell stage and RNA extraction from the corresponding fractions. CMFSW, Ca^{2+}- and Mg^{2+}-free seawater. (*B* and *C*) Northern blot of total RNA from entire 16-blastomere embryos (lanes 1), isolated micromeres (lanes 2), isolated meso- and macromeres (lanes 3), hybridized either with 18S rRNA (control), or with *bep1* (*B*) and *bep4* (*C*) probes. Hybridization bands of each probe used are indicated by arrowheads. (*D* and *E*) Immunoblotting of proteins extracted from meso- and macromere (lanes 1) and micromere (lanes 2) fractions incubated with anti-bep1 (*D*) and anti-bep4 (*E*) polyclonal antibodies. Molecular mass of the proteins is indicated on the left.

stage blastomere RNA. The presence of 18S rRNA, which was used as a control, is, however, evident in the micromere lane in the same figure.

To investigate whether the proteins produced by the *bep1* and *bep4* RNAs were also localized in the same territories, we carried out a Western blot analysis of proteins extracted from isolated micromeres or from meso- and macromeres. As shown in Fig. 3 *D* and *E*, the bep1 (Fig. 3*D*) and bep4 (Fig. 3*E*) proteins were found to have the same spatial distribution as their corresponding RNAs. These results were confirmed by immunostaining unfertilized eggs with polyclonal antibodies to these proteins (Fig. 4). Immunostaining with either anti-bep1 (Fig. 4*A*) or anti-bep4 (Fig. 4*B*) antibody was detected only in that half of the egg identified as the animal half.

At what stage of oogenesis are these mRNAs synthesized? To answer this question, we isolated oocytes from gonads and separated them according to size in a Ficoll gradient by the method described by Giudice *et al.* (15). The RNAs were separately extracted from the small oocytes ranging in diameter from 30 to 50 μm and from larger ones ranging in diameter from 50 to 100 μm. Northern blot analysis (Fig. 5*A*) demonstrated that *bep1* and *bep4* RNAs were not yet detectable in the small oocytes, although these oocytes hybridized with the control probe for the 18S rRNA. On the other hand, clear hybridization bands were obtained with the larger oocytes, indicating that the synthesis of *bep1* and *bep4* RNAs

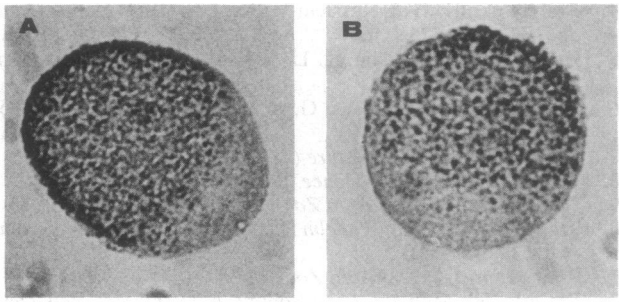

FIG. 4. Immunostaining of section of unfertilized eggs using polyclonal antibodies against bep1 (*A*) and bep4 (*B*).

commenced during the vitellogenic period. Differences in the intensity of 18S rRNA hybridization between the small and the larger oocytes were due to different amounts of RNA (20 and 10 mg, respectively) loaded on the gel.

Are the *bep1* and *bep4* RNAs synthesized directly in the oocytes or in the follicular cells and then transferred into oocytes? The results of *in situ* hybridization performed on entire ovaries using *bep1* as probe (Fig. 5*B*) favor the first possibility, because of the localization of these RNAs in the oocyte nuclei—i.e., presumably at the site of their synthesis (Fig. 5*Ba*). As expected from the experiments with isolated oocytes, the radioactivity was observed mainly in the larger oocytes and, in some of these, it has already been transferred almost completely from the nucleus into the cytoplasm (Fig. 5*B b* and *c*). In some of the larger oocytes, there appeared to be an asymmetric localization of these RNAs in the cytoplasm (Fig. 5*Bb*).

DISCUSSION

We found that in the egg of the sea urchin *P. lividus* there is an asymmetric distribution of two mRNAs that correspond to cell surface proteins even in the unfertilized egg. At fertilization, these mRNAs become localized in the cortical zone at the egg pole identified as the animal pole. These mRNAs are confined mostly to the animal parts of the embryo until the gastrula stage, at which time they disappear. They are not detected in the micromeres, vegetal plate, mesenchyme cells, and early intestine. Also, the corresponding proteins show the same localization pattern in the unfertilized egg and at the 16-blastomere stage (where they have been studied so far). The synthesis of these mRNAs appears to start during oogenesis during the vitellogenic period. As mentioned in the Introduction, axis-determining substances were demonstrated in the *Drosophila* egg (1) to be the products of maternal genes and of a cascade of processes including cell surface receptors such as products of the genes torso (21) and Toll (5). The resulting chain of events culminates in the differential activation of zygotic genes that bring about the formation of different structures in the embryo.

We propose that a fundamentally similar chain of events occurs also in the sea urchin. It is known that certain genes are preferentially activated in different territories of the embryo. The earliest genes whose activation was demonstrated in animal blastomeres are the VEBs (22). Later, the genes for the hatching enzyme and for a tolloid-like protein are turned on (23, 24) and expressed only in the animal territories. The Spec genes follow (25) and are preferentially expressed in the aboral ectoderm cells and their precursors, while the SM50 (26) and Endol (27) genes are expressed only in the mesenchyme and endoderm, respectively. The CyIIIa (28) gene also is expressed in the aboral ectoderm. Still, other sea urchin genes have territorial expressions (for a review, see ref. 29) that correspond to the specific morphogenesis of those territories. However, what is presently unknown is the nature of the initial egg asymmetry that starts the cascade of gene activations leading to axis determination and morphogenesis. We hypothesize that the territorial location of the two maternal mRNAs and their products described here in one-half of the unfertilized egg represents the asymmetry that starts such a cascade of gene activation. It is of potential interest that the proteins produced by these two maternally derived mRNAs appear to be associated with the cell surface (13).

Assuming that the territorial localization of *bep1* and *bep4* genes generates a localized expression of cell surface proteins, it is conceivable that these proteins function in reception of specific developmental signals derived, perhaps, from the micromeres. Davidson (30, 31) suggested that mi-

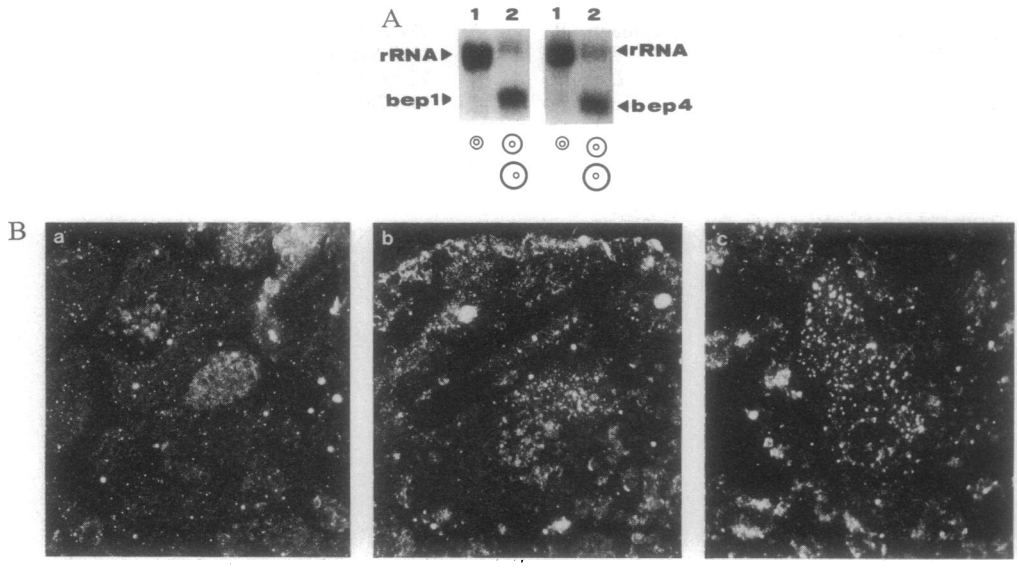

FIG. 5. (*A*) Temporal expression of *bep1* and *bep4* mRNAs during oogenesis. Total RNA extracted from small (20 mg) (lanes 1) and large (10 mg) (lanes 2) oocytes hybridized either with 18S rRNA or with *bep1* and *bep4* probes. Schematic drawing of oocytes of different sizes used is presented under the corresponding lanes. Hybridization bands of each probe are indicated by arrowheads. (*B*) Spatial expression of *bep1* mRNA during oogenesis. *In situ* hybridization using *bep1* probe reveals localization of the corresponding mRNA in the nuclei (*a*), in the nuclei and in the cytoplasm (*b*), and only in the cytoplasm (*c*) of some of the large oocytes.

cromeres are responsible for a cascade of cell interaction-activated events, leading to the early morphogenesis of the sea urchin embryo. That the bep1 and bep4 proteins may have a role in cell interactions was suggested by experiments in which treatment of dissociated embryo cells with Fabs against bep1 and bep4 proteins caused inhibition of cell reaggregation (13). On the other hand, these proteins may have other roles because, as previously shown (13), they are found not only on the cell surface but also within the cytoplasm. Analysis of the primary structure of these proteins, inferred from the nucleotide sequence, has not revealed any resemblance to known proteins. Their amphipathic structure, however, shows similarities to that of the gp80 cell surface protein of *Dictyostelium*, which is known to have a role in cell adhesion during the aggregation stage (32). Experiments to verify the suggested role of the asymmetric distribution of these two mRNAs and proteins in embryonic axis determination by treatment of eggs with antibodies against bep1 and bep4 proteins remain to be done.

The finding that after fertilization these mRNAs are detected only in the cortical zone of the animal pole suggests movement of these mRNAs to that region. This suggestion is supported by a count of the radioactive grains that showed a 40% increment of grain concentration in the egg cortex versus the endoplasm after fertilization. Although other hypotheses cannot be presently excluded, movement of the mRNAs seems the most plausible possibility that is also consistent with the translocation of Vg1 mRNA to the vegetal pole during the maturation of *Xenopus* oocytes (7). Since this translocation apparently involves microtubules and microfilaments (33, 34), whether these structures play a role in the movement of *bep1* and *bep4* RNAs to the animal pole of the sea urchin egg and whether any part of their nucleotide sequences is relevant to their translocation and anchoring need to be investigated.

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