Maternal mRNAs of *PEM* and *macho 1*, the ascidian muscle determinant, associate and move with a rough endoplasmic reticulum network in the egg cortex

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Summary

Localization of maternal mRNAs in the egg cortex is an essential feature of polarity in embryos of *Drosophila*, *Xenopus* and ascidians. In ascidians, maternal mRNAs such as *macho 1*, a determinant of primary muscle-cell fate, belong to a class of postplasmic RNAs that are located along the animal-vegetal gradient in the egg cortex. Between fertilization and cleavage, these postplasmic RNAs relocate in two main phases. They further concentrate and segregate in small posterior blastomeres into a cortical structure, the centrosome-attracting body (CAB), which is responsible for unequal cleavages.

By using high-resolution, fluorescent, in situ hybridization in eggs, zygotes and embryos of *Halocynthia roretzi*, we showed that *macho 1* and *HrPEM* are localized on a reticulated structure situated within 2 μm of the surface of the unfertilized egg, and within 8 μm of the surface the vegetal region and then posterior region of the zygote. By isolating cortices from eggs and zygotes we demonstrated that this reticulated structure is a network of cortical rough endoplasmic reticulum (cER) that is tethered to the plasma membrane. The postplasmic RNAs *macho 1* and *HrPEM* were located on the cER network and could be detached from it. We also show that *macho 1* and *HrPEM* accumulated in the CAB and the cER network. We propose that these postplasmic RNAs relocalized after fertilization by following the microfilament- and microtubule-driven translocations of the cER network to the poles of the zygote. We also suggest that the RNAs segregate and concentrate in posterior blastomeres through compaction of the cER to form the CAB. A multimedia BioClip 'Polarity inside the egg cortex' tells the story and can be downloaded at www.bioclips.com/bioclip.html

Key words: Ascidian embryo, Maternal mRNA, Egg cortex, RNA localization, Endoplasmic reticulum

Introduction

Localization of specific mRNAs is essential for cell polarity, asymmetric division and differentiation in several cells, including yeast, fibroblasts, neurons and oocytes, and in embryos (Bassell et al., 1999; Jansen, 2001; Kloc et al., 2002). In oocytes, eggs and embryos, maternal mRNA determinants that are localized in the egg cortex – *oskar* and *bicoid* in *Drosophila*, *VegT* and *Xdazl* in *Xenopus*, and *macho 1* in the ascidians *Halocynthia* and *Cion* – specify embryonic axes and cell types (King et al., 1999; Lasko, 1999; Mowry and Cote, 1999; Nishida, 2002; Riechmann and Ephrussi, 2001). It is not well understood how these mRNAs translocate and are anchored to the cortex or how they relocate within the cortex, as they do in oocytes and eggs of ascidians and *Xenopus*.

Ascidians are urochordates that develop into simple tadpoles of ~3000 cells (Satoh, 1994). They display a profound degree of autonomous development (Nishida, 1997; Satoh, 2001). Removal and transfer of small fragments of the periphery of the egg show that the formation of three kinds of tissue (tail muscle, endoderm and epidermis) involves peripherally localized maternal determinants that are repositioned in two main phases of reorganisation that occur between fertilization and first cleavage (Nishida, 1997; Roegiers et al., 1999). An essential element of the muscle determinant is a cortically localized maternal mRNA that encodes a zinc-finger transcription factor called *macho 1* (Nishida and Sawada, 2001). The mRNA for *macho 1* is one of a large number of maternal mRNAs (called postplasmic RNAs) that concentrate at the posterior pole of the cleaving embryos. The original, most abundant posteriorly-localized mRNA, called *pem* (for posterior end mark), was identified in *Ciona* (Yoshida et al., 1996). This was followed by the discovery of several mRNAs (*pem-2-pem-6*) with similar localization (Satou and Satoh, 1997). To date, 28 postplasmic mRNAs have been identified in *Halocynthia*, in the MAGEST maternal mRNA database (http://www.genome.ad.jp/magest/) (Sasakura et al., 2000; Makabe et al., 2001; Nakamura et al., 2003).

The *Halocynthia* homologue of *Ciona pem*, *HrPEM*, plus *macho 1* mRNA and some postplasmic mRNAs in *Halocynthia* are already located before fertilization in an animal-vegetal gradient (classified as type I postplasmic RNAs) (Nishida and Makabe, 1999; Sasakura et al., 2000; Makabe et al., 2001;

Nishida and Sawada, 2001; Nakamura et al., 2003) (the present study). These type I postplasmic mRNAs then become concentrated in the vegetal cortex after fertilization and relocalize to the posterior cortex before first cleavage (see Fig. 1 for *HrPEM* and *macho 1*). Co-ordinated relocalization of cortical and cytoplasmic domains has been well documented in ascidians since 1905, when Conklin described the myoplasm in the egg. This is a pigmented peripheral cytoplasmic domain that is rich in mitochondria and inherited by muscle cells (Jeffery, 1995; Nishida, 1997; Satoh, 2001). The concentration and translocation of the myoplasm occurs between fertilization and first cleavage in two main phases that consists of several subphases of cytoplasmic and cortical reorganizations (Sardet et al., 1989; Chiba et al., 1999; Roegiers et al., 1999). These reorganizations (historically called ooplasmic segregation) are driven by the interplay of microfilaments and/or astral microtubules in the cortex and subcortex. In this way, at least five cortical and cytoplasmic domains are stratified in the vegetal hemisphere by the time meiosis ends (Roegiers et al., 1999). After meiosis, two of the domains, the cortical ER-rich domain and the bulk of the mitochondria-rich subcortical-myoplasm domain, move posteriorly and are distributed equally between the first two blastomeres.

By the 8-cell stage, the bulk of these adjacent cortical and subcortical domains is segregated into the posterior-vegetal blastomere pair (Roegiers et al., 1999). These blastomeres are fated to give rise to 24 primary muscle cells in the tadpole tail (Nishida, 1997). During these early cleavages, postplasmic mRNAs are concentrated in a particular cortical structure, the CAB, that is located in the posterior-vegetal blastomere pair (see Fig. 1D,E) (Hibino et al., 1998; Nishikata et al., 1999). The CAB is a 10-μm-thick cortical specialization at the posterior pole of the cleavage-stage embryo. It is clear that the CAB is a dual-function complex that mediates both unequal cleavages and localization of specific maternal RNAs (Nishida, 2002).

We have shown that it is possible to isolate the cortex of eggs of the ascidian *Phallusia mammillata* by gently shearing eggs and embryos fixed to a polycationic surface using a stream of isotonic solution that mimicks the cytoplasm of the egg (Sardet et al., 1992). Isolated cortices are constituted of characteristic networks of cER, microfilaments and some microtubules, all of which remain strongly attached to the plasma membranes during the isolation procedure (Sardet et al., 2002). In unfertilized ascidian eggs the cER network is a monolayer distributed along the animal-vegetal gradient (Sardet et al., 1992). It is characterized by the presence of numerous sheets and tubes in the vegetal hemisphere and by a sparse distribution of tubes in the animal hemisphere. After fertilization, this gradient distribution of the cER network increases via microfilament-driven cortical contractions which concentrate the cER into a 2- to 5-μm-thick layer around the contracted vegetal pole (Gualtieri and Sardet, 1989; Speksnijder et al., 1993). The vegetal cER network is then displaced towards the posterior pole via microtubule-driven translocations mediated by the sperm aster (Speksnijder et al., 1993). The bulk of the cER network and the myoplasm are distributed equally in the posterior region of the first two blastomeres after amphimixy, mitosis and cleavage (Roegiers et al., 1999). The cER is of particular interest because at low resolution its location and displacement after fertilization seem to correspond closely to the position and relocalizations of type I postplasmic RNAs.

In the present work, we examined the precise localization of *HrPEM* and *macho 1* in eggs, zygotes, embryos and isolated cortices using high-resolution in situ hybridization. Our results showed that *HrPEM* and *macho 1* mRNAs could be isolated with the cortex and that both were associated with the network of rough cER. We propose that some postplasmic mRNAs translocate and relocalize with the cER network in the egg cortex following fertilization and accumulate with cER in the CAB during cleavages. A multimedia BioClip 'Polarity inside the egg cortex' (www.bioclips.com/bioclip.html) describes this in more detail.

Materials and methods

Eggs and embryos

Adults of the ascidian *Halocynthia roretzi* were collected near the Asamushi Marine Biological Station, Aomori, Japan, and the Otsuchi Marine Research Center of the Ocean Research Institute, Iwate, Japan. *Halocynthia* spawns eggs ~280 μm in diameter. Naturally spawned eggs were fertilized artificially and raised in seawater at 11°C. They start first cleavage 2.3 hours after fertilization and reach the 8-cell-stage at 4.5 hours. Eggs were fertilized with sperm activated by NaOH treatment, and the vitelline membrane was removed chemically either before or after fertilization as described previously (Mita-Miyazawa et al., 1985).

Isolation of cortices from eggs and embryos

Isolated cortices were prepared using cortex solutions (Buffer X, EMC and CIM buffer) and observed fixed or live as described (Sardet et al., 1992). Devitellinated eggs (15-30), synchronously developing zygotes and embryos were deposited in a Ca^{2+}-free medium (EMC solution) onto polylysine-coated coverslips. Unfertilized eggs that were attached, fertilized eggs (20-30 minutes after fertilization) and 8-cell-stage embryos (4-5 hours) were immediately sheared with a gentle stream of Millipore-filtered isotonic cortex solution (Buffer X) to which was added 1 unit μl^{-1} RNase inhibitor (Toyobo, Osaka, Japan). The cortices were fixed within 1-2 minutes after isolation in a freshly prepared mixture of paraformaldehyde (4%) and glutaraldehyde (0.1%) in CIM buffer. After 30 minutes fixation in a humid chamber at 11°C, cortices were washed in CIM buffer and stored for <6 hours in phosphate-buffered saline (PBS) to which was added 1 unit μl^{-1} RNase inhibitor. The ER network in isolated cortices was labelled with carbocyanine dyes, DiIC16(3) (Molecular Probes, Eugene, OR, USA) emulsion or a solution of DiOC6(3) (Molecular Probes) in either CIM buffer or Buffer X as described (Sardet et al., 1992). Each cortex-loaded coverslip was inverted and mounted on a microscope slide using two strips of double-sided sticky tape spacers, then fixed firmly with epoxy glue on the left and right sides to form a perfusion chamber. Several cortices were identified by their position on the coverslip and photographed to record the cER distribution. These cortices were identified again after in situ hybridization to record the distribution of specific mRNAs. Cortices were observed and photographed by fluorescence microscopy (Nikon) and confocal microscopy using a Nipkow disc-based apparatus (Yokogawa, Tokyo, Japan) coupled to a fluorescence microscope (Zeiss).

In situ hybridization

Specimens were hybridized in situ with digoxigenin (DIG)-labelled *HrPEM* and *macho 1* antisense probes that cover the entire cDNAs (GenBank accession numbers, AB045129 for *HrPEM* and AB045124 for *macho 1*). Arginyl-tRNA synthetase was used as a probe for ubiquitously distributed maternal mRNA (GenBank accession

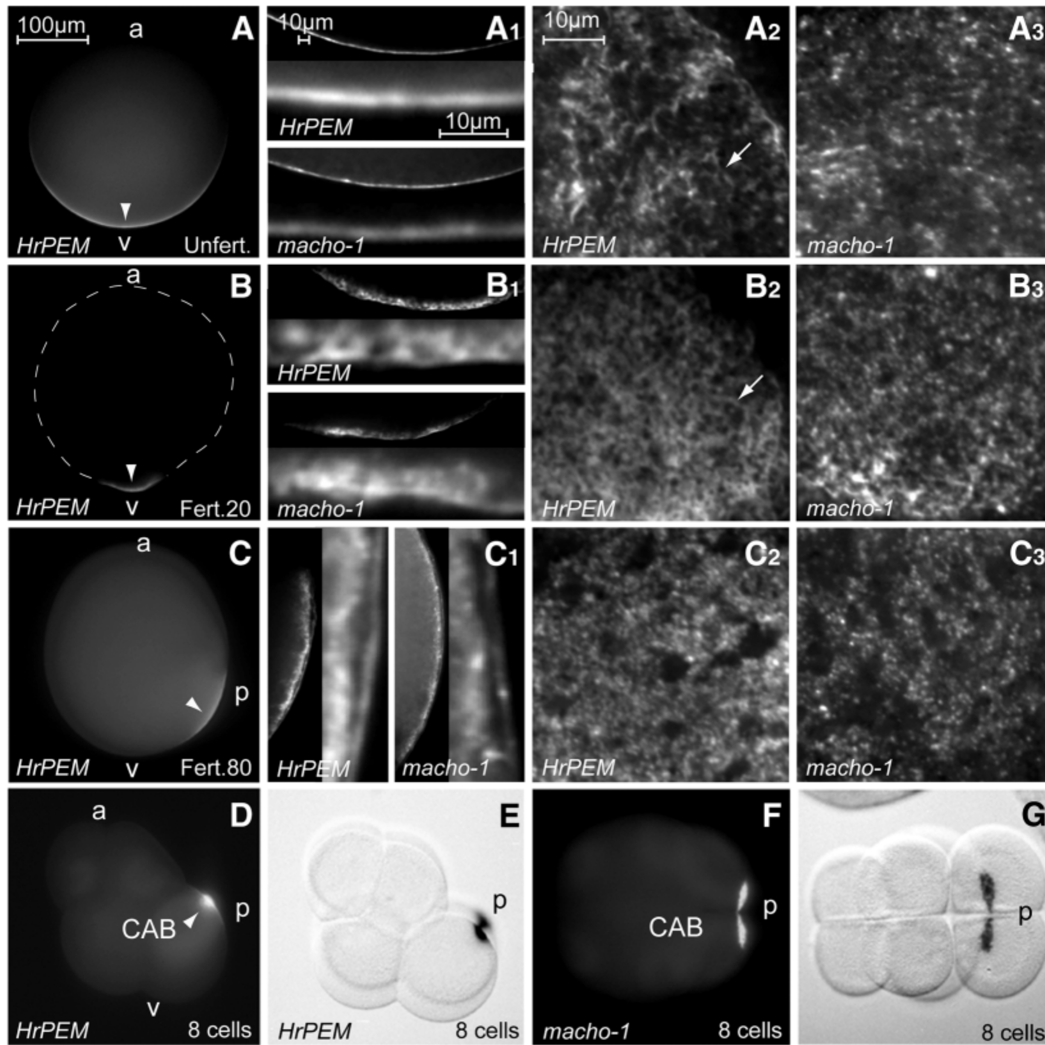

Fig. 1. Localization of *HrPEM* and *macho 1* mRNAs in eggs, zygotes and embryos. mRNAs are visualized in fluorescent (A-D,F), confocal (A1-A3,B1-B3,C1-C3) and DIC microscopy (E,G), by fluorescent (TSA) (A-D,F) and chromogenic (E,G) methods. (A) Unfertilized egg (Unfert.). (B,C) Zygotes after first (Fert.20, 20 minutes after fertization) and second (Fert.80, 80 minutes after fertilization) major relocalization phases. (D,E) Lateral views of 8-cell embryos. (F,G) Animal-posterior views of 8-cell embryos. (A1,B1,C1) Median sections, the thickness of the labelled zone is ~2 µm in unfertilized eggs and 5-8 µm in fertilized eggs. (A2-A3,B2-B3,C2-C3) Confocal tangential sections below the surface. Probes are indicated in the lower-left corner of each panel. a, animal; v, vegetal; p, posterior; CAB, centrosome-attracting body. Arrowheads in A,B,C show mRNA-rich cortical regions. Arrows in A2,B2 show that this mRNA-rich structure is reticulate.

number, AV383566). Detection of mRNA in whole-mount specimens was carried out essentially as described previously (Miya et al., 1994).

For cortex specimens, the in situ protocol was carried out without treating fixed samples with detergent such as Tween 20, solvent such as EtOH, and proteolytic enzymes such as proteinase K. Solutions between the coverslip and the microscope slides of the perfusion chamber were changed by drawing solutions from one side with a piece of filter paper whilst perfusing the next solution from the opposite side. The slides were maintained in handmade humid chambers throughout the in situ hybridization protocol. After washing with PBS, samples were prehybridized for 1 hour at 42°C. Then the specimens were allowed to hybridize with the DIG-labelled antisense probe (approximately 0.1 µg ml^{-1}) overnight at 42°C. The specimens were treated with 20 µg ml^{-1} RNase A (Sigma) for 30 minutes at 37°C and washed. The conventional coloring using alkaline-phosphatase conjugated anti-DIG antibody was performed as described (Miya et al., 1994).

The tyramide signal amplification method (TSA Biotin System, NEN Life Science Products, Boston) was used to obtain high-resolution fluorescent images of mRNA localization (Wilkie and Davis, 1998). This technique depends on peroxidase-mediated deposition of biotin-labelled tyramide at the location of the antisense RNA probe labelled with DIG. After washing of hybridized embryos and cortices, samples were treated with an HRP-conjugated anti-DIG antibody Fab fragment (1:2000, Boehringer Mannheim, Mannheim, Germany) overnight at 4°C. The TSA reaction was carried out for 10 minutes according to the supplier's instructions. Samples were then washed with PBS for 30 minutes and reacted with Alexa green 488-conjugated streptavidin (1:100, Molecular Probes, Eugene, OR, USA) for 60 minutes. After washing with PBS for 60 minutes, samples were perfused with 80% glycerol and observed under a fluorescent microscope or a confocal microscope. This method gives high resolution because the peroxidase reaction produces tyramide radicals that react covalently with proteins at the site of HRP activity, which prevents appreciable diffusion of the signal and fluorescently labelling the structures surrounding the target mRNA.

Electron microscopy of isolated cortices

Isolated cortices fixed as described above were treated briefly with OsO_4, fast-frozen and processed for deep-etching and replication as described (Sardet et al., 1992). Replicas were examined in a Hitachi electron microscope.

Results

HrPEM and *macho 1* mRNAs are located within 2-8 µm of egg and zygote surfaces

The fluorescent TSA-biotin system provides a level of detection of hybridized mRNAs with sensitivity close to that of the conventional chromogenic method using the alkaline phosphatase-based substrate-precipitation strategy.

Such comparisons are displayed for *HrPEM* and *macho 1* mRNAs localization in 8-cell embryos in Fig. 1D-G. In addition, the use of fluorescence allows high-resolution examination of RNA localization in confocal median (Fig. 1A-C,A1-C1) and tangential sections (Fig. 1A2-C2,A3-C3).

The changing localization of *HrPEM* and *macho 1* maternal mRNAs is shown in Fig. 1. In unfertilized eggs, *HrPEM* mRNA, the most abundant postplasmic RNA and *macho 1* (not shown) formed a thin cortical layer along the animal-vegetal gradient, with a maximum concentration in the vegetal hemisphere (Fig. 1A). High-resolution examination of the surface by confocal microscopy showed that *HrPEM* and *macho 1* are located in a reticulated structure <2 μm thick situated beneath the plasma membrane (Fig. 1A1). The mRNAs became further concentrated in the vegetal pole region as a consequence of the first major phase of ooplasmic segregation, which occurs 20-30 minutes after fertilization (Fig. 1B,B1). After completion of meiosis (40-50 minutes), postplasmic mRNAs moved to the future posterior pole during a second major phase of relocalization (80-100 minutes, Fig. 1C,C1). As a result of these cortical relocalizations, the cortical structure decorated by *HrPEM* and *macho 1* thickened to 5-8 μm (Fig. 1A1-C1). This mRNA-rich cortical structure generally appears reticulate (see particularly the tangential section shown in Fig. 1A2,B2) and sometimes punctate (possibly because the formaldehyde fixation does not preserve the continuity of the structure). Some labelled structure apparently detached from the surface after the second phase of relocalization (Fig. 1C1).

Zygotes of *Halocynthia* start to cleave at about 140 minutes. The first cleavage partitioned the posteriorly and cortically localized mRNAs, such as *HrPEM* and *macho 1,* equally between the first two blastomeres (data not shown). At the 8-cell stage, only posterior-vegetal (B4.1) blastomeres inherited postplasmic mRNAs. These RNAs are concentrated in a tiny area of the posterior region of the B4.1 blastomeres (Fig. 1D-G). This region corresponded to the CAB, the cortical structure that attracts the centrosome and nucleus towards the posterior pole of 8- to 64-cell-stage embryos (Nishikata et al., 1999). Posterior-animal views of the 8-cell embryos displayed the moustache-like shape of the mRNA localization, which corresponds to the characteristic shape of the CAB just before unequal cleavage starts (Fig. 1F,G).

HrPEM and *macho 1* mRNAs are retained in cortices isolated from eggs and embryos

Because the appearance and movements of the reticulated structure decorated by *HrPEM* and *macho 1* reminded us of the cER situated beneath the egg surface (Roegiers et al., 1999; Speksnijder et al., 1993), we isolated cortices from eggs, zygotes and embryos of *Halocynthia* using the methods previously described for unfertilized eggs of *Phallusia* (Sardet et al., 1992). We isolated cortices after the first phase of ooplasmic segregation, when postplasmic RNAs are concentrated in the vegetal-pole region (Fig. 2A-D), and from 8-cell embryos where they make a small posterior mark (Fig. 2E-I). Characteristic fields of pancake-like imprints that represent fragments of cortices could be observed on the coverslip (Fig. 2A,C,E). To investigate the localization of mRNAs at the subcellular level, it was essential to preserve the structures of these isolated cortices and, in particular, their membranes (plasma membrane, cER network and occasional

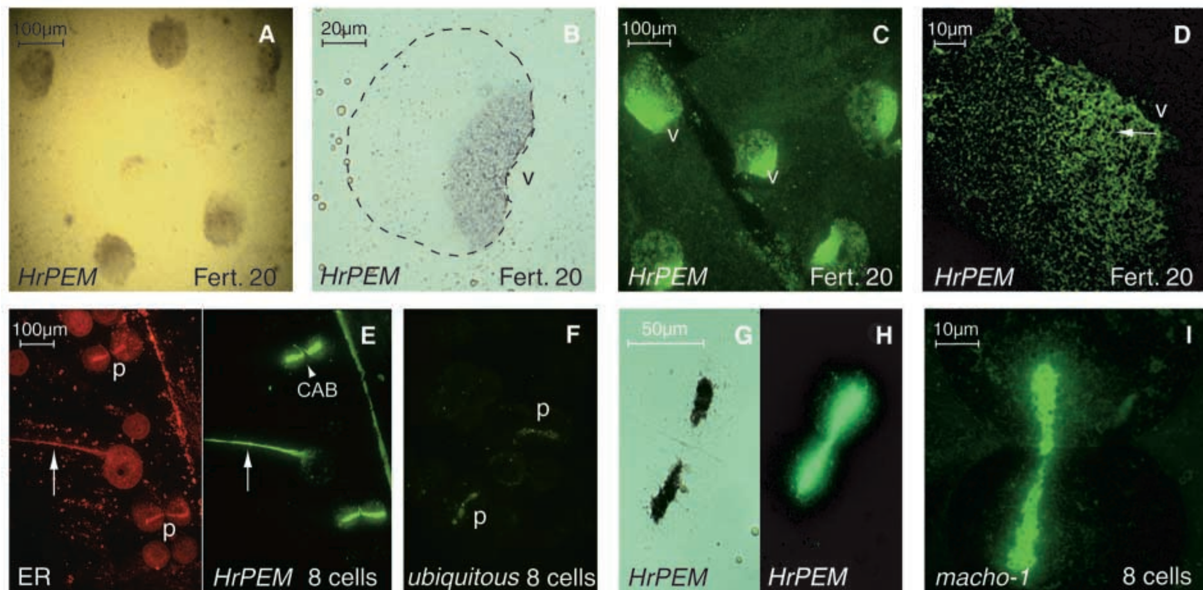

Fig. 2. Isolated cortices from zygotes and 8-cell-stage embryos retain *HrPEM* and *macho 1* mRNA. Low magnification (A,C) and higher magnification (B,D) views of cortices isolated from zygotes attached and sheared 20 minutes after fertilization. *HrPEM* is visualized by the chromogenic (A,B) or fluorescent (TSA) methods (C,D). v, region of the isolated cortex corresponding to the vegetal pole; arrow in D indicates that mRNA-labelled network is reticulate. (E-I) Cortices isolated from 8-cell embryos. (E) Visualization of ER with the lipid dye DiIC16(3) (left) and of *HrPEM* mRNA with Alexa green 488 (right). Arrows show a stretched-out CAB. (F) Overexposed image of ubiquitous mRNA (*arginyl-tRNA synthetase)* visualized by the TSA method. In E and F, p indicates 2 posterior quartet patches with the characteristic mustache-shaped CAB (arrowhead) present in 2 of the 4 patches. (G-I) Characteristic mustache-shaped CAB in which *HrPEM* (G,H) and *macho 1* (I) mRNAs are concentrated.

membranous organelles). To this end, we omitted the permeabilization, digestion and extractions steps (using Triton X100, proteinase and ethanol) that are generally included after fixation in conventional in situ hybridization procedures.

Initially, we labelled cortices with the lipophilic dye DiIC16(3) to observe and photograph the cER network in isolated cortices. After localizing mRNAs with the chromogenic method (alkaline-phosphatase-generated precipitate) (Fig. 2A,B), we relocated and rephotographed the same cortices. At low resolution there was a good correspondence between the presence of the *HrPEM* mRNA-rich zone and the DiIC16(3)-labelled area that corresponded to the cER-rich zone in isolated cortices (data not shown). We also compared the chromogenic and fluorescence (Alexa) visualization of *HrPEM* mRNA in isolated cortices fixed with a mixture of paraformaldehyde and glutaraldehyde to preserve the continuity of the cER network. Consistent labelling of isolated cortices was obtained by both methods (Fig. 2A,C). As seen in Fig. 2B,D, the fluorescent-visualization method provides finer details than the chromogenic method. As in the fertilized egg (Fig. 1,B2) an *HrPEM*-rich reticulated network could be discerned in the isolated cortex (Fig. 2D). It is clear from both chromogenic and fluorescent methods that the cortical mRNAs are not distributed homogeneously over the entire isolated cortex (Fig. 2A-D), which indicated that the polarized distribution of cortical mRNAs in the egg is preserved in isolated cortical fragments.

Cortices could also be isolated from the 8-cell embryos, which are cube-like. Groups of four, cortical, pancake-like fragments were detected (Fig. 2E,F). In roughly 1 in 6 cases, which represented embryos that fell on the posterior side, isolated cortices retained the characteristic mRNA-rich moustaches (Fig. 2E-I). These moustaches correspond to the postplasmic RNA-rich CAB in Fig. 1D-G. By contrast, pieces of cytoplasm that occasionally adhered to isolated cortices did not hybridize appreciably to *HrPEM* and *macho 1* maternal mRNAs (data not shown).

To verify the specificity of the localization of *HrPEM* and *macho 1* mRNAs in the cortex, we examined whether a ubiquitously distributed maternal mRNA in egg cytoplasm (*arginyl-tRNA synthetase*; T. Okada and H. Nishida, unpublished) was retained in isolated cortices. The prevalence of this mRNA in eggs is thought to be comparable to that of *macho 1*, based on the EST data of MAGEST, an EST database of maternal mRNA in *Halocynthia* (Makabe et al., 2001). When cortices isolated from eggs and 8-cell embryos were examined, the signal from *arginyl-tRNA synthetase* mRNA is very weak compared with *HrPEM* and *macho 1* mRNAs (see Fig. 2F, in which overexposure was used to increase the background signal), which indicates that noncortical mRNAs are not retained appreciably in isolated cortices.

Isolated cortices are characterized by a polarized cER network that adheres to the plasma membrane

The cER network can be made visible in live cortices with the lipophilic dyes DiIC16(3). As in *Phallusia*, the unfertilized egg cortex of *Halocynthia* was characterized by the presence of a continuous monolayer of cER tubes and sheets, which form a carpet that adheres firmly to the underside of the plasma membrane (Fig. 3A-C). Outside the attached cortices, the inner cytoplasmic tubes of ER that connected with the cER were usually projected onto the glass surface by the stream of cortex

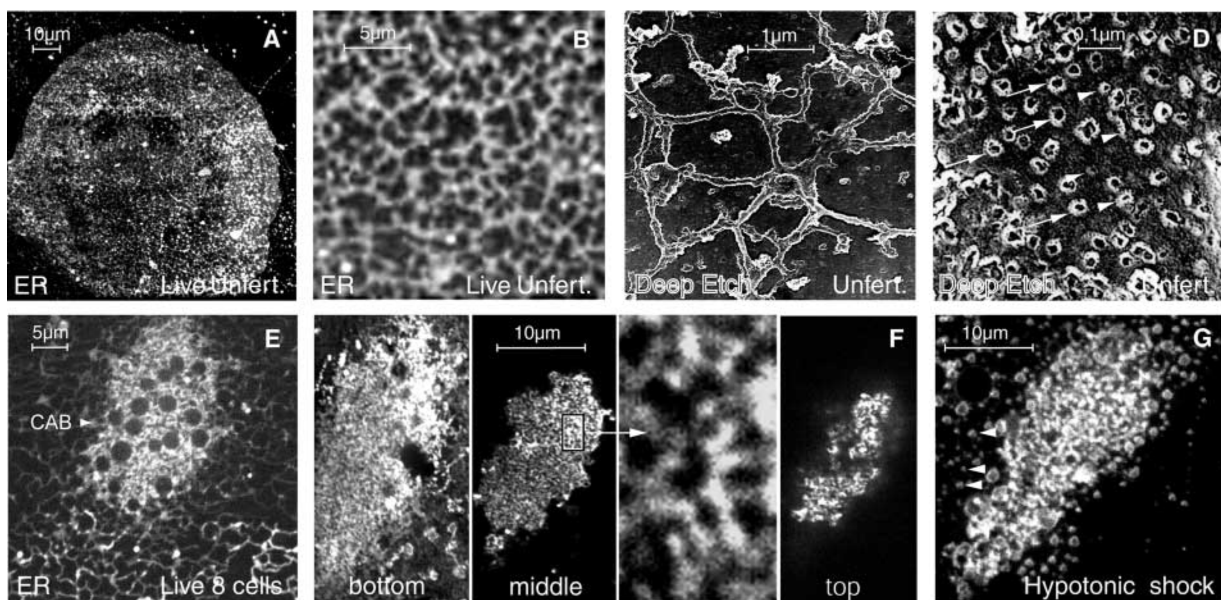

Fig. 3. The cER network in cortices isolated from eggs and 8-cell-stage embryos. (A-D) ER networks from cortices isolated from live unfertilized eggs labelled with the lipophilic dye DiIC16(3) and viewed with fluorescence microscopy (A,B), or prepared for electron microscopy using the fast-freeze deep-etch method (C,D), revealing ribosomal particles (arrows in D) mixed with smaller or odd-shaped particles (arrowheads). (E-G) High-magnification confocal sections of the DiOC6(3)-labelled CAB region in live cortices isolated from 8-cell embryos. (E) Confocal section close to the plasma membrane. The ER network in the CAB (arrowhead) is contiguous with the surrounding cER network. Vesicles are present in the CAB. (F) Three, evenly-spaced confocal sections (bottom, middle and top) through a CAB isolated with the cortex. The third image is an enlarged view of the ER network. (G) The ER network vesiculates (arrowheads) when a live labelled cortex is exposed to hypotonic solution.

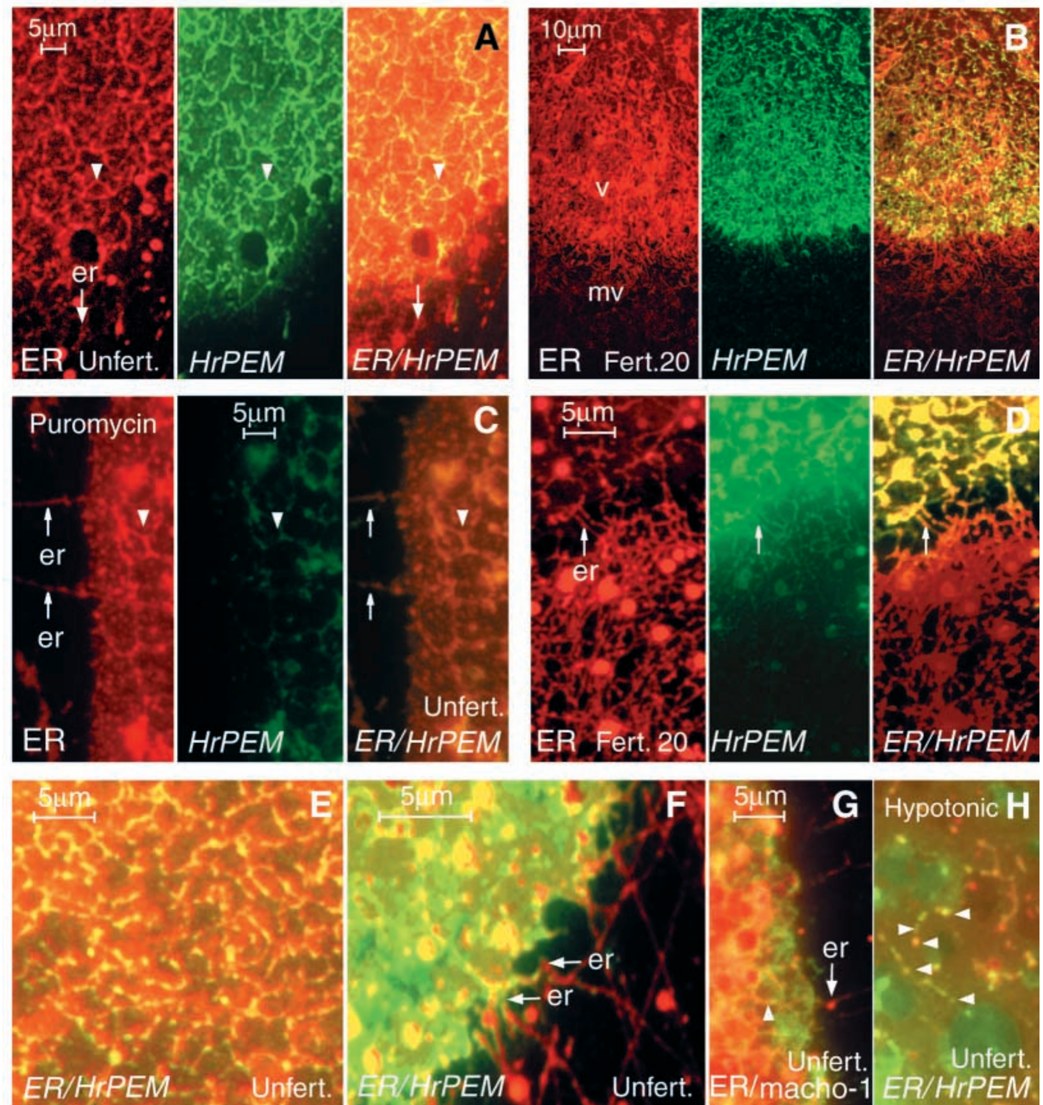

Fig. 4. *HrPEM* and *macho 1* mRNA localization on the cER network in eggs and zygotes. (A) Edge of a cortex isolated from an unfertilized egg, labelled to visualize the cER network (red, left panel), and the network of *HrPEM* (green, middle panel). The right panel shows an overlay of red and green images and the colocalization (yellow) of ER and mRNA on the cortex (arrowhead). Most ER tubes outside the cortex are devoid of *HrPEM* (er arrow). (B) Vegetal pole region (v) of cortex isolated from a fertilized egg 20 minutes after fertilization (this area corresponds to the densely labelled regions of isolated cortices seen in Fig. 2C,D. Microvillated region characteristic of the vegetal pole region is indicated (mv). (C) Edge of an unfertilized egg cortex treated with puromycin-KCl to detach particles from the cER. Only a weak signal of *HrPEM* remains, and partial colocalization (arrowheads) is shown. ER tubes outside the cortex are indicated (er arrow). (D) Edge of a fertilized egg cortex showing the continuity between the *HrPEM*-labelled cER network (upper part) and the nonlabelled cytoplasmic ER network projected outside the cortex during the shearing process (lower part). The arrows (er arrow) show the zone of transition between cER and cytoplasmic ER outside the cortex. (E) Colocalization of cER and *HrPEM* in the central region of an isolated unfertilized egg cortex. The yellow colour results from superposed green and red images as in panel A. (F) Edge of an unfertilized egg cortex showing the continuity between the cER network (arrowhead) and the cytoplasmic ER tubes projected outside the cortex (red). Note the lack of *HrPEM* signal outside the cortex. The arrows (er arrow) show the zone of transition between cER and cytoplasmic ER outside the cortex. (G) Edge of an isolated unfertilized egg cortex colabelled for ER (red signal) and *macho 1* mRNA (green signal). Co-localization is seen on the cortex (yellow; arrowhead), but not outside the cortex (red, er arrow). (H) Fragment of isolated cortex exposed to hypo-osmotic shock showing colocalization (yellow) of ER and *HrPEM*. Most of the cER network is vesiculated (arrowheads).

solution, forming a characteristic comet tail in the direction of shear (Fig. 4A,C,D,F,G). High-resolution electron microscopy of replicas of cortices generated by the fast-freeze deep-etch technique showed that the tubes and sheets of the cER were studded with particles (Fig. 3C,D). Most particles on the cER had the same size and shape, and were most likely ribosomes (Fig. 3D), as shown for *Phallusia* cortices (Sardet et al., 1992). Therefore, the cER of *Halocynthia* cortices is rough ER. However, a few particles were smaller or had elongated shapes, different from those of ribosomes.

When the first phase of ooplasmic segregation was complete (~20 minutes after fertilization), many isolated cortices showed an area where the cER was highly concentrated (Fig. 2C,D, Fig. 4B). This area corresponds to the region at the vegetal pole of the egg where cER accumulates as a result of the cortical microfilament-driven contraction (Roegiers et al., 1999; Speksnijder et al., 1993). At higher magnification, the edge of the cortex in the zone of ER accumulation was characterized by microvilli (Fig. 4B). This is a hallmark of the vegetal pole region and results from the cortical contraction. As mentioned above, there was a good correspondence between the *HrPEM* and *macho 1* mRNA-rich zone and the vegetal region where ER and microvilli were most concentrated (Fig. 2A-D, Fig. 4B). In this study, we focused mainly on *HrPEM* localization because it is more abundant than *macho 1* mRNA and images obtained using the *HrPEM* probe had less background. However *macho 1* mRNA had similar localization, as shown in Fig. 4G.

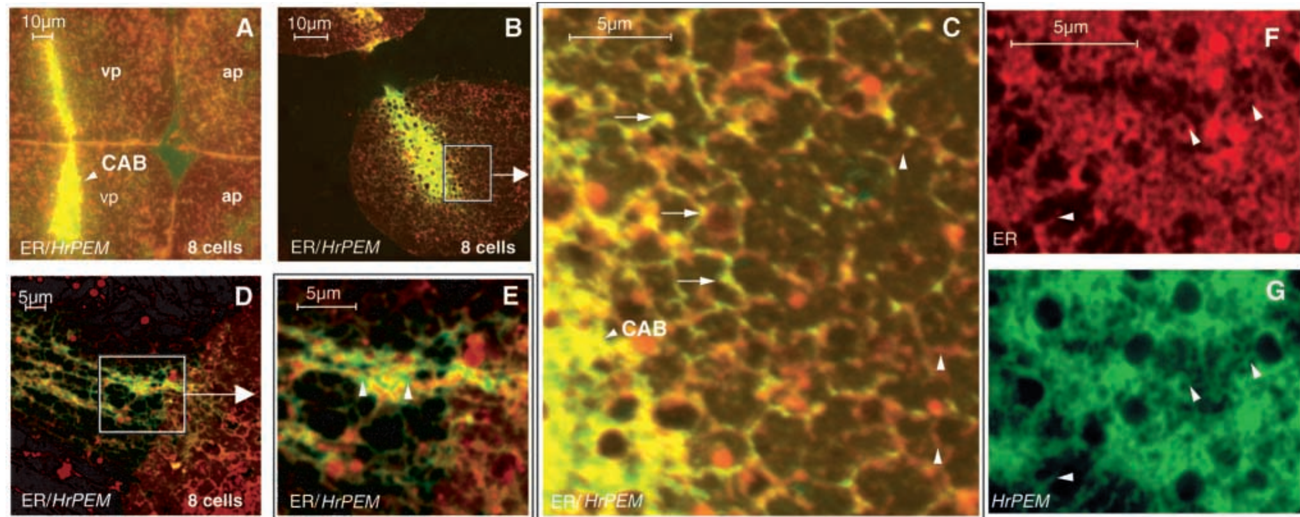

Fig. 5. *HrPEM* mRNA concentrates in the ER-rich CAB region. (A-E) Merged images of ER (red) and *HrPEM* (green) in cortices isolated from *Halocynthia* 8-cell-stage blastomeres. Areas where ER and *HrPEM* mRNA are perfectly colocalized appear yellow. (A) Quartet of cortices isolated from an 8-cell embryo attached by the posterior pole to a coverslip. Cortices of vegetal-posterior (vp) blastomeres are rich in cER and in *HrPEM* in and around the CAB (CAB arrowhead). Animal-posterior (ap) blastomeres have a cER network that lacks *HrPEM*. (B,C) Views of the CAB and of the cER network that surround the CAB. C is a higher magnification of the boxed region (edge of CAB) in B. In C, large patches of *HrPEM* mRNA localize on the cER network in the immediate vicinity of the CAB (CAB arrowhead), shown by arrows. The cER network away from the CAB (arrowheads in C) displays only occasional and small mRNA-rich patches. (D,E) These merged images show that the ER network, which is normally compacted in the CAB, has stretched away from the attached cortex under the force of shear. E is a higher magnification view of the boxed region in D. Large patches of *HrPEM* mRNAs are detected both on and inbetween stretched ER tubes (arrowheads show green RNA-rich patch). (F,G) Nonmerged images. Maximum magnification of confocal images of the inside of the CAB showing colocalization of ER [ER is labelled with DiIC(16)3 in F, and *HrPEM* mRNA labelled by Alexa green 488 in G] in some regions of the CAB (arrows). In other regions, differences in the relative intensity of mRNA and ER labelling can be seen.

HrPEM and *macho 1* bind to the cER network in cortices isolated from eggs and zygotes

The cER network in the unfertilized egg is a monolayer of interconnected tubes and sheets attached to the plasma membrane, so this is the easiest stage at which to analyse colocalization of fluorescently labelled mRNAs and cER. At the highest resolution possible with the fluorescent microscope, mRNAs for *HrPEM* and *macho 1* were distributed in a network that coincided precisely with the cER network (Fig. 4A,E-G). It was also clear that there was no appreciable hybridization of the probes for *HrPEM-1* and *macho 1* on ER tubes that originated in deeper cytoplasmic regions. Such inner cytoplasmic strands of ER often lie outside the cortex in the comet tails produced during shearing of the eggs, and are in continuity with the cER network (Fig. 4A,D,F). The lack of signal on noncortical ER indicates that the mRNA signal detected on cER is not caused by nonspecific binding of the postplasmic mRNAs to the ER of the whole egg. Further evidence for the specificity of localization of *HrPEM* and *macho 1* on the cER network was provided by the low amount of binding of the sense probes for *HrPEM* and *macho 1,* and the antisense probes for the ubiquitously distributed maternal mRNA of arginyl-tRNA synthetase on the cER network (Fig. 2F). To further test the binding of mRNAs for *HrPEM* and *macho 1* to the cER network, we exposed live cortices to hypotonic treatment, which vesiculates the cER network. Previously, we showed that many of the ER vesicles remain attached to the plasma membrane even after hypotonic treatment (Sardet et al., 1992). The *HrPEM* mRNA hybridization pattern in this case appeared as lines of fluorescently double-labelled spots that coincided with vesiculated ER (Fig. 4H). These observations, therefore, provide strong evidence that cortical postplasmic mRNAs, such as *HrPEM* and *macho 1*, are localized on the monolayer of cER that is attached to the cytoplasmic face of the plasma membrane in the unfertilized egg.

We have previously shown that we could strip the cER of particles (ribosomes and others) by treating isolated *Phallusia* cortices with a solution of puromycin and KCl (Sardet et al., 1992). The puromycin-KCl treatment dramatically decreased the signal for *HrPEM* RNA on the cER network of unfertilized *Halocynthia* eggs (Fig. 4A,C).

The colocalization of *HrPEM* and *macho 1* mRNA with the cER is also evident in egg cortices after the cortical contraction triggered by fertilization, at the end of the first phase of ooplasmic segregation, 20 minutes after fertilization (Fig. 4B,D). However because the cER network in the vegetal region of isolated cortices is much thicker and compacted by the cortical contraction (Fig. 1B), precise colocalization is seen only using confocal microscopy (Fig. 4B,D). In these isolated cortices, microvilli at the vegetal pole, which resulted from fertilization-induced cortical contraction, were labelled with the lipophilic dye DiIC16(3) but did not contain significant *HrPEM* mRNA. This indicates that RNA localization is restricted to the cER network (Fig. 4B).

HrPEM and *macho 1* mRNA is concentrated in the CAB together with the cER

As described previously (Fig. 2E-I) and shown at higher

resolution in Fig. 5A, cortices isolated from mid-8-cell-stage embryos typically contained striking patches of cER accumulation in the shape of moustaches. The two sides of the moustaches correspond to the two CABs present in the cortex of the two B4.1 posterior-vegetal blastomeres (Fig. 5A). Our observation that tight packing of cER filled most of the volume of the CAB that were isolated with *Halocynthia* cortices fits with the previous detection of many vesicles in the CAB by electron microscopy (Iseto and Nishida, 1999). The CAB of 8-cell-stage *Halocynthia* embryos is a macroscopic cortical structure ~4-7 μm thick, 5-10 μm wide and 30-50 μm long. Both DiIC16(3) and DiOC6(3) gave identical labelling patterns that revealed the topography of the ER network in isolated cortices (Fig. 3E,F, Fig. 5F). The ER in the CAB seems to be a continuous network of tightly packed tubes (Fig. 3E,F), which are unfolded occasionally by shearing force (Fig. 5D). The ER network compacted in the CAB was connected to tubes of the cER network that extended as a monolayer to line the plasma membrane outside the CAB (Fig. 3E, Fig. 5C). In some cases, large vesicles were embedded in the ER-rich CAB; we presume that this represents an early stage of compaction of the CAB (Fig. 3E). As expected, the tubular ER network inside and outside the CAB vesiculated when live, 8-cell-stage cortices were perfused with hypo-osmotic solutions (Fig. 3G).

A low-resolution inspection of fields of cortices probed for *HrPEM* and *macho 1* indicated that these transcripts were highly concentrated in the CAB (Fig. 2E,G-I, Fig. 5A,B), as expected from whole-mount specimens of 8-cell-stage embryos labelled for *HrPEM* and *macho 1* (Fig. 1D-G). We also noticed that with *HrPEM*, which gives a strong signal using fluorescent in situ hybridization, very little mRNA was detected in 8-cell-stage cortices outside the CAB (Fig. 5B,C). The *HrPEM* mRNA signal decreased with increasing distance from the CAB (Fig. 5B,C). Fig. 5C shows that *HrPEM* mRNA formed ER-localized patches that only partially covered the cER network at the periphery of the CAB. Although highly concentrated in the CAB, the *macho 1* signal was less intense than that of *HrPEM*, and labelled patches outside the CAB were less obvious than with *HrPEM* (Fig. 2I). Although a cER network was also present in animal or anterior blastomeres, it did not bind significant amounts of *HrPEM* mRNA (Fig. 5A).

At the highest possible magnification of the CAB with confocal microscopy, we observed partial colocalization of ER labelled with DiIC16(3) and of mRNA fluorescently labelled with Alexa green 488. Inside the CAB, the ER network was so tightly compacted that it was difficult to determine the exact degree of colocalization of the ER and mRNAs (Fig. 5F,G). Fortunately, the network of ER tubes and sheets compacted in the CAB occasionally stretched like spaghetti under the shear force applied to prepare cortices (Fig. 2E, Fig. 5D,E). In these 'stretched' CABs, the large patches of colocalization of ER and *HrPEM* mRNA were evident. However, although some mRNA patches coincided with the ER network, others seemed to fill the space between ER sheets and tubes (see green regions indicated by arrowheads in Fig. 5E).

Discussion

Using conditions that preserved the substructure of the isolated cortices and high-resolution fluorescent in situ hybridization in eggs, zygotes, embryos and their cortices, we showed that two postplasmic RNAs, *HrPEM* and *macho 1*, were mainly associated with a polarized network of cER. Control experiments, using sense probes and a probe for a ubiquitously distributed mRNA, indicated that binding to the cER was specific. Moreover, the fact that signals for *HrPEM* and *macho 1* were present only on the cER and not on the inner cytoplasmic ER network indicated that nonspecific binding of probes to ER was unlikely.

The association of *HrPEM* and *macho 1* mRNAs with the cER network, which is present initially as a gradient in the unfertilized egg, provides a simple explanation for the polarization and relocalization of at least some type I postplasmic RNAs, and their ultimate accumulation in the CAB, the structure responsible for unequal cleavages at the 8-64-cell stage. Our observations also indicate that the primary muscle determinant – the cortical maternal mRNA *macho 1* – probably reaches its site of localized accumulation in the CAB as a passenger of the cER network. Localization and translocation of cER therefore appear to provide a simple mechanism for the localization of specific cortical mRNAs to a subset of blastomeres.

Postplasmic mRNAs are associated with the cortical ER network of eggs

Microtubules, microfilaments and ER have been implicated in the translocation and anchoring of mRNAs to the cortex in yeast, somatic cells, oocytes and embryos (reviewed in Bassell et al., 1999; Lasko, 1999; Jansen, 2001; Kloc et al., 2002; Palacios, 2002). However, it has been difficult to directly visualize the association between mRNAs and these subcellular structures, because fixation, digestion and permeabilization procedures used for high-resolution in situ localization studies alter the integrity of cellular membranes and subcellular organization. High-resolution localization studies of *HrPEM* and *macho 1* in isolated cortices (an open-cell preparation that needs no permeabilization) provided direct evidence that the rough cER network that is attached to the plasma membrane is a main site of localization for some cortical mRNAs in unfertilized ascidian eggs. We cannot presume, however, that all type I postplasmic RNAs associate with the cER. At present, we do not know how type I postplasmic mRNAs reach their cortical location during oogenesis. In *Xenopus* oocytes it has been suggested that that localizing mRNAs (such as maternal *Veg1* mRNA) might move with the ER to the vegetal cortex during oogenesis (Deshler et al., 1997; Schnapp, 1999). It will be interesting to see whether the cER network and cortical mRNAs such as *HrPEM* and *macho 1* reach their cortical location separately or together during oogenesis and maturation, and whether this happens at the same time as the polarized mitochondria-rich myoplasm becomes established as a subcortical basket (Swalla et al., 1991). Another important question is the nature of the localization signal in these postplasmic RNAs. Recently, Sasakura and Makabe (Sasakura and Makabe, 2002) showed that small elements in the 3′-UTR region are sufficient for posterior localization of *Halocynthia* type I postplasmic mRNAs such as *HrWnt-5* and *HrPOPK-1*, as is the case for localized *Drosophila* and *Xenopus* mRNAs (Betley et al., 2002).

For the moment we can only speculate about the nature of the binding of *HrPEM* and *macho 1* to the cER network. We

have examined the distribution of cER, microtubules and microfilaments in the isolated cortex (Sardet et al., 1992). The fine distribution of microtubules and microfilaments is very different from that of cER. Therefore, the possibility that these cytoskeletal elements mediate association of cER and mRNAs is unlikely. Sasakura at al. (Sasakura at al., 2000) observed the localization of some type I postplasmic RNAs (*HrZf-1*, *HrPOPK-1* and *HrWnt-5*) in fertilized eggs treated with cytoskeletal inhibitors. Treatment with cytochalasin and nocodazole (which disassemble microfilaments and microtubules) disturbed the translocation of those postplasmic RNAs as well as the first and second phases of ooplasmic segregation respectively, as observed previously (Sardet, 1989; Speksnijder et al., 1993). However, these inhibitors do not cause postplasmic RNAs to detach from the cortex. This indicates that cytoskeletal elements are required for translocation of cER but not for the association of mRNAs to cER. At present there is no way to disrupt intermediate filaments in ascidian embryos.

One possibility is that these mRNAs bind to ribosomes or other particles present at the surface of the rough cER network. This is indicated by the fact that puromycin-KCl treatment detaches the bulk of *HrPEM* and *macho 1* from the cER network in isolated cortices. This treatment was used originally to detach ribosomes from the rough ER-microsome fraction (Sabatini et al., 1966) and also detaches ribosomes and other particles from the cER in the isolated cortex of *Phallusia* eggs (Sardet et al., 1992). In *Phallusia*, the cER network can be labelled with the RNA dye thiazole orange and the staining is abolished by the puromycin-KCl treatment (Sardet et al., 1992). Because rough ER is generally thought to be a site of localization for mRNAs that encode membrane or secreted proteins, it might seem surprising to find that mRNAs such as *HrPEM* and *macho 1*, which do not code for such proteins, are also associated with the cER. However, using DNA microarrays for the large-scale identification of secreted and membrane-associated gene products has revealed recently that many mRNAs encoding cytosolic proteins are associated with microsomal membranes. This indicates that the rough ER might be a major site of mRNA localization (Diehn et al., 2000). Localization of specific mRNAs to a cER network has been shown most clearly in rice seeds, in which a specialized subdomain of rough ER is the major localization site for mRNAs that encode prolamin-storage proteins (Choi et al., 2000). It has also been hypothesized that in *Xenopus* oocytes, developmentally important mRNAs which code for secreted proteins, such as *Vg1*, are associated with ER during their transport to the cortex, possibly via Vera, the conserved RNA zipcode-binding protein (Deshler et al., 1997; Mowry and Cote, 1999). Although anchoring of *Vg1* to the cortex seems to depend principally on the presence of keratin filaments in isolated *Xenopus* cortices, it might also depend on the cER because *Vg1* mRNA is lost from isolated cortices after detergent treatment (Alarcon and Elinson, 2001).

In yeast, fibroblasts and nerve cells, translocation and anchoring of mRNAs involves large irregular particles (0.2-0.7 μm) that, apparently, contain many types of mRNAs, proteins, a large number of ribosomes and, in some cases, ER (reviewed in Jansen, 2001; Kloc et al., 2002). This is also apparently the case in *Drosophila* oocytes, in which translocation and localization of *bicoid* mRNA in the cortex seems to be mediated by large particles, in particular by ER-containing ‘sponge bodies’ (Wilsch-Brauninger et al., 1997). A role for intracellular membranes in the localization of *Oskar* has also been recently proposed (Jansen, 2001; Dollar et al., 2002). In addition, there are several indications that rough ER may play a role in mRNA localization in neuronal dendrites because the translocating particles contain the RNA-binding protein Staufen, which is clearly associated with rough ER in these cells (Kohrmann et al., 1999). Because Staufen is also essential for the MT-dependent localizations of the determinants *oskar* and *bicoid* to the poles of the *Drosophila* oocyte, and for the microfilament-mediated cortical localization of the homeobox-containing transcription factor *prospero* in neuroblasts (Roegiers and Nung Jang, 2000), it is possible that cortical mRNA localization in *Drosophila* also relies on binding to the ER and on its translocation.

Postplasmic RNAs are relocated with the cER network after fertilization

It is well established that in all ascidians examined, the cortically localized postplasmic RNAs, and the determinants for muscle and endoderm formation, concentrate in the vegetal cortex under the influence of the cortical microfilament-driven contraction that is triggered by the fertilizing sperm (Nishida, 1997; Chiba et al., 1999; Roegiers et al., 1999). We have shown that, in *Phallusia,* this contraction is a consequence of the Ca^{2+} wave that is triggered at the site of sperm entry, and that it results in the accumulation of cER into a 2-5 μm-thick cortical patch that is located in a microvillus-rich contraction pole in the vegetal hemisphere (Roegiers et al., 1995; Roegiers et al., 1999). In the present work we showed that in the much larger egg of *Halocynthia*, a cER accumulation also forms in the vegetal pole region and that it is retained in the isolated cortex. This cER accumulation concentrates the postplasmic RNAs in a 5-8 μm-thick layer in the vegetal pole region. If contraction is inhibited by cytochalasin, type I postplasmic RNAs do not concentrate vegetally, but form multiple cortical patches (Sasakura et al., 2000), a behaviour we also noted in the cER network of *Phallusia* eggs (Speksnijder et al., 1993).

In the *Phallusia* zygote, the vegetal accumulation of cER moves to a posterior equatorial location after meiosis completion. This second major phase of reorganization is initiated by a displacement of the sperm aster and of its microtubules with respect to the cortex, and is completed by the subsequent microfilament-mediated wave of cortical relaxation initiated in the vegetal pole just before cleavage (Roegiers et al., 1999). We have not yet analysed this posterior translocation of cER in *Halocynthia*. However, because cortical and cytoplasmic relocalizations and cleavage patterns appear well conserved in ascidian species, we assume that some postplasmic RNAs move posteriorly with the cER accumulation that formed first in the vegetal pole region after fertilization.

Postplasmic mRNAs and cER concentrate in the CAB

Our present work indicates that the extreme concentration of postplasmic RNAs in a small posterior region of the B4.1 posterior-vegetal blastomeres is related to the packing of most of the cER in the unfertilized egg into one tight mass that fills a large part of the volume occupied by the CAB (Figs 3, 5). It has been observed by the electron microscopy that a dense

matrix containing vesicles is concentrated in the CAB of *Halocynthia* (Iseto and Nishida, 1999).

Confocal light microscopy sectioning of either live or fixed cortices isolated at the 8-cell stage showed that the cER formed a tight network in the CAB, and that this network is connected with a cER network that surrounds the CAB and lines the plasma membrane. How compaction of the cER occurs during early cleavages is unknown. Microtubules are, apparently, not involved in the formation of the CAB, whose precursors first appear as a number of small cortical particles at the 2-cell stage (Hibino et al., 1998; Nishikata et al., 1999). Cortical microfilaments or 'adhesion complexes' on the surface of the ER might participate in the progressive aggregation of cER into one compact CAB domain.

We observed that the cER near the CAB is covered with patches of *HrPEM* mRNA, and that the contiguous cER in the same blastomere and the cER network in the animal or anterior blastomeres is devoid of *HrPEM* and *macho 1* mRNA. This argues for the presence of a specific mRNA-rich cER domain in the unfertilized egg that is inherited selectively by posterior-vegetal blastomeres. This cER could also be considered the precursor of the CAB.

The egg cortex as a repository of spatio-temporal information

Our observations have interesting implications for the localization of some type I postplasmic RNAs in the cortex, and in particular of the muscle determinant *macho 1*. It is clear that the compaction of the cER network underlying CAB formation represents a way to segregate important cortical mRNAs, such as *macho 1,* in a small localized area of the cortex in specific blastomeres. By analogy with what is known of the large particles that localize mRNA in many cells, including yeast, neurons and oocytes (reviewed in Jansen, 2001; Kloc et al., 2002), we suspect that in the 8-cell-stage ascidian embryo, a translational machinery is concentrated in the mRNA patches that we observed on the cER in and near the CAB. Localization and concentration of the translational machinery and of *macho 1* mRNA could be essential for the activation of the translation of the muscle determinant *macho 1*. Indeed, in order for *macho 1* mRNA to be the primary muscle determinant, macho 1 protein must be produced before or at the time the compact CAB is formed, i.e. before the unequal cleavage of the 8-16-cell stage separates the ER-rich CAB from the mitochondria-rich myoplasm domain inherited by primary muscle cell blastomeres.

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