

# Reorganization of Cytoplasm in the Zebrafish Oocyte and Egg During Early Steps of Ooplasmic Segregation

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The aim of this work is to determine when and how ooplasmic segregation is initiated in the zebrafish egg. To this end, the organization of the ooplasm and vitelloplasm were examined in oocytes and eggs shortly after activation. Ooplasmic segregation, initiated in the stage V oocyte, led to the formation of ooplasmic domains rich in organelles, and ribonucleoproteins. A linear array of closely arranged peripheral yolk globules separated an outer domain of ectoplasm from an inner domain of interconnected endoplasmic lacunae. The structure of this yolk array and the distribution of microinjected labeled tracers suggests that it may provide a barrier limiting ooplasm transit. Loosely arranged yolk globules at the animal hemisphere allow wide connections between the endoplasm and a preblastodisc domain. Activation caused further segregation of ooplasm, reorganization of endoplasmic lacunae, and blastodisc growth. The presence of an endoplasmic cytoskeleton suggests that these changes may be driven by microtubules and microfilaments.

**Key words:** zebrafish; oocyte; egg; ooplasmic segregation

## INTRODUCTION

Distinctive features of egg cytoplasm are the presence of numerous organelles, yolk inclusions, maternal mRNAs, proteins, and sometimes lipid droplets and pigments. These cytoplasmic components are at first intermingled and preparation of the egg for development is often accompanied by separation of the organelles and maternal factors from the yolk, which in turn leads to establishment of ooplasmic domains. Orderly partition and funneling of these domains into different blastomeres during cleavage is necessary for normal embryogenesis to take place. The process is called "ooplasmic segregation." Its initiation, usually, is triggered by egg activation. Activation may be elicited by sperm

penetration or by chemical or mechanical stimulation of the egg. The eggs of many invertebrate and some vertebrate phyla have been used as models for the study of ooplasmic segregation as for example the eggs of nematodes (Strome and Wood, 1983; Bowerman, 1999), ascidians (Reverberi, 1971; Jeffery and Meier, 1983; Sardet et al., 1989), annelids (Shimizu, 1982; Fernández and Olea, 1982; Fernández et al., 1998), mollusks (Hess, 1971), amphibians (Elinson and Rowning, 1988; Danilchik and Denegre, 1991), and fish (reviewed by Hart and Fluck, 1995). Although every cell model has advantages and limitations, the teleost fish egg has particular advantages for studying ooplasmic segregation. First, the egg is translucent and

poleward transportation of ooplasm can be traced in live eggs using regular light microscopy (Roosen-Runge, 1938; Devillers, 1961; Ivanenkov et al., 1990; Abraham et al., 1993; Kimmel et al., 1995; Leung et al., 2000). Second, eggs are amenable for experimental manipulation such as microinjection, ablation of parts, development within wide range of temperatures, ultraviolet irradiation, drug treatment, and so on (Oppenheimer, 1936; Tung et al., 1945; Jesuthasan and Strähle, 1996). In addition, information is available about the location and manner of transportation of maternal factors during ooplasmic segregation (reviewed by Pelegri, 2003). Another interesting feature of the fish egg is that yolk may be sequestered within a

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membrane-bound compartment at the center of the egg, as in Medaka, or may be intermingled with ooplasm, as in zebrafish (reviewed by Hart and Fluck, 1995). This situation determines important differences on how ooplasmic segregation is achieved.

The zebrafish egg has been used extensively to understand the mechanisms of ooplasmic segregation. Early studies by Roosen-Runge (1938), Lewis and Roosen-Runge (1943), Hisaoka and Firlit (1960), and Beams et al. (1985) were descriptive, whereas that of Katow (1983), using cytochalasin B, aimed to understand the role of actin in ooplasmic segregation. Leung et al. (1998, 2000) have focused on the role of calcium and cortical actin filaments in ooplasmic segregation, whereas Jesuthasan and Strähle (1996) and Gore and Sampath (2002) have shown that cortical microtubules transport foreign microinjected particles as well as cytoplasmic determinants. Information is now available from *in situ* hybridization and pharmacological approaches about location and transportation of maternal mRNAs (reviewed by Pelegri, 2003).

Despite the importance of the ooplasm in the construction of the zebrafish blastodisc, and consequently of the embryo (Lewis and Roosen-Runge, 1943; Leung et al., 2000), little is known on the fine structure of its components, particularly of the endoplasm. Katow (1983) briefly described the structure of endoplasm streamers in zebrafish early fertilized eggs using transmission electron microscopy. He found mitochondria and 30-nm granules but no cytoskeletal elements. A few years later, Beams et al. (1985) described the possible presence of microtubules and microfilaments across the endoplasm using scanning electron microscopy. Numerous studies of the fine structure of the ectoplasm have been made, particularly of the cortical actin-rich region and its participation in fertilization (Wolenski and Hart, 1987; Hart et al., 1992), cortical reaction (Hart et al., 1977; Hart and Yu, 1980), and formation of the blastodisc (Katow, 1983).

In this study, we show that ooplasmic segregation is initiated in the stage V oocyte by separation of yolk from ooplasm. This process leads to the establishment of 3 ooplasmic domains: ectoplasm, endoplasm, and preblastodisc. The ooplasm encloses numerous or-

ganelles scattered across a granular matrix of ribonucleoproteins. It is presumed that ooplasmic domains are formed as result of concerted clustering of yolk globules across the oocyte cytoplasm. At most of the oocyte periphery, this process generated a palisade of closely arranged yolk globules that separated ecto- and endoplasm, while at the center of the oocyte yolk clustering gave rise to irregularly shaped endoplasmic lacunae. Narrow pathways, or canaliculi, between peripheral globules may impose restrictions to the transit of ooplasm between ecto- and endoplasm. Loosely arranged peripheral yolk globules at the top of the animal hemisphere, on the contrary, left wide gaps, allowing endoplasm flow into the preblastodisc. Activation caused further narrowing of the canaliculi between peripheral yolk globules, a situation that probably imposed further restrictions to the transit of organelles and ribonucleoproteins between ecto- and endoplasm. This conclusion is supported by results dealing with the distribution of labeled dextrans. Narrowing of canaliculi between central yolk globules provoked enlargement of endoplasmic lacunae due to translocation of granular ooplasm from between the yolk globules. These processes as well as the gathering of endoplasmic lacunae along the animal/vegetal axis of the egg, the rearrangement of organelles and ribonucleoprotein granules in the endoplasm of many lacunae, and the blastodisc growth are likely to be driven by an ubiquitous internal cytoskeleton of microtubules and microfilaments.

## RESULTS

### Comparison of Early Development Between Fertilized and Unfertilized Eggs at Different Temperatures

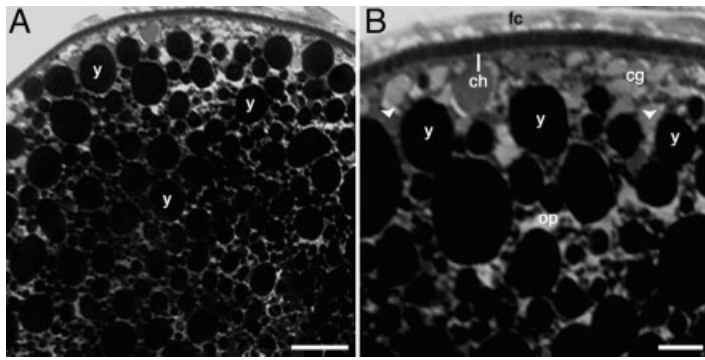
Tests were made to determine whether there were differences on how ooplasmic segregation progressed in fertilized and unfertilized eggs. For this purpose, we considered two developmental parameters: (1) rate of growth of the blastodisc, as a result of ooplasm accumulation; and (2) time at which the animal actin ring underwent the first contraction, as shown by the appearance of a constriction ring at the outer border of

the blastodisc. Adequate visualization of the growing blastodisc and contraction ring was achieved by following individual properly oriented chorionated eggs. At the normal developing temperature of 28°C, the blastodisc of the fertilized and unfertilized eggs grew at the same rate and the first contraction of the actin ring occurred at approximately the same time: 26–29 min of development (data for 16 unfertilized and 15 fertilized eggs).

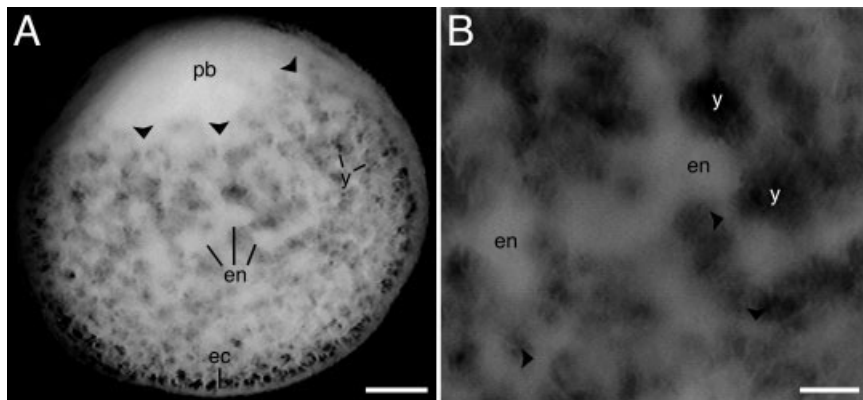
At 28°C, ooplasmic segregation in the egg and zygote occur too rapidly to be followed in detail. For this reason, it was important to determine whether early development at lower temperatures could alter the subsequent steps. In fertilized and unfertilized eggs at 20°C, we found that the blastodisc grew similarly and the first contraction of the actin ring also occurred at approximately the same time: 45–49 min of development (data for 18 unfertilized and 12 fertilized eggs). Comparison of fertilized and unfertilized eggs developing at either 28°C or 20°C indicated that the lower temperature slowed the developmental rate approximately 85%, yet under these conditions, ooplasmic segregation appeared to progress normally. Gross abnormalities were not detected in fertilized eggs developed at 20°C until cleavage had produced several blastomeres. Results confirmed the work of Schirone and Gross (1968) in that at 20°C zebrafish eggs developed at a reduced rate and normally until the 32-cell stage embryo.

### Ooplasm and Yolk Are Intermingled in the Stage III Oocyte

Formation of ooplasmic domains as result of ooplasm and yolk separation may be conveniently studied in acid-fixed cells. Unfortunately, this technique failed to show the distribution of ooplasm in a nontranslucent cell like the stage III oocyte. However, examination of stained thick sections of resin-embedded medium and large sized stage III oocytes showed that ooplasm and yolk globules were distributed evenly across the oocyte cytoplasm (Fig. 1). Therefore, separation of ooplasm from yolk has not yet started in the stage III oocyte. For reasons explained in the Experimental Procedures sec-



**Fig. 1.** **A,B:** Low- (A) and high- (B) magnification photomicrographs of a stained plastic-embedded thick section of a late stage III oocyte. The cell was not translucent, had a central meiotic nucleus, and measured approximately 600  $\mu\text{m}$  in diameter. Its cytoplasm consists of darkly stained yolk globules (y) evenly distributed across a lightly stained ooplasm (op). The loose arrangement of peripheral globules allow communication between central and peripheral ooplasm (arrowheads). The latter encloses numerous cortical granules (cg). A layer of follicular cells (fc) extends over the chorion (ch). Scale bars = 50  $\mu\text{m}$  in A, 10  $\mu\text{m}$  in B.



**Fig. 2.** Z-sections of acid-fixed whole-mounted stage V oocytes that show the distribution of ooplasmic domains that appear bright after inverting their contrast with the Metamorph program. **A:** Optical section 32 of a stack of 56 images taken every 2  $\mu\text{m}$ . Separation of ooplasm from yolk has generated three ooplasmic domains: ectoplasm (ec), endoplasm (en), and preblastodisc (pb). The ectoplasm forms an outer band of cytoplasm continuous with the cap-shaped preblastodisc at the top of the animal hemisphere. Endoplasmic lacunae appear scattered among yolk globules (y), are interconnected with another and with the base of the preblastodisc (arrowheads). **B:** Deconvoluted image 33 of a stack of 38 images taken at 2- $\mu\text{m}$  intervals. It shows endoplasmic lacunae and their interconnections (arrowheads) at higher magnification. y, yolk globules. Scale bars = 100  $\mu\text{m}$  in A, 40  $\mu\text{m}$  in B.

tion, it was not possible to examine stage IV oocytes.

### Separation of Ooplasm From Yolk and Establishment of Ooplasmic Domains in the Stage V Oocyte Constitute an Early Step in Ooplasmic Segregation

#### *Examination of whole-mounted acid-fixed stage V oocytes by video light microscopy.*

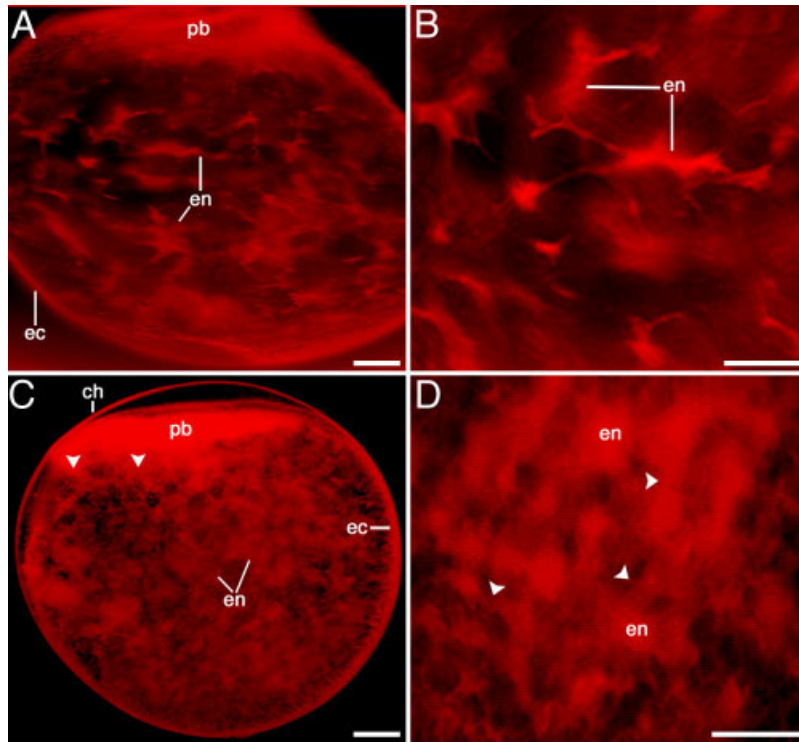
Microscopic examination of nonactivated acid-fixed stage V oocytes showed that their ooplasm was segregated from

the yolk and formed three domains: (a) a peripheral domain, or ectoplasm (yolk cytoplasmic layer), that formed a thin layer surrounding the oocyte; (b) a central domain, or endoplasm, that consisted of a network of irregularly shaped foci (lacunae) of ooplasm scattered among transparent yolk globules; and (c) a polar domain, or preblastodisc, that constituted a thin cap at the top of the animal hemisphere (Fig. 2). The endoplasm together with the yolk globules will later constitute the yolk cell. The amount of segregated endoplasm and its pattern of distribution was similar in oocytes removed from the same mother but different in oocytes collected from

different mothers. This finding suggests that development of stage V oocytes is more or less synchronized, and its progress varies in different pregnant mothers. Thus, oocytes with few small endoplasmic lacunae (Fig. 3A,B) are considered to be less advanced than those with numerous large endoplasmic lacunae (Fig. 3C,D). This observation suggests that a great part of the endoplasmic segregation takes place in the stage V oocyte. Animation or montages of Z-sectioning stacks of images, prepared at 2- to 5- $\mu\text{m}$  intervals, showed that lacunae of endoplasm accumulation were interconnected to one another and to the preblastodisc by strands of ooplasm (Figs. 2B, 3B,D). The ectoplasm, on the other hand, was connected with the entire outer perimeter of the preblastodisc. Connections between ecto- and endoplasm were more difficult to determine in acid-fixed eggs.

#### *Examination of sectioned plastic-embedded oocytes by video light microscopy.*

Further information on ooplasm distribution in the stage V oocyte was obtained by examination of stained JB-4, Epon 812, or Epon-Araldite-embedded thick sections. It was found that the broad 15- to 20- $\mu\text{m}$ -thick ectoplasm consisted of lightly stained cytoplasm containing small vesicles, granular material, and numerous vacuoles (3–20  $\mu\text{m}$  in diameter) with a lightly or darkly stained core. The latter corresponded to intact cortical granules and demonstrated that oocytes were not activated in the fixative. The most peripheral yolk globules, underlying the ectoplasm, were closely apposed to each other forming a palisade between ecto- and endoplasm. The space between these yolk globules, called canaliculi, could not be properly resolved with the light microscope or appeared filled with a thin layer of ooplasm (Fig. 4A). There were, however, few places where the space between the yolk globules was greater forming ectoplasmic recesses (Fig. 4B). In those cases wide connections between ecto- and endoplasm could be present. At the preblastodisc/endoplasm boundary zone, yolk globules were loosely arranged with broad connections between the two ooplasmic domains (Fig. 4C). The endoplasm formed a network extending through-



**Fig. 3.** Pseudo-colored Z-sections of acid-fixed whole-mounted stage V oocytes that show the progressive formation of endoplasmic lacunae. **A:** Image 53 of a stack of 57 images taken at 5  $\mu\text{m}$  intervals showing the initial formation of ectoplasm (ec), endoplasm (en) and preblastodisc (pb) domains. At this early stage of ooplasmic segregation, oocytes are flaccid and easily deformed. **B:** Image 56 of the same stack of images showing thin and elongated nascent endoplasmic lacunae at higher magnification. **C:** Image 61 of a stack of 72 images taken at 5- $\mu\text{m}$  intervals showing an advanced stage of endoplasm formation. The chorion (ch) is visible at the animal pole due to retraction of the preblastodisc. Endoplasmic lacunae have increased in size and number and appear connected to the preblastodisc (arrowheads). **D:** Image 40 of a stack of 77 images showing the multiple interconnections (arrowheads) between endoplasmic lacunae. Scale bars = 100  $\mu\text{m}$ .

out branched clusters of yolk globules. It consisted of thin canaliculi and irregularly shaped lacunae that ranged in size from a few micrometers to more than 50  $\mu\text{m}$  (Fig. 4D). Lacunae corresponded to the foci of endoplasm accumulation seen in whole-mounted acid-fixed oocytes (Fig. 4D). A distinctive feature of these lacunae was the presence of irregularly shaped empty vacuoles scattered across a lightly stained, sometimes granular, endoplasm (Fig. 4E,F). The preblastodisc enclosed the meiotic nucleus and was structurally similar to the ectoplasm.

Our evidence suggests that a great part of the first step of ooplasmic segregation takes place in the stage V oocyte. However, since stage V oocytes do not normally stay in the ovary lumen for too long before discharge (Li et al., 1993) presents the possibility that this process may be initiated in the stage IV oocyte, about which little information is available.

#### *Examination of oocytes under transmission electron microscopy.*

The electron microscope showed that the ectoplasm and preblastodisc domains consisted of cortical and subcortical regions. The cortical region was approximately 0.5  $\mu\text{m}$ -thick and contained granulofibrillar profiles approximately 7 nm in diameter that probably corresponded to actin filaments (see also Hart and Fluck, 1985). The subcortical region included cortical granules, numerous organelles, and 20- to 30-nm granules. The structure and distribution of the cortical granules were described in detail by Hart and Yu (1980). Mitochondria were 0.5–1  $\mu\text{m}$  in diameter, enclosed parallel or multidirectional cristae, and appeared scattered singly, in small groups or aligned at the surface of the yolk globules. Some mitochondria had swollen cristae-free region. The distribution of tubulovesic-

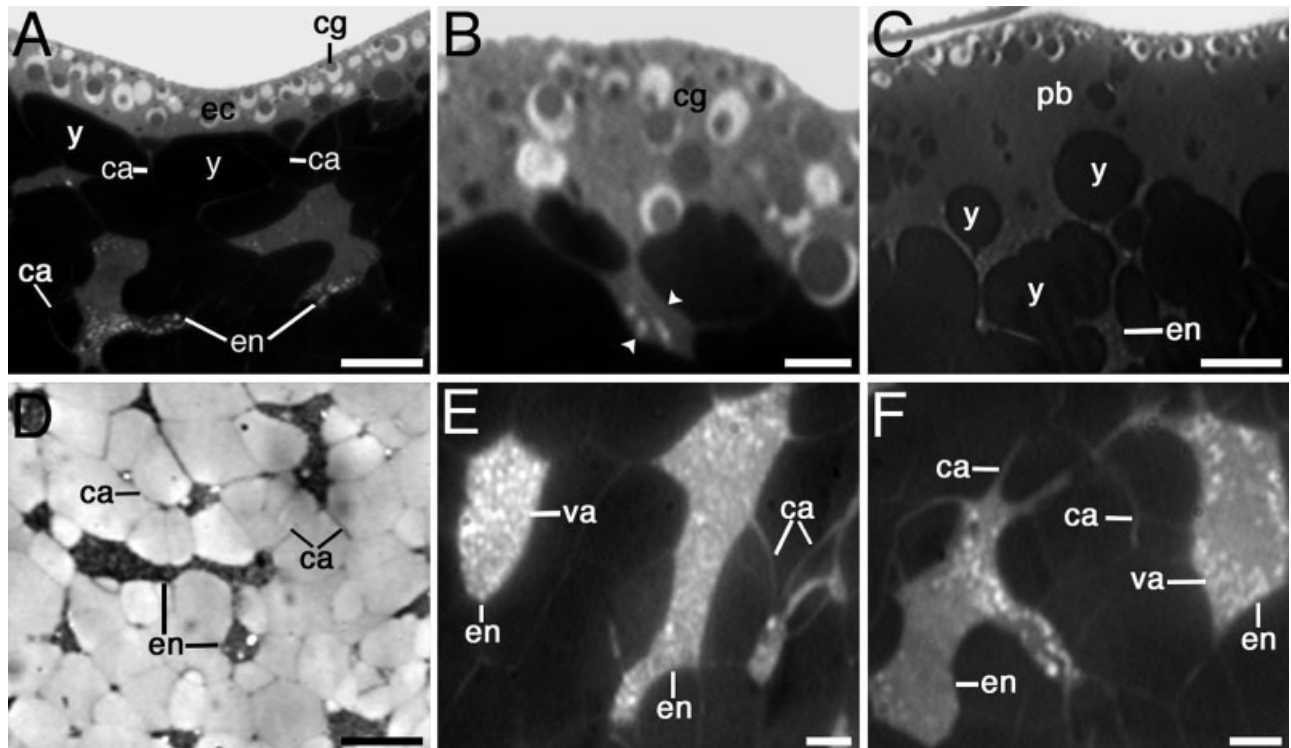
ular profiles of sER was similar to that of mitochondria. The organization of the peripheral yolk globules, and particularly the structure of the space between them, was of especial interest for understanding the relationships between two compartments of the mature oocyte. Membrane-bound peripheral yolk globules formed an orderly array of closely packed bodies that left narrow spaces between them. Some of the spaces were empty 20-nm clefts, whereas the majority were granule-filled canaliculi reaching up to 0.5  $\mu\text{m}$  in diameter (Fig. 5A,B).

Endoplasmic lacunae were bordered by membrane-bound yolk globules, enclosed 20–30 nm electron-dense granules, tubulovesicular profiles of smooth endoplasmic reticulum (sER), mitochondria, Golgi complexes, and vacuoles (Fig. 5C). Granules were scattered across the lacunae and their size and electron density, together with the fact that they could be digested by RNase, indicated that they corresponded to ribonucleoproteins (Fig. 5G). Rounded or oval mitochondria were 0.5–1  $\mu\text{m}$  in diameter, had multidirectional cristae and were scattered across the lacunae and lined up at the surface of the yolk globules. The sER had a similar distribution. Irregularly shaped vacuoles reached several micrometers in diameter and enclosed flocculent material. They were concentrated at the periphery of the lacunae. Small yolk globules, multivesicular bodies, and dense core vesicles were also found in the lacunae (Fig. 5C–F). Canaliculi connecting endoplasmic lacunae were similar to those found between peripheral yolk globules. The majority contained ribonucleoprotein granules only. These findings indicate that organelle segregation leading to lacunae formation appears to be completed by stage V of oogenesis.

#### **Activation Provoked Reorganization of the Endoplasm Lacunae, Poleward Endoplasmic Flow, and Blastodisc Formation**

##### *Examination of whole-mounted acid-fixed eggs by video light microscopy.*

A remarkable consequence of activation is the growth of the blastodisc at the animal pole of the egg. This structure grows as a consequence of the



**Fig. 4.** Light photomicrographs of stained plastic-embedded thick sections of stage V oocytes showing the structure of ooplasmic domains (compare with Fig. 1). **A:** Peripheral yolk globules (y) are closely apposed to one another forming a palisade separating a continuous outer ectoplasm (ec) from a system of inner endoplasmic lacunae (en). The ectoplasm encloses intact cortical granule (cg). Notice that ooplasm-filled canaliculi (ca) are present between peripheral and central yolk globules. **B:** Ectoplasmic recess (arrowheads) formed between peripheral yolk globules may sometimes be connected to endoplasmic lacunae. The ectoplasm encloses several layers of cortical granules. **C:** Loosely arranged yolk globules at the animal pole region of the oocyte allow continuity between the endoplasm and the preblastodisc (pb). **D:** Low-magnification photomicrograph showing the distribution of endoplasmic lacunae and canaliculi connecting them. **E,F:** High-magnification photomicrographs showing that endoplasmic lacunae enclose empty vacuoles (va) and a lightly stained, sometimes granular, matrix. Canaliculi between yolk globules interconnect endoplasmic lacunae and are filled with lightly stained ooplasm. Scale bars = 10  $\mu\text{m}$  in B,E,F, 30  $\mu\text{m}$  in A,C,D.

accumulation of endoplasm from the yolk cell region of the activated egg. This process is particularly well shown when acid-fixed stage V oocytes are compared with activated acid-fixed eggs (Fig. 6). In the stage V oocyte, the preblastodisc is thin and transparent, when viewed from the animal pole, showing the profiles of underneath yolk globules. The preblastodisc is 50- to 90- $\mu\text{m}$ -thick and 250–350  $\mu\text{m}$  in diameter. Three minutes after activation, the blastodisc has lost its transparency becoming thicker (approximately 130  $\mu\text{m}$ ) and wider (approximately 450  $\mu\text{m}$ ). Five minutes after activation, the blastodisc became highly opaque reaching 180  $\mu\text{m}$  in thickness and up to 550–650  $\mu\text{m}$  in diameter. Therefore, activation was followed by quick accumulation of ooplasm at the animal pole. Evidence presented below demonstrates that poleward flow of endoplasm is also accompa-

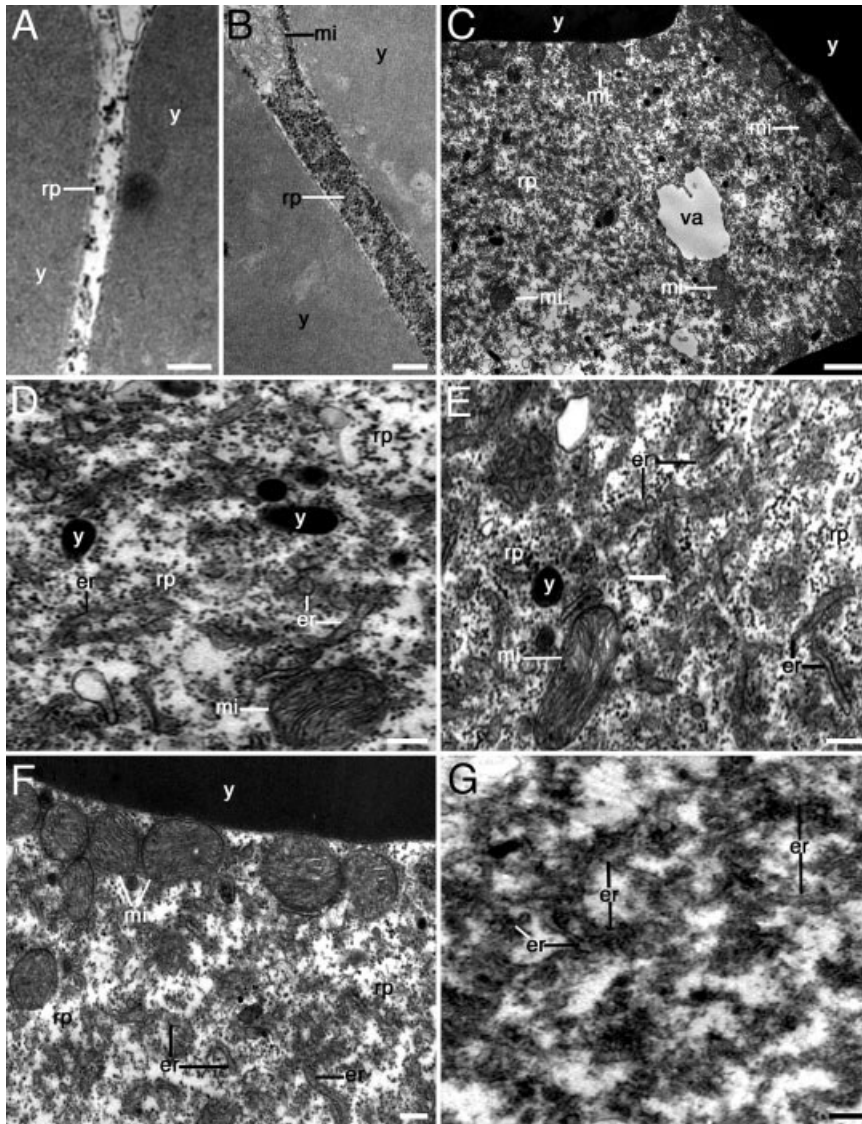
nied by reorganization of the endoplasmic lacunae generated in the stage V oocyte.

The redistribution of the endoplasmic lacunae was also studied in activated whole-mounted acid-fixed eggs prepared every 10 sec during the first minute of development and every 30 sec during the following 4 min. Stacks of Z-sectioned eggs allowed examination of digitally processed optical sections and preparation of animations or reconstructions. Activation produced changes in the distribution of endoplasmic lacunae (Fig. 7) as a result of endoplasm flowing into the blastodisc. Because the blastodisc and ectoplasm have multiple connections (Fig. 8A) ectoplasm may also flow into the embryonic disc. In many eggs, the network of endoplasmic lacunae accumulated at the animal/vegetal axis of the egg, in close proximity to the growing blastodisc. Here, lacunae were seen to extend into the blastodisc

forming short vertical channels reminiscent of the axial streamers formed in the zygote (Fig. 8B). Higher resolution microscopic observation of acid-fixed eggs showed that the enlarged endoplasmic lacunae were interconnected forming a complex network (Fig. 8C).

#### *Examination of sectioned plastic-embedded eggs by video light microscopy.*

Plastic sections of fertilized and unfertilized eggs examined by video microscopy during the first 5 min after activation confirmed results obtained in acid-fixed eggs. First, it was difficult to visualize the lateral border between adjacent peripheral yolk globules (Fig. 9A). This observation suggested that canaliculi between yolk globules narrowed. Second, loosely arranged yolk globules persisted at the blastodisc/endoplasm boundary zone (Fig. 9B),



**Fig. 5.** Electron photomicrographs that illustrate the fine structure of the stage V oocyte. **A,B:** These photomicrographs show the most common type of canaliculi present between peripheral yolk globules (y). Small canaliculi (A) only include electron-dense granules (rp), whereas large canaliculi (B) may also have organelles such as mitochondria (mi). **G:** Granules are digested by RNase A and, hence, are considered to be ribonucleoproteins. The ectoplasm opening of the canaliculi is at its upper end. **C–F:** These photomicrographs illustrate the structure of the endoplasmic lacunae. **C:** Low-magnification lacuna showing that the endoplasm includes numerous mitochondria that are scattered across a matrix of ribonucleoprotein granules or line up at the surface of the yolk globules (y). **D,E:** Higher-magnification photomicrographs of endoplasmic lacunae showing the abundance of smooth endoplasmic reticulum (er), mitochondria and small yolk globules scattered across a granular matrix. **F:** Closely apposed mitochondria lining up the surface of a yolk globule. **G:** A substantial number of electron-dense granules disappeared from the endoplasm after RNase A digestion. This finding caused the appearance of empty spaces surrounded by organelles. va, vacuole. Scale bars = 0.25  $\mu\text{m}$  in A,B,D–G, 1  $\mu\text{m}$  in C.

maintaining spacious pathways for poleward endoplasmic flow. Third, endoplasmic lacunae enlarged and the outlines of the central yolk globules were in general difficult to detect (Fig. 9C). Canaliculi had narrowed, presumably as result of ooplasm flowing into the lacunae. Detailed microscopy

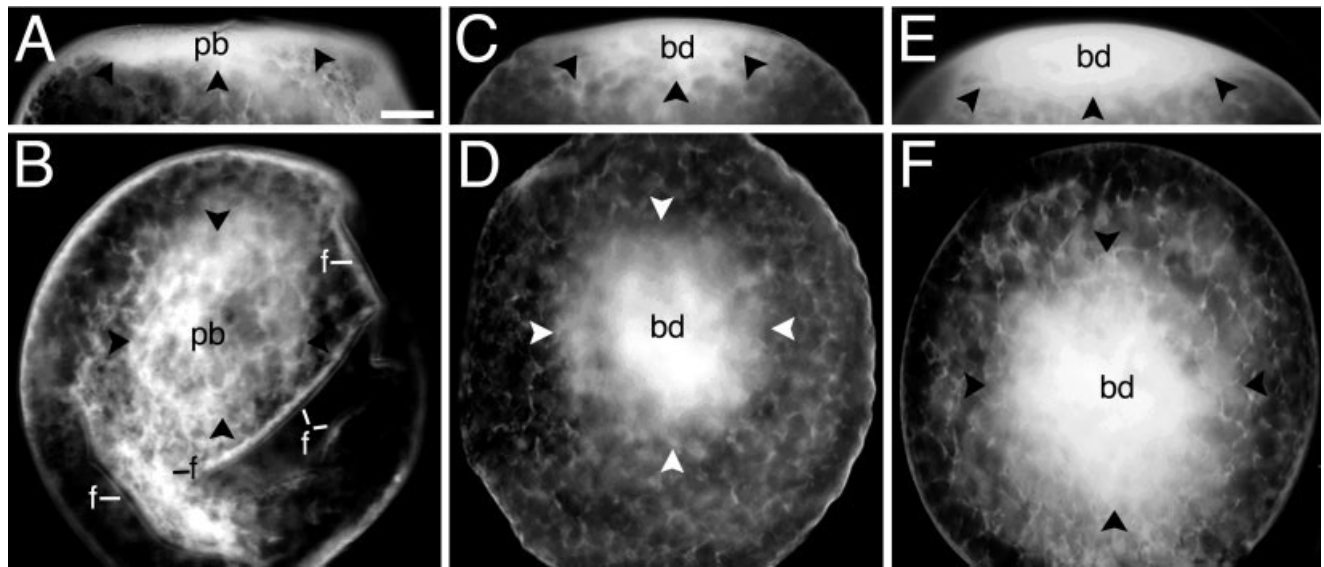
of activated eggs revealed that some endoplasmic lacunae had undergone structural changes. Thus, two types of endoplasm lacunae could be detected in the activated egg. Type I lacunae were similar to those seen in stage V oocytes (Fig. 9D), whereas type II lacunae presented a different distribu-

tion of their endoplasmic components (Fig. 9E,F). Vacuoles diminished in number, and darkly stained bodies, 2–7  $\mu\text{m}$  in diameter, appeared scattered across the lacunae. Type I and II lacunae may be interconnected, and some displayed a mixed structure. In some eggs, type II lacunae were abundant in the blastodisc neighborhood.

#### *Examination of eggs by transmission electron microscopy.*

Observations with the transmission electron microscope confirmed that activation led to a reduced width of the canaliculi running between peripheral yolk globules. Figure 10A–C shows junctions between peripheral yolk globules of activated eggs. Junctions were 20-nm-wide granule-free clefts or under 0.25  $\mu\text{m}$ -wide ooplasm-filled canalicules. In this manner, activation imposed further restriction in the size of the pathways connecting ecto- and endoplasm. Although preservation of the endoplasm was difficult, the two type of lacunae were easily recognized across the yolk cell. Type I lacunae appeared similar to those found in the stage V oocyte. Their endoplasm consisted of mitochondria and profiles of sER scattered across a matrix of electron-dense granules (Fig. 11A,B). The electron microscope also showed that dark bodies in type II lacunae were clusters of medium sized electron-dense granules surrounded by mitochondria, profiles of sER, and often dense core vesicles. These clusters were dispersed across a matrix of regularly spaced highly electron-dense granules (Fig. 11C–E). As it happened in type I lacunae, numerous mitochondria and profiles of sER lined up at the surface of the yolk globules facing the lacunae (Fig. 11F). RNase digestion demonstrated that electron-dense granules in type II lacunae were also ribonucleoproteins (Fig. 12).

The cytoskeleton of the zebrafish egg was poorly preserved after regular fixation in the Karnovsky solution. However, prefixation treatment of the eggs with cytoskeleton stabilizers such as Taxol and phalloidin allowed visualization of microtubules and actin filaments throughout the ecto- and endoplasm as well as blastodisc. We



**Fig. 6.** A–F: Lateral (A,C,E) and animal pole (B,D,F) views of whole-mounted acid-fixed cells showing development of the blastodisc from the early stage V oocyte (A,B) to the egg shortly after activation (C–F). The early stage V oocyte (A and B) is a flaccid cell exhibiting numerous folds (f). Its preblastodisc (pb) is thin and remains largely translucent after fixation. At 3 (C,D) and 5 (E,F) min after activation, the blastodisc (bd) has enlarged and thickened considerably. Arrowheads mark the outer border of the preblastodisc and blastodisc. Scale bar = 100  $\mu$ m.

put particular attention to the endoplasmic cytoskeleton, because its existence has been controversial. Loose bundles of microtubules were seen along the endoplasmic lacunae in proximity to organelles. Bundles of microfilaments, approximately 7 nm in diameter, were also seen scattered throughout the endoplasmic lacunae near microtubules (Fig. 13A,B). These bundles of microfilaments closely resembled those present at the egg cortex (Fig. 13C), suggesting that they also consisted of actin filaments. Definitive identification of actin filaments in endoplasmic bundles awaits decoration with heavy meromyosin. The structure of the cytoskeleton could not be investigated in the stage V oocyte, because these flaccid cells were difficult to microinject with cytoskeleton stabilizers. However, it was possible to explore in the egg whether changes in the structure and distribution of endoplasmic lacunae, as result of activation, were accompanied by changes in the structure of their cytoskeleton. Observations with the electron microscope have not yet provided sufficient information on this matter. However, it is clear that during early remodeling of the endoplasmic lacunae microtubule and microfilament bundles remain scattered across the endoplasm. It is expected

that immunocytochemical staining of the cytoskeleton during ooplasmic segregation will provide the needed information.

#### **Distribution of Microinjected Labeled Tracers Suggests That a Transport Barrier Is Present at the Ectoplasm/Endoplasm Boundary Zone**

To determine whether there was a relationship between the organization of peripheral yolk globules and transport between different ooplasmic domains, labeled proteins and dextrans were microinjected into the yolk cell. Transport of fluorescent tracers was monitored by time-lapse fluorescence video and confocal microscopy of whole-mounted eggs microinjected shortly after activation. Distribution of the tracer, on the other hand, was analyzed in video Z-series of similar eggs microinjected in different regions of the yolk cell.

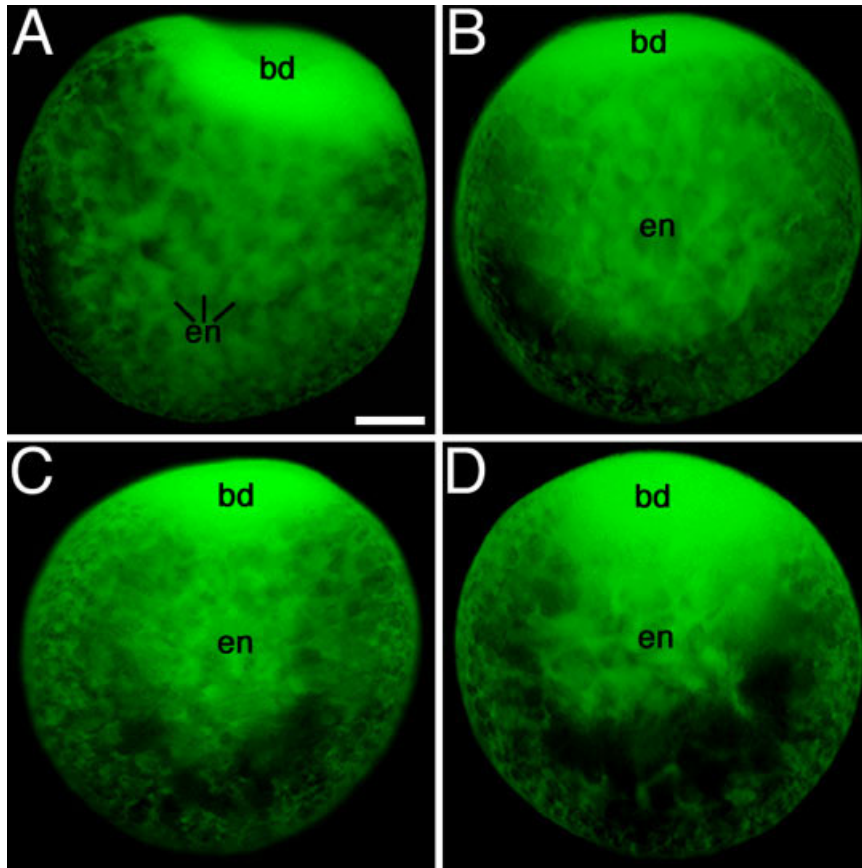
#### *Distribution of labeled proteins.*

Labeled proteins, ranging between 21.5- and 66-kDa, diffused throughout the endoplasmic lacunae and entered both into the blastodisc and ectoplasm within the first minutes after microinjection. Of interest, the layer of peripheral ectoplasm became more fluo-

rescent than the endoplasmic lacunae and blastodisc (not shown). These results indicated that mid molecular weight proteins microinjected in the endoplasm crossed the closely apposed layer of peripheral yolk globules at the endoplasm/ectoplasm boundary zone. Brighter fluorescence of the ectoplasm may be indicative that microinjected labeled proteins were more concentrated in the ectoplasm than in other ooplasmic domains.

#### *Distribution of labeled dextrans.*

Distribution of 10- to 70-kDa dextrans was similar to that of labeled proteins, but their rate of diffusion across the ooplasm was slower. It took no less than 5–15 min for these probes, microinjected at the center of the yolk cell, to become distributed across the endoplasmic lacunae, blastodisc and ectoplasm (Fig. 14A,B). In the case of microinjected 500-kDa dextrans, the time of diffusion of the probe across the endoplasm and blastodisc was even slower, sometimes extending up to 20–25 min. Substantial amount of the probe reached the blastodisc indicating that the 500-kDa dextran crossed the blastodisc/yolk cell boundary zone. However, very little high molecular weight dextran en-



**Fig. 7.** Pseudo-colored Z-sectioned acid-fixed whole-mounted early unfertilized eggs showing the redistribution of endoplasm during the first minutes after activation. Optical sections were obtained at 5- $\mu$ m intervals. **A:** Image 37 of a stack of 41 images of an egg 10 sec after activation. The distribution of endoplasmic lacunae (en) is similar to that of a late stage V oocyte (compare with Fig. 3C). **B:** Image 26 of a stack of 37 images of an egg 30 sec after activation. Endoplasmic lacunae have started accumulating at the center of the egg. **C:** Image 26 of a stack of 36 images of an egg 2 min after activation. Endoplasmic lacunae are seen congregated at the animal/vegetal axis. **D:** Image 35 of a stack of 46 images of an egg 5 min after activation. Endoplasmic lacunae are congregated closer to the growing blastodisc (bd). Scale bar = 100  $\mu$ m.

tered into the ectoplasm, even after placing the probe injection close to the egg periphery (Fig. 14C–E). For comparison purposes, some eggs received a peripheral microinjection of the high molecular weight dextran that infiltrated both the ecto- and endoplasm. Under these conditions, the ectoplasm was heavily labeled with the 500-kDa dextran (Fig. 14F). To examine in more detail the distribution of this probe, microinjected eggs were examined under the confocal microscope. To this end, gallery of sections were prepared from eggs microinjected in different regions of the yolk cell. It was found that substantial amounts of the 500-kDa dextran crossed the blastodisc/endoplasm boundary zone (Fig. 15A), but little or

no dextran crossed the endoplasm/ectoplasm boundary zone (Fig. 15B,C). The ectoplasm, however, was heavily loaded with dextran when the probe was microinjected at the egg periphery (Fig. 15D). Although results on the distribution of the 500-kDa labeled dextran exceeded the time frame chosen for other observations (until 5 min after activation), they are indicative that transport of high molecular weight dextran is restricted at the endoplasm/ectoplasm boundary zone but not at the endoplasm/blastodisc boundary zone. Another interesting observation on microinjected eggs is that the 500-kDa dextran that had reached the blastodisc did not flow back into the ectoplasm (see Figs. 14C, 15A).

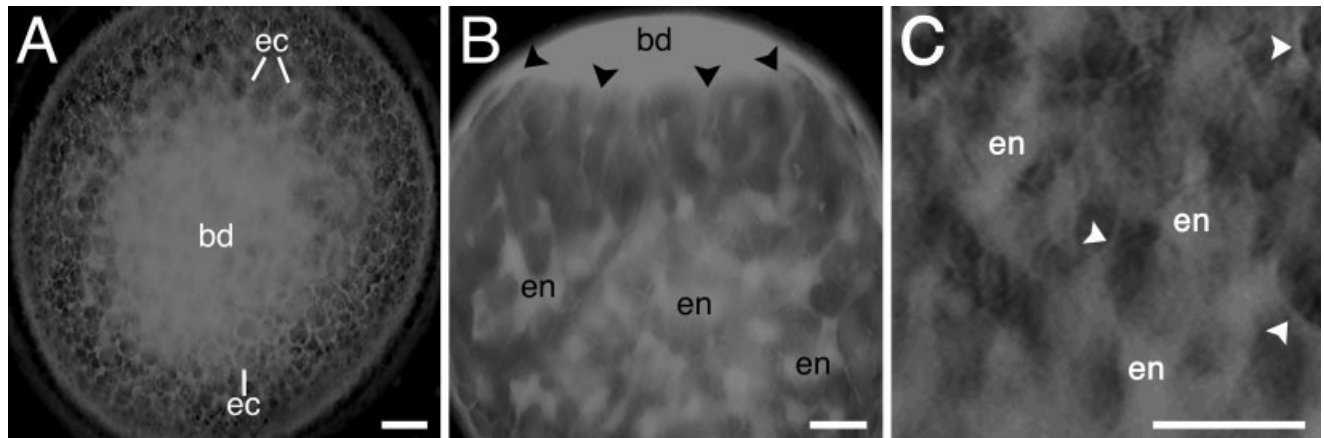
## DISCUSSION

### Preactivation Phase of Ooplasmic Segregation Is Initiated in the Ovary and Mainly Concerns With the Establishment of Ooplasmic Domains

Ooplasmic segregation in the zebrafish egg is considered to be triggered by activation, as indicated by the appearance of a blastodisc (Ho, 1992; Kimmel et al., 1995; Lee et al., 1996). Results reported in this study demonstrate, however, that a first step of ooplasmic segregation indeed starts earlier during oogenesis. This early step of ooplasmic segregation involves physical separation of yolk from ooplasm and local accumulation of the latter to form three transient ooplasmic domains: ectoplasm, endoplasm, and preblastodisc. Our observations confirmed and extended previous findings (Katow, 1983) that the ooplasm encloses numerous organelles and granular material. Mitochondria and profiles of sER are found in large number across ooplasmic domains. Golgi complexes are found with less frequency. The presence of large number of mitochondria is typical of the ooplasm of invertebrate eggs (Fernández et al., 1998), but the significance of this finding is not yet clear. Profiles of sER, and, hence, calcium reservoirs are abundant across all ooplasmic domains. This finding indicates that free calcium transients may be produced in many regions of the zebrafish egg besides known places such as the ring of contraction at the outer border of the blastodisc (Leung et al., 1998). There are three reasons for considering that the 20- to 30-nm electron dense granules are ribonucleoproteins. First, their size is similar to that of ribosomes. Second, they are digested by RNase and third zebrafish eggs contain abundant maternal mRNA distributed across the three ooplasmic domains (reviewed by Pelegri, 2003).

Results have not revealed the possible mechanisms involved in the separation of yolk and ooplasm. However, comparison of stage III and V oocytes allows one to speculate on how such separation might have occurred and how ooplasmic domains formed during oogenesis. It is presumed that the





**Fig. 8.** Z-sectioned acid-fixed whole-mounted early unfertilized eggs showing the interconnections between ooplasmic domains. **A:** Image 18 of a stack of 54 images that shows an animal pole view of an egg 5 min after activation. The ectoplasm (ec) surrounding the outer surface of the peripheral yolk globules is continuous with the growing blastodisc (bd). **B:** Image 29 of a stack of 40 images that show the blastodisc/endoplasm boundary zone of an egg 4 min after activation. Endoplasmic lacunae (en) appear connected to the blastodisc by numerous short channels (arrow heads). **C:** Image 44 of a stack of 64 images of an egg 5 min after activation, showing the growth and close gathering of endoplasmic lacunae. Interconnection between lacunae are marked by arrowheads. Scale bars = 100  $\mu$ m.

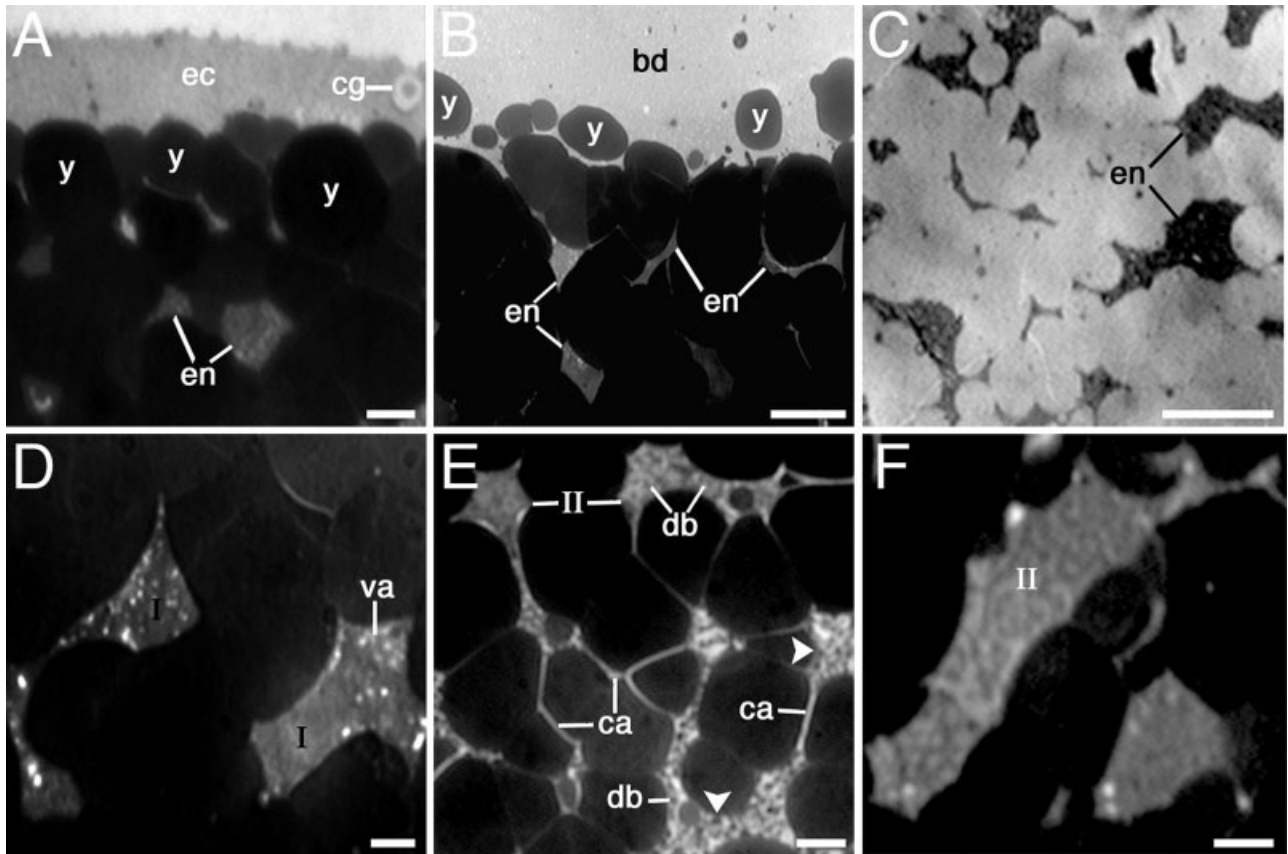
driving force for ooplasm segregation and compartmentalization may be the clustering or close aggregation of yolk globules in certain regions of the oocyte. This process may start with the orderly linear clustering of yolk globules at the oocyte periphery, a process that would give rise to the yolk palisade separating an outer ectoplasm compartment from an inner endoplasm compartment. Focal clustering of yolk globules across the oocyte interior, on the other hand, would give rise to the system of interconnected endoplasmic lacunae. The fact that narrow canaliculi predominate between peripheral yolk globules in the stage V oocyte, and perhaps also in the stage IV oocyte, suggests that this situation may generate restrictions to the transit of ooplasm between the two compartments. This situation is probably strengthened after activation, due to progressive narrowing of the canaliculi present between peripheral yolk globules. Distribution of microinjected dextrans of different molecular weight provides further support to the idea that the peripheral yolk palisade may function as a barrier regulating translocation of ribonucleoproteins and organelles between ectoplasmic and endoplasmic domains. However, that some high molecular weight dextran reaches the ectoplasm from the endoplasm suggests that a limited number of wider pathways between peripheral yolk

globules do exist. It seems likely that ectoplasmic recesses described in this study form part of such pathways. Although the number and properties of these pathways are completely ignored, they may provide shunts that in some parts of the egg may turn endoplasmic into ectoplasmic flow and vice versa. These findings demonstrate the way in which maternal factors become distributed during oogenesis and translocated during ooplasmic segregation. It is known that numerous maternal mRNAs accumulate in the zebrafish oocyte during oogenesis. Moreover, such mRNAs may be subjected to an orderly process of redistribution (reviewed by Pelegri, 2003). In situ hybridization studies of zebrafish oocytes at different stages of development show that relocation of many maternal mRNAs occur before the oocyte reaches the stage V of development, that is, before establishment of a yolk palisade separating ecto- from endoplasm. This is the case for *peu2*, *cyclin B* (Howley and Ho, 2000), *zorba*, *Vg 1* (Bally-Cuif et al., 1998), and *brul* (Susuki et al., 2000). Many maternal mRNAs become located to the animal pole during oogenesis (Bally-Cuif et al., 1998), whereas others do so as result of ooplasmic flow triggered by egg activation (Howley and Ho, 2000; Suzuki et al., 2000). Our findings suggest that the bulk of the transcripts sequestered in ectoplasmic or endoplasmic domains are translocated pole-

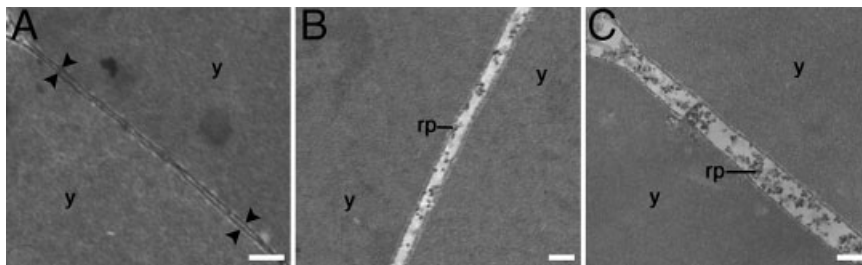
ward by separated peripheral or central routes. The fact that, by the end of oogenesis, maternal mRNAs are ubiquitously distributed across ecto- or endoplasm or are anchored to vegetal or animal regions of the oocyte suggests the existence of cues that determine specific patterns of localization of maternal factors across the ooplasm. Effect of drugs suggests that the cytoskeleton may (Suzuki et al., 2000; Gore and Sampath, 2002) or may not (Bally-Cuif et al., 1998) be involved in mRNA localization.

Maternal genes probably play an important role in the control of ooplasmic segregation. Unfortunately, very little is known on maternal-effect mutations that might affect this process (see Pelegri, 2003). Findings reported in this study will be valuable to interpret results of such mutations.

The organization of peripheral yolk globules is different at the animal pole of the stage V oocyte. Here, loosely arranged yolk globules partly separate endoplasm and preblastodisc domains, thus providing spacious pathways for animal pole-directed flow of organelles and ribonucleoproteins. Transport of high molecular weight dextran from the yolk cell into the blastodisc in eggs supports this conclusion. The ooplasm accumulated at the preblastodisc probably has a dual origin: part would come from the cytoplasm originally surrounding the germinal vesicle and the rest would be



**Fig. 9.** Light photomicrographs of stained plastic-embedded thick sections showing reorganization of the cytoplasm in the early unfertilized egg (2 min after activation). **A:** Boundaries between peripheral yolk globules (y) are barely visible, because ooplasm between them have probably been squeezed out. In this manner, ectoplasm (ec) and endoplasm (en) are more separated than before activation. Most of the cortical granules (cg) have been released. **B:** Loosely arranged yolk globules remain at the blastodisc (bd)/endoplasm (en) boundary zone, thus ensuring persistence of wide transport pathways between the two ooplasmic domains. **C:** Photomicrograph taken at the center of the egg showing that the boundaries between internal yolk globules are barely visible, while endoplasmic lacunae have enlarged (compare with Fig. 4D). **D:** Photomicrograph taken at the center of the egg showing that type I (I) endoplasmic lacunae have numerous vacuoles (va) scattered across a homogenous cytoplasmic matrix. **E:** Type II (II) endoplasmic lacunae have less vacuoles and numerous dark bodies (db) scattered across a moderately stained matrix. In mildly preserved type II lacunae (arrowheads) dark bodies appear scattered across an empty matrix. Notice that canaliculi (ca) linking these lacunae look empty and wider. They are considered to have suffered artifactual expansion. **F:** High-magnification of type II lacuna. Scale bars = 10  $\mu\text{m}$  in A, D–F, 50  $\mu\text{m}$  in B, C.



**Fig. 10.** Electron photomicrographs that illustrate the structure of the most-common type of junctions between peripheral yolk globules in early unfertilized eggs. **A:** There are numerous 20 nm in diameter ooplasm-free clefts that extend between parallel running membranes (arrowheads) enveloping neighbor yolk globules (y). **B, C:** Ooplasm-filled canaliculi are less than 0.25  $\mu\text{m}$  in diameter and include variable number of ribonucleoprotein granules (rp) but lack organelles. The ectoplasm opening of the cleft and canaliculi are situated at the upper end. Scale bars = 0.25  $\mu\text{m}$ .

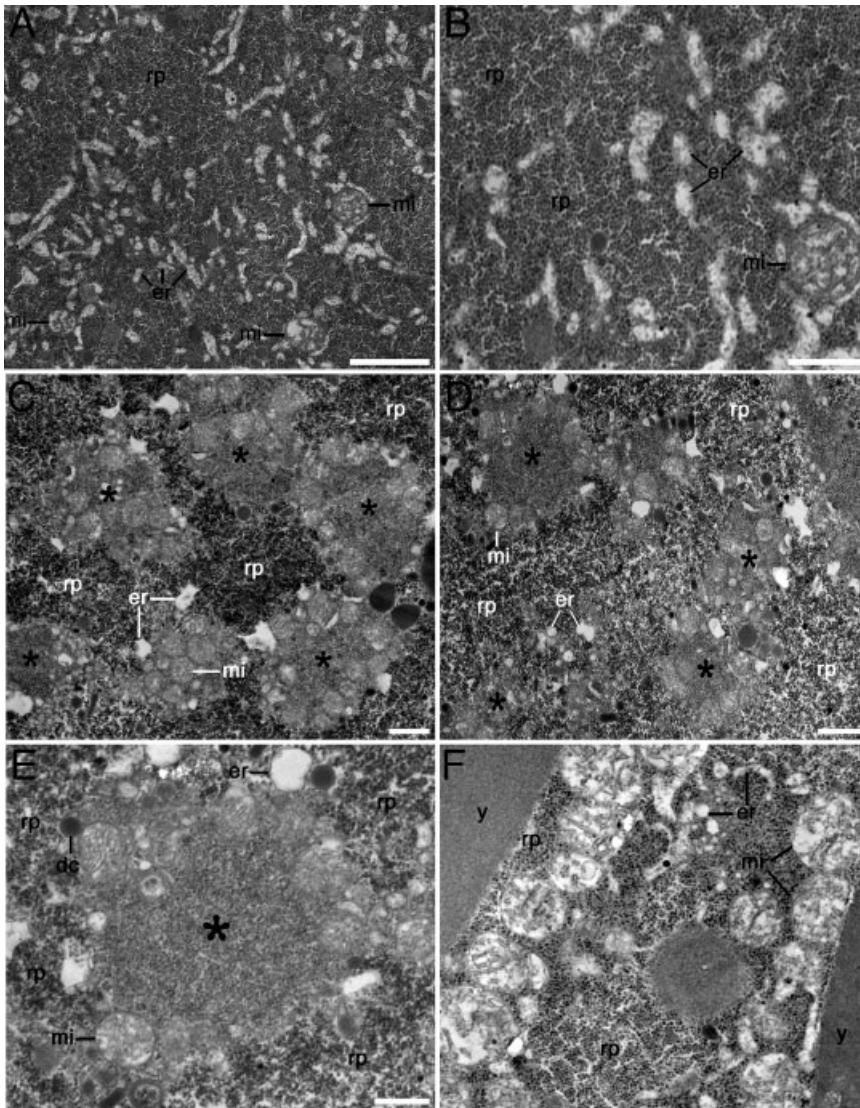
mRNA-rich ooplasm translocated to the animal pole during oogenesis (see Bally-Cuif et al., 1998). There are reasons to suspect that separation of oo-

oplasm from yolk and establishment of ooplasmic domains are cytoskeleton-dependent processes. Although little information is available on the orga-

nization of the cytoskeleton in developing oocytes, that the early activated egg has an ubiquitous cytoskeleton suggests that a similar one might be present in the stage V oocyte.

### Postactivation Phase of Ooplasmic Segregation Is Manifested by Poleward Flow of Endoplasm and Blastodisc Growth

The available information indicated that activation provoked lifting of the vitelline envelope, formation of the blastodisc, and discharge of the second pole cell (see Devillers, 1961). We have provided further information showing that a blastodisc precursor forms in the stage V oocyte and that blastodisc growth is initi-



**Fig. 11.** Electron photomicrographs that show the fine structure of endoplasmic lacunae in early unfertilized eggs. **A,B:** Low- (A) and high- (B) magnification photomicrographs of a type I lacuna having profiles of smooth endoplasmic reticula (sER; er) and mitochondria (mi) scattered across a matrix of ribonucleoprotein (rp) granules. This type of lacuna is similar to those found in the stage V oocyte (see Fig. 5D,E). Differences in the appearance of the sER may be due to swelling. **C,D:** Low-magnification photomicrographs showing the structure of type II lacunae. Dark bodies found in the light microscope correspond to clusters of mitochondria and profiles of sER (asterisks) scattered across a matrix of ribonucleoprotein granules. **E,F:** High-magnification photomicrographs showing the distribution of organelles and ribonucleoprotein granules in type II lacunae. **E:** Each cluster of organelles consists of a central sector (asterisk) with medium electron-dense ribonucleoprotein granules and a peripheral sector with mitochondria, sER, and dense core vesicles (dc). The cluster of organelles is surrounded by highly electron-dense ribonucleoprotein granules. **F:** Mitochondria, profiles of sER, and ribonucleoprotein granules also gather at the periphery of the lacuna, in close contact with the surface of the yolk globules (y). Scale bars = 0.5  $\mu\text{m}$  in B,E (applies to F), 1  $\mu\text{m}$  in A,C,D.

ated immediately after activation as result of enlargement and redistribution of endoplasmic lacunae. Enlargement of endoplasmic lacunae is probably the combined result of further endoplasm accumulation and coalescence of neighboring lacunae. Evidence for further endoplasm accumulation comes from the observa-

tion that enlargement of lacunae is accompanied by narrowing of the ooplasm-filled canaliculi present between central yolk globules. This process is the result of granular ooplasm being squeezed out from between the yolk globules and added to lacunae. We suggest further that ooplasm translocated to lacunae after

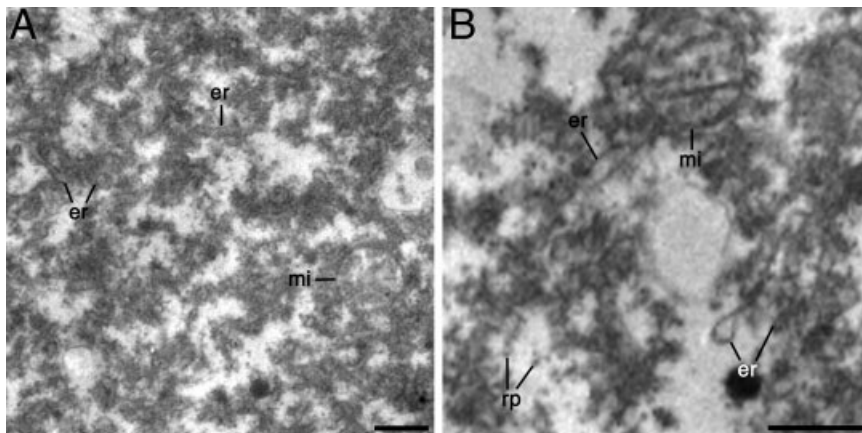
activation includes ribonucleoprotein granules, whereas ooplasm accumulated in lacunae before activation includes both organelles and ribonucleoprotein granules. Early workers of zebrafish eggs such as Lewis and Roosen-Runge (1943) and Beams et al. (1985) proposed that poleward squeezing of endoplasm trapped among the yolk globules was the mechanism involved in ooplasmic segregation. However, they were not aware that an earlier step of this process, consisting in the formation of ooplasmic domains, took place in the ovary. Centripetal accumulation and alignment of enlarged lacunae along the animal/vegetal axis of the egg probably favors formation of axial streamers later in development. Axial streamers are vertically running pathways of endoplasm flowing toward the blastodisc (Lewis and Roosen-Runge, 1943; Leung et al., 2000). Several lines of evidence indicate that ectoplasm also moves toward the blastodisc after activation. First, polystyrene beads microinjected in the ectoplasm move toward the animal pole (Jesuthasan and Strähle, 1996). Second, maternal mRNAs, such as *vasa* (Howley and Ho, 2000), reach the blastodisc by mean of an ectoplasmic flow. Third, labeled mitochondria move poleward across the ectoplasm to reach the blastodisc (our unpublished observations). Therefore, activation triggers both ecto- and endoplasmic flow. Resumption of ooplasmic segregation, as well as other processes triggered by egg activation (cortical reaction and second polar body release), are not dependent on sperm penetration (Wolenski and Hart, 1987) and, hence, occur in both fertilized and unfertilized eggs. It seems likely, then, that the wave of free cytosolic calcium generated upon activation of the fertilized or unfertilized egg is the main signal for resumption of ooplasmic segregation (Lee et al., 1996; Leung et al., 1998; Lee et al., 1999). Free calcium transients, probably generated simultaneously in many regions of the egg, would activate signaling pathways acting upon internal and external components of the cytoskeleton engaged in ooplasmic flow.

### Activation Provokes Redistribution of Organelles and Ribonucleoprotein Granules in Endoplasmic Lacunae

A surprising effect of activation was the appearance, across the yolk cell, of a different type of lacuna called type II lacuna. Type I and type II lacunae have similar structures but differ in the way that their components are arranged. In type I lacunae, ribonucleoprotein granules and most organelles are evenly distributed, whereas in type II lacunae, granules and organelles form conspicuous clusters. Type II lacunae could be newly formed

structures or modified type I lacunae. The last alternative is probably correct, because by the time of activation, most organelles have already been accumulated in type I lacunae and the ooplasm remaining among the yolk globules is essentially granular. In addition, there are transitional lacunae having a mixed arrangement of their organelles. It is not known how activation provokes such redistribution of organelles and ribonucleoprotein granules. We suspect, however, that  $[Ca^{2+}]$  transients accompanying activation might stimulate a cascade of internal signals affecting the function of an internal system of motors and cytoskeleton components. The latter

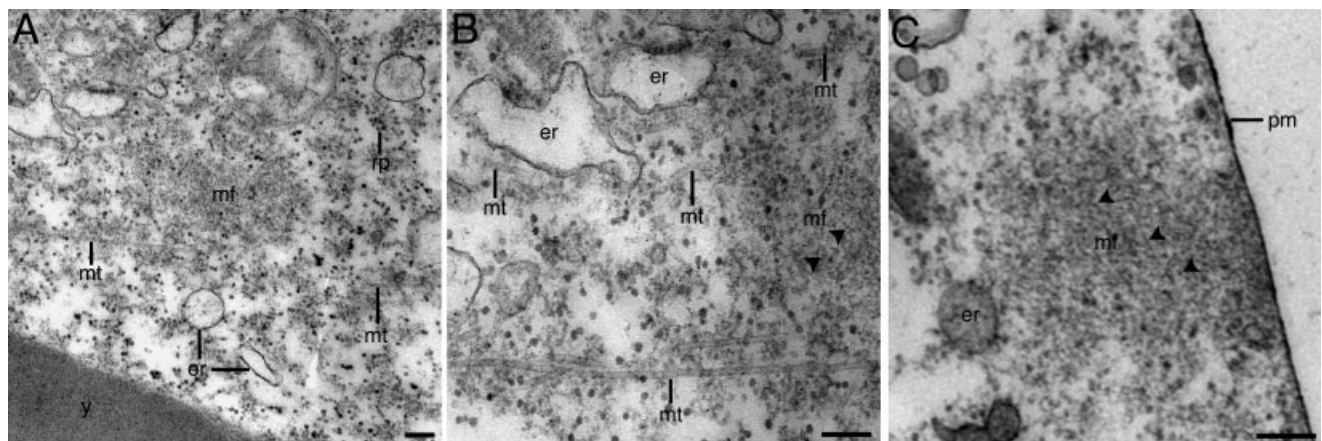
have been described in this study and, hence, microtubules and microfilaments may be involved in the rearrangement of organelles and granules across endoplasmic lacunae. That type II lacunae are sometimes concentrated in the animal sector of the yolk cell may be taken as indicative that these lacunae may be more actively involved in ooplasmic flow than type I lacunae. If this presumption is correct, aggregation of organelles and ribonucleoprotein granules may expedite endoplasmic flow. Assembly of mitochondria and granular material in clusters along transport pathways is also seen in the leech zygote at the peak of ooplasmic segregation (Fernández et al., 1998).



**Fig. 12.** **A,B:** Low- (A) and high- (B) magnification photomicrographs showing type II lacunae of egg fragments digested with RNase A. A great number of electron-dense granules have been removed by the enzyme causing formation of numerous empty spaces. Mitochondria (mi), smooth endoplasmic reticula (sER; er), and some granules (rp) persist at the periphery of the empty spaces. Scale bars = 0.5  $\mu$ m.

### Ubiquitous Distribution of Microtubules and Microfilaments Across the Egg Suggests That Ooplasmic Segregation Is Driven by Both Peripheral and Central Cytoskeletons

Findings reported in this study show that microtubules and microfilaments are present throughout the three ooplasmic domains. These results confirm the work of others in that actin filaments (Hart et al., 1977, 1992; Hart and Yu, 1980; Kato, 1983; Wolensky and Hart, 1987) and microtubules (Jesuthasan and Strähle, 1996; Gore and Sampath, 2002) are present across the ecto-



**Fig. 13.** **A,B:** Low- (A) and high- (B) magnification electron photomicrographs that show presence of microtubules (mt) and microfilaments (mf) in endoplasmic lacunae. Microtubules are scattered singly or in small loose bundles running into different directions near profiles of sER (er) and ribonucleoprotein particles (rp). Microfilaments are seen forming bundles adjacent to microtubules. **C:** Microfilament bundles in the endoplasm are similar to those seen in the cortical ectoplasm. Individual microfilaments are indicated by arrowheads. pm, plasma membrane. Scale bars = 0.2  $\mu$ m.

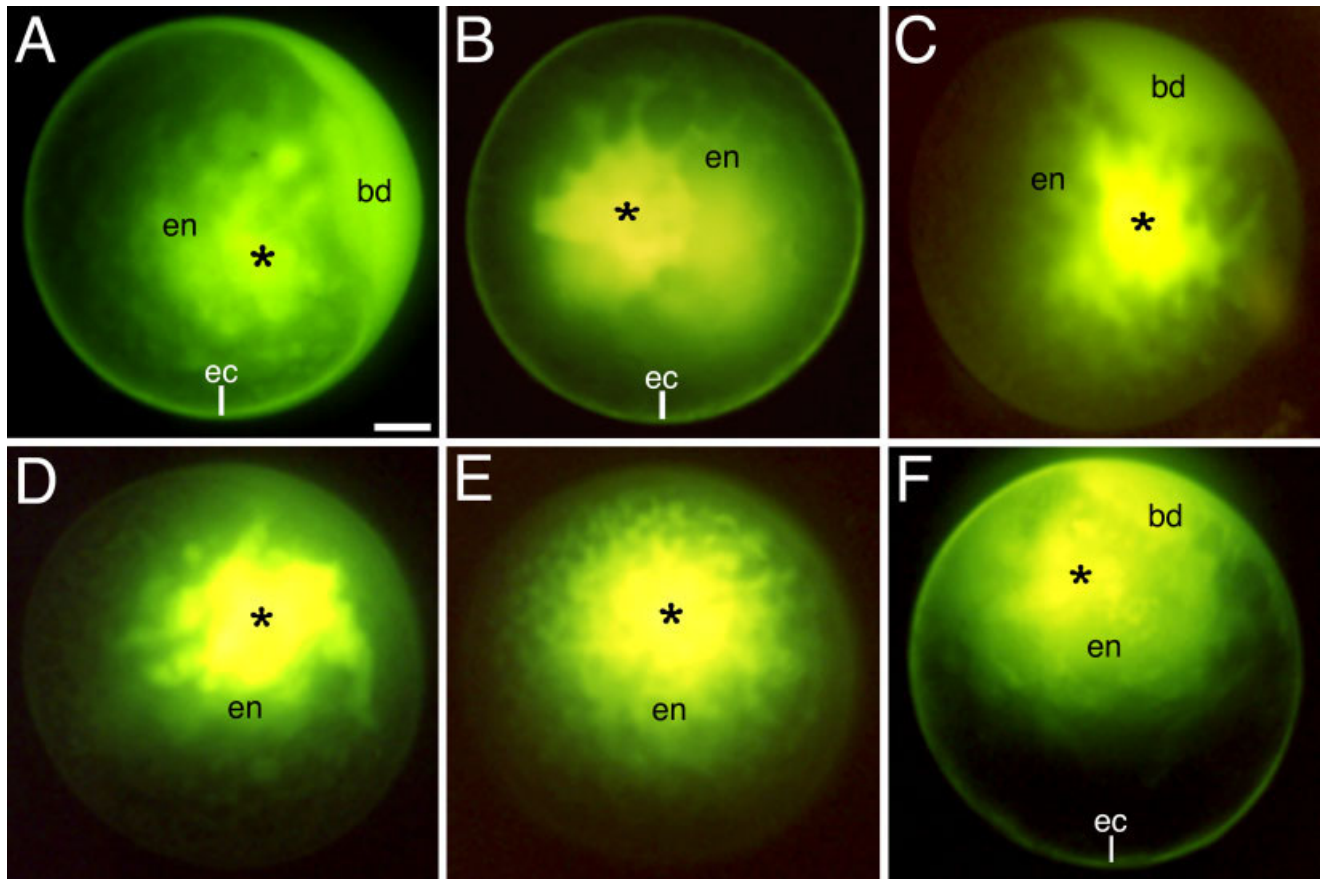


Fig. 14.

plasm. However, there is only one report (Beams et al., 1995) based on the observation of zebrafish eggs under the scanning electron microscope, in which the authors interpreted lineal elements as belonging to microtubules or microfilaments. For reasons explained in the text, it was not possible to compare the cytoskeleton of stage V oocytes with that of early developing eggs. For that reason, we could not trace changes in the cytoskeleton related to the first steps of ooplasmic segregation. However, that the cytoskeleton of developing eggs remains embedded across the ooplasm indicates that sliding of microtubules and actin filaments may provide the force for ooplasmic flow across both ecto- and endoplasm. This alternative does not exclude the possibility that microtubules and actin filaments may also function as tracks along which organelles and ribonucleoproteins would move thanks to the activity of molecular motors.

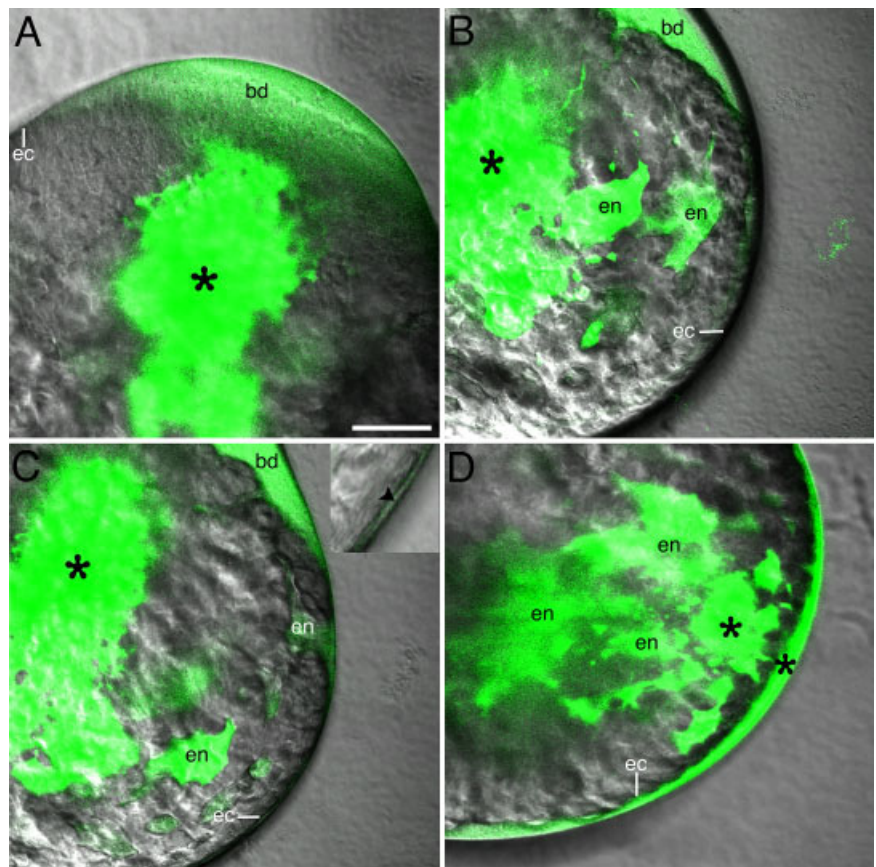


Fig. 15.

## EXPERIMENTAL PROCEDURES

### Collection and Handling of Oocytes and Eggs

Adult zebrafish (*Danio rerio*) were maintained at 28°C in aerated aquaria subjected to a 14-hr light/10-hr dark photoperiod. Males and females were separated a day before and crossed the next day. Nontranslucent medium and large sized stage III oocytes (400–600  $\mu\text{m}$  in diameter), and translucent stage V oocytes (approximately 750  $\mu\text{m}$  in diameter), were surgically removed from the ovaries. Selected stage III oocytes had a centrally located meiotic nucleus (see Selman et al., 1993). Stage V oocytes were also procured by gently pressing the abdomen of pregnant mothers anesthetized with tricaine (Sigma). Stage IV oocytes are difficult to identify, because they overlap in size with large stage III oocytes and are rapidly converted in stage V oocytes (Selman et al., 1993; Li et al., 1993). For this reason, they were not included in this work. Early activated stage V oocytes (unfertilized eggs) were obtained by briefly soaking the cells in filtered tank water. A small fraction of the stage V oocytes removed from the ovary failed to be activated. Fertilized eggs were obtained by natural or arti-

ficial procedures. Fertilized and unfertilized eggs developing in conditioned tank water at 20°C were removed for microscopic examination at different time points during the first 5 min after activation. To determine the effect of temperature and fertilization on ooplasmic segregation, development of fertilized and unfertilized eggs were compared at 20°C and 28°C.

### Light Microscopy

#### *Preparation of whole-mounted acid-fixed oocytes and eggs.*

Stage V oocytes and eggs are translucent, but their ooplasm may be rendered opaque by fixing the cells in tissue culture dishes (35  $\times$  10 mm, Sarstedt) using freshly prepared neutralized 10% formaldehyde, to which a few droplets of glacial acetic acid (2–3 droplets in 4–5 ml of formaldehyde) were added at the beginning of the fixation (Fernández, 1980). The chorion did not interfere with the acid fixation, but it could be removed to facilitate positioning of the oocyte during microscopic examination. For more detailed observation, acid-fixed cells may be further cleared by transferring them to a 1:1 mixture of glycerol and formaldehyde/acetic acid. Study of the displayed images were improved by converting opacity into

brightness, using the reverse contrast facility of the Metamorph program.

#### *Loading cells with labeled proteins and dextrans.*

To test the permeability of the ectoplasm/endoplasm yolk barrier the transport of proteins and dextrans were studied in eggs microinjected shortly after activation. The probes were injected with micropipettes prepared from borosilicate glass capillaries (Harvard Apparatus Ltd.) using a Narishige IM 300 microinjector. A total of 4 to 8 nl of the probes were microinjected into different regions of the yolk cell: close to the blastodisc, close to the ectoplasm or at the egg center. The amount of injected tracer represented less than 5% of the egg volume (approximately 200 nl). Labeled proteins were column-purified and its purity tested by electrophoresis and Western blot. The following labeled proteins were used: rhodamine-labeled dimeric tubulin (approximately 45-kDa, see Fernández et al., 2002, 2004), Alexa fluor 594-labeled bovine serum albumen (approximately 66-kDa, Molecular Probes). These probes were dissolved in glutamate buffer (Hyman et al., 1991) to a final concentration at the pipette of 12–90  $\mu\text{M}$ . Dextrans were also dissolved in glutamate injection buffer to a final concentration at the pipette of 25–50 mM. Fluorescein-labeled dextrans (Molecular Probes) of the following molecular weight were tested: 10 kDa, 70 kDa, and 500 kDa. All microinjected proteins and dextrans.

**Fig. 14.** Low-magnification micrographs of whole-mounted live zygotes showing the distribution of fluorescein-labeled dextrans microinjected shortly after activation. The site of injection is marked by an asterisk. **A:** Zygote microinjected with 10-kDa dextran close to the cell center. The probe diffused across endoplasmic lacunae (en) and reached the ectoplasm (ec) and blastodisc (bd). **B:** Zygote microinjected with 70-kDa dextran close to the cell center. The probe also diffused across the endoplasmic lacunae and reached the ectoplasm that became labeled. **C:** Egg microinjected with 500-kDa dextran under the blastodisc. The probe diffused across the endoplasm and blastodisc but the ectoplasm remained unlabeled. For this reason, a fluorescent rim is absent at the zygote periphery. **D,E:** Zygotes microinjected at the cell center with 500-kDa dextran. In both cases, the probe diffused across the endoplasm but did not accumulate in the ectoplasm. **F:** Eggs were microinjected with 500-kDa dextran at the periphery of the cell, so that the probe infiltrated both the ecto- and endoplasm. Under these conditions, the heavy molecular weight dextran diffused across the endoplasm and ectoplasm, where it formed a fluorescent rim. Scale bar = 100  $\mu\text{m}$ .

**Fig. 15.** Combined phase contrast and confocal images of whole-mounted live zygotes showing the distribution of 500-kDa fluorescein-labeled dextran microinjected shortly after activation. The site of injection is marked with an asterisk. **A:** The probe was microinjected close to the cell center and entered the blastodisc (bd). Notice that dextran accumulated in the blastodisc did not diffuse into the ectoplasm (ec). **B,C:** Optical section 20 (B) and 35 (C) of a stack of 37 images taken every 3.3  $\mu\text{m}$ . The probe was microinjected close to the zygote center from where it diffused across endoplasmic lacunae and entered the blastodisc. Very little of the probe gained direct access to the ectoplasm (arrow in the inset). **D:** Zygote microinjected at the periphery of the cell so that the probe infiltrated both the ecto- and endoplasm. Notice transportation of abundant labeled dextran across both the endoplasm and ectoplasm. White spots in the images are not empty spaces but phase contrast effects. Scale bar = 100  $\mu\text{m}$ .

#### *Preparation of stained sections of resin-embedded oocytes and eggs.*

Cells were fixed and postfixed as for electron microscopy (see below) and embedded in JB-4 (Polysciences), a soft mixture of Epon-Araldite or hard Epon 812 (Pelco) resins. In the first case, 1- to 2- $\mu\text{m}$ -thick sections were stained with methylene blue and basic fuchsin and in the second case, with 1% toluidine blue in borate buffer. Sections obtained with a MT 5000 Sorvall ultramicrotome were floated and dried in an aqueous diluted ammonia solution.

Video microscopy and image processing. Live, microinjected and acid-fixed cells were examined in a Zeiss

135 inverted fluorescence microscope equipped with a Z motor (Prior) and a Hamamatsu chilled CCD camera (Model C5985). For lower magnification, Achromplan objectives ( $\times 10$ – $40$ ) were used. For higher magnification, a Plan Neofluar objective ( $\times 100$ , NA 1.4) and the Optovar ( $\times 1.6$ – $2.5$ ) allowed the projection of images onto the camera chip at 160–250 magnification. Image grabbing and analysis were performed on a PC computer using the Metamorph (6.1 version) program. This strategy allowed preparation of stacks of images for time lapse and Z-sectioning analysis, elaboration of superficial topographic maps, best focus, and deconvolution. Assignment of pseudo coloring was possible in 24 bit-converted images. Further processing of the images was performed with a 7.0 version of Adobe Photoshop. For observation, cells were placed in Falcon 3072 tissue culture wells or between two coverslips separated by plasticine stoppers. The coverslips were mounted on the window of a metallic slide and the edges sealed with Vaseline. This type of cell mounting allowed better resolution of the images and inspection of the two sides of the cells by turning the preparation 180 degrees.

### Confocal Microscopy

Whole-mounted eggs microinjected with labeled proteins or dextrans were mounted as for video microscopy and examined in a Zeiss LSM 510 META laser scanning microscope, equipped with argon and He-Ne lasers. Time-lapse series of microinjected eggs were taken every 5–10 min, and Z-sections were performed every 3–5  $\mu\text{m}$ . Gallery of images allowed preparation of animations or reconstructions.

### Transmission Electron Microscopy

Preparation and observation of samples. Use of several fixative solutions and embedding media indicated that the fine structure of the zebrafish oocyte and egg was difficult to preserve. The best results were obtained with the use of the Karnovsky fixative and the application of a slow resin-embedding procedure. The fixative contained

0.1% tannic acid (Merck), 1  $\mu\text{g}/\text{ml}$  of phalloidin (Sigma), and 1  $\mu\text{g}/\text{ml}$  of Taxol (Molecular Probes). Whole oocytes and mechanically dechorionated eggs were fixed for 2–2:30 hr at room temperature. During fixation, cells were sliced to favor fixative and resin penetration. After rinsing in cacodylate buffer (containing the same concentration of phalloidin and Taxol) for the same amount of time, cells were postfixated for 1:30 hr in 1%  $\text{OsO}_4$  in the cacodylate buffer at room temperature and darkness. After dehydration in graded ethanol, cells were embedded in Epon 812 under vacuum. For this purpose, cells remained for approximately 12 hr in each of the following Epon/propylene oxide mixtures: 1:2, 1:1, and 2:1. They finally remained for 36 hr in pure Epon replaced every 12 hr. Thin sections were double stained with uranyl acetate and lead citrate and examined with a Jeol 100 SX electron microscope. Photographic negatives were scanned with an Epson Expression 1600 Professional scanner and processed with Adobe Photoshop in a G3 McIntosh computer.

Preservation of microtubules and microfilaments was greatly improved by pretreatment of the eggs with a mixture of cytoskeleton stabilizers. For this purpose, chorionated activated eggs were microinjected at the equatorial region with a mixture of 5 mM Taxol (4 nl) and 0.8  $\mu\text{M}$  phalloidin (10 nl) in dimethyl sulfoxide. After 10 min, eggs were fixed, rinsed, dehydrated, and embedded as outlined above.

### RNase Digestion

Egg fragments fixed as for electron microscopy were rinsed for 2 hr in cacodylate buffer and then incubated for 45 min in RNase A (Sigma) with continuous agitation. RNase was diluted in cacodylate buffer pH 7.6 at a concentration of 10  $\mu\text{g}/\text{ml}$ . After buffer rinsing, digested egg fragments were post-fixed and embedded in Epon resin.

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