Formation of polar cytoplasmic domains (teloplasms) in the leech egg is a three-step segregation process

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ABSTRACT Segregation and proliferation of mitochondria, leading to formation of the teloplasms (pole plasms), were studied in eggs of the leech T. rude by immunocytochemistry, fluorescent time lapse video imaging, confocal and electron microscopy. The translocation of mitochondria was analyzed after loading the egg with either Rhodamine 123 or a Mitotracker. Mitochondrial proliferation was assessed after pulse labeling with BrdU. The involvement of the cytoskeleton in the segregation process was determined by drug action. The teloplasms form during the first interphase as consequence of a 3-step sequential process of mitochondrial redistribution throughout the egg cytoplasm. The first step is a microtubule dependent process of ectoplasm thickening due to centrifugal mitochondrial transportation from the neighboring endoplasm. The furrowing pattern of the egg during this step can be modified by cold treatment and seems to be determined during oogenesis. During the third step the ectoplasm flows to either of the poles in conjunction with bipolar displacement of the polar rings and shortening of the contraction bands. This step depends on both microtubules and microfilaments. Mitochondria of first interphase eggs have three special features: (1) they move in clusters, (2) their movement depends on both microtubules and microfilaments and (3) they proliferate continuously. During the first interphase the polarized meiotic egg becomes a bipolar cell.

KEY WORDS: egg development, ooplasmic segregation, mitochondrial segregation, egg cytoskeleton

Introduction

Preparation of eggs for embryonic development starts in the ovary as a primary oocyte enters meiosis. This process is often completed after fertilization and before initiation of cleavage, days or years afterwards. During this long meiotic period the oocyte begins to build up its cytoplasmic supply of nutrients, represented by the yolk platelets, RNAs, proteins and organelles. Precise localization of some RNA transcripts is known to take place during oogenesis in Drosophila (reviewed by: St Johnston and Nüsslein-Volhard, 1992; Suprenant, 1993; Theurkauf, 1994; Lehmann, 1995) and Xenopus (reviewed by Suprenant, 1993; Klymkowsky and Karnovsky, 1994; Forristal et al., 1995; Kloc and Elkin, 1995). In animals such as leeches (Fernández et al., 1992) and insects (reviewed by Mahajan-Miklos and Cooley, 1994), each oocyte is the privileged cell of a clone whose remaining cells (nurse cells) furnish the oocyte with most of the RNAs, proteins, and organelles needed to support early development. In other animals, such as fish and amphibia (Wallace and Selman, 1980), the developing oocyte relies on its own metabolic machinery to fulfill this purpose.

It is known that fertilization triggers important changes in the egg. Besides formation, approximation and fusion of pronuclei and assembly of the cleavage spindle, the cytoplasmic components of the egg may be subjected to an orderly process of redistribution called ooplasmic segregation. This cytoskeleton-dependent phenomenon allows translocation of RNAs, proteins, pigments, lipid droplets, granules, organelles, or mixture of these, to specific regions of the cytoplasm. It is well documented that ooplasmic segregation takes place in the egg of amphibia (Elinson and Rowning, 1988; Perry and Capco, 1988; Houliston, 1991; Larabell et al., 1996), fish (Ivanenkov et al., 1990; Abraham et al., 1993), nematodes (Strome and Wood, 1982, 1983), ascidian (Reverberi, 1971; Jeffery and Meier, 1983; Sardet et al., 1989) and annelids (Fernández and Olea, 1982; Shimizu, 1982a).

Abbreviations used in this paper: ar, animal polar ring; at, animal teloplasma; ec, ectoplasm; fu, furrow; go, Golgi complex; gr, granules; is, inner sector of the ectoplasm; mi, mitochondria; mt, microtubules; mv, microvilli; os, outer sector of the ectoplasm; pc, pole cell; pp, perinuclear plasm; sc, smooth endoplasmic reticulum; ve, vesicle; vt, vegetal teloplasma; yp, yolk platelet.

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The leech egg is a good example of a cell that undergoes a dramatic series of cytoplasmic rearrangements after fertilization and before cleavage. These rearrangements lead to establishment of 3 prominent cytoplasmic domains: the perinuclear plasm at the center and the teloplasms at the poles (Fernández and Olea, 1982). These domains enclose numerous organelles and their asynchronous formation is a cytoskeleton-based process (Fernández et al., 1987, 1994; Fernández and Olea, 1995). The perinuclear plasm starts forming during the second meiotic division (1.30-2.30 h of development) and attains its full size by the end of the first interphase (4.30-5.00 h of development). It encloses the zygote nucleus and the sperm centrosome (Fernández and Olea, 1995), and is responsible for the assembly of the cleavage spindle. The teloplasms are established by the end of the first interphase but their formation is initiated earlier. They are inherited by the SM and SNOPQ blastomeres (proteloblasts) that give rise to the teloblasts. These are stem cells that use the teloplasm for the manufacture of the mesodermal and ectodermal precursor blast cells (Fernández and Stent, 1980; Fernández and Olea, 1982; Weisblat et al., 1984).

In this paper we monitored formation of the teloplasms by (a) tracing live mitochondria under video fluorescence microscopy and (b) determining proliferation of mitochondria based on the incorporation of BrdU and electron microscopy. The teloplasms form throughout the first interphase as consequence of a 3-step sequential process of mitochondria redistribution accompanied by proliferation of the organelle. The first step is characterized by thickening of the ectoplasm as result of organelle accumulation. During the second step furrowing is accompanied by accumulation of mitochondria at the walls of polar rings and bands of contraction. During the third step, poleward displacement of the polar rings and shortening of the contraction bands lead to bipolar accumulation of mitochondria. We have analyzed the role of microtubules and microfilaments in these steps.

Results

Events to be described below took place during the first interphase, that extended for about 2 h at 20°C (2.30-3.00 to 4.30-5.00 h of development). Based on the initiation and extinction of furrowing activity this period may be subdivided into early, mid and late interphase. Early first interphase takes about 45 min and occurs between release of the second pole cell and the initiation of furrowing activity. Mid first interphase takes about 15 min and ends by the time furrowing activity has reached a peak. Late first interphase takes about 60 min and terminates by the time furrows disappear from the egg surface and formation of the teloplasms concludes.

The cytoplasm of the early first interphase egg consists of a peripheral ectoplasm, deficient in yolk platelets, and a central endoplasm, with numerous yolk platelets. Organelles are present throughout both types of cytoplasm.

First step: Ectoplasm thickening and mitochondrial accumulation

At the initiation of the first interphase the egg exhibited marked animal/vegetal polarity and its layer of ectoplasm was thin.

Observations of whole eggs with the scanning electron microscope and of sectioned eggs with the light or electron microscope showed that the cell at this stage displayed polarity along the animal/vegetal axis. Thus, short microvilli were highly concentrated at the top of the animal hemisphere and substantially decreased in number toward the vegetal pole. Transverse folds of the plasmalemma decreased in number from the vegetal to the animal pole. Rounded bumps of the egg surface (produced by outpocketing of the most peripheral yolk platelets) were mainly seen at the equatorial region of the egg (Fig. 1a-c). The ectoplasm (Fig. 2a) was thicker at the animal (4 μm) than at the vegetal pole (2-3 μm) and very thin at the equator (about 1 μm). It enclosed granular material, smooth endoplasmic reticulum, Golgi complexes and mitochondria (Fig. 2b). The latter were rounded (0.3-0.5 μm in diameter) or elongated (about 1 μm in length) and appeared scattered singly or grouped in small clusters. Small islands of ectoplasm-like cytoplasm that included similarly arranged mitochondria were seen at the ectoplasm/endoplasm junction zone (Fig. 3). The endoplasm contained many rounded yolk platelets of different size, but the largest were present at the vegetal hemisphere. Organelles and granules appeared less concentrated in the endoplasm than in the ectoplasm.

Gradual thickening of the ectoplasm, as result of organelle accumulation, took place during early first interphase

At the initiation of the first interphase eggs loaded with a mitotracker exhibited diffuse red fluorescence. This aspect changed gradually as result of the appearance of a growing bright red halo at the egg periphery (Fig. 4a). Optical sections produced in the confocal microscope showed that the halo was produced by numerous bright fluorescent spots scattered across the egg surface (Fig. 4b). Due to the small size of mitochondria these spots are considered agglomerations of the organelle.

Sectioned eggs showed that the layer of ectoplasm thickened elsewhere (Fig. 5a,b), particularly at the animal pole where it reached more than 8 μm. At the equatorial regions the accumulation of ectoplasm provoked internalization of the peripheral yolk platelets, and this in turn made the egg surface smoother. Ectoplasm thickening was accompanied by accumulation of not only mitochondria but also of granules and other organelles. Elongated figure of eight mitochondria could be seen forming clusters or rows (Fig. 5c.d). Large rounded mitochondria (about 1 μm in diameter) with short curved cristae were also seen across the ectoplasm (Fig. 7). Bundles of microtubules were frequently visualized in the ectoplasm and adjacent endoplasm (Fig. 6a,b).

Ectoplasm thickening involved both peripheraward displacement and proliferation of mitochondria

Results presented above suggested that mitochondria as well as other organelles moved centrifugally from the adjacent endoplasm. However, the appearance of numerous elongated and figure of eight mitochondria in the growing ectoplasm also indicated that they might correspond to proliferating forms of the organelle. Hence, accumulation of organelles might be the result of combined transport and proliferation. To get insights into the process of mitochondrial proliferation eggs were pulse labeled with BrdU during early first interphase. Immunofluorescence showed that during this period the ectoplasm became labeled (Fig. 8), probably indicating that in preparation for their division ectoplasmic mitochondria replicated their DNA.

Effect of drugs indicated that the first step of teloplasm formation was a microtubule-based process

Ectoplasm thickening was prevented in eggs microinjected with colchicine at the initiation of the first interphase (Fig. 9a). However,
continuous incubation of the egg with high concentrations of cytochalasin B from 0 h of development did not prevent ectoplasm thickening (Fig. 9b). It may be argued that cytochalasin B probably did not enter the egg or reached a sufficient cytoplasmic concentration to affect ectoplasmic thickening. The fact that 0 h eggs incubated in cytochalasin B did not exhibit meiotic deformation movements and failed to discharge the pole cells (see Fernández et al., 1990) was taken as indicative that the drug indeed reached a critical cytoplasmic concentration that affected the actin meshwork. Therefore, microtubules but not microfilaments were engaged in ectoplasmic thickening, presumably by allowing centrifugal transportation of the organelles from the neighboring endoplasm.

Second step: Furrowing and mitochondrial redistribution

A furrowing pattern was generated during mid first interphase

The second step of teloplasm formation was initiated by furrowing activity leading to establishment of a contraction ring (polar ring) at each egg hemisphere and several contraction bands between the rings. The animal ring formed first, often at the upper third of the animal hemisphere, and the vegetal ring last, usually at the lower third of the vegetal hemisphere. The furrowing pattern between the rings depended on the temperature at which the gravid leeches were maintained and not on the temperature at which the fertilized egg developed. Leeches maintained at room temperature (about 20°C) usually laid eggs that generated a meridional furrowing pattern (Fig. 10a) consisting of about a dozen bands of contraction linking the two rings (see also Fernández et al., 1987). Eggs from leeches maintained at 12-14°C for several weeks often exhibited variable furrowing patterns. Some eggs showed meridional furrowing patterns while in others meridional furrows appeared interconnected by transverse contraction bands (intermediate furrowing pattern). Other eggs generated a complex system of anastomosed channels forming a sort of reticulum (reticulated furrowing pattern, see Fig. 10b). Bands of contraction were always continuous across the egg surface with no interruption at the equator. Eggs with different furrowing patterns developed normally.

Furrowing was accompanied by accumulation of mitochondria at the wall of rings and bands of contraction

Eggs loaded with a mitotracker demonstrated that mitochondria moved in the plane of the ectoplasm to become concentrated at sites of furrowing. Walls of the animal polar ring were, therefore, the first regions to be invaded by mitochondria, followed by the walls of the vegetal polar ring and those of the bands of contraction. Mitochondria destined to the polar rings came mostly from the ectoplasm of the respective polar region of the egg. Those destined for the bands of contraction derived from the ectoplasm between the polar rings. As expected, the pattern of mitochondrial accumulation depended on the arrangement of the contraction bands (Fig. 11a-e). Furrowing seemed to be closely followed by mitochondrial accumulation. However, double labeling experiments (mitochondria plus actin filaments) are needed to disclose the precise temporal relationship between the two events.

Examination of sectioned eggs by light and electron microscopy confirmed previous observations and additionally showed that microtubules and mitochondria, as well as other organelles, were particularly abundant in the inner sector of the ectoplasm lining the rings and bands of contraction (Figs. 12 and 13).

Proliferation of mitochondria continued during their redistribution across the ectoplasm

Numerous elongated and figure of eight mitochondria, together with small rounded mitochondria, were found across the ectoplasm lining the rings and bands of contraction (Fig. 13b). Moreover, large rounded mitochondria were now seen in greater number than before. The surface of these large mitochondria appeared associated with small mitochondria, some of which formed rows (Fig. 13c). It may be argued that such mitochondrial polymorphism and size variation may not be real but the result of random sectioning of the organelle. This is undoubtedly true for many of the small mitochondria, a great proportion of which may be sections across elongated or figure of eight mitochondria. Random sectioning of large mitochondria is considered to generate few small and elongated mitochondria and probably fewer figure of eight ones. Although examination of serial sections is needed to settle this
question, we favor another explanation for these findings. Large and figure of eight mitochondria are considered to be proliferating forms of the organelle. Furthermore, the peculiar relationships between small and large mitochondria strongly suggests that the latter produce the former by a sort of budding process, similar to that seen in yeast.

Incubation of mid interphase eggs with BrdU revealed incorporation of the precursor in the wall of rings and bands of contraction (Fig. 14). Double labeling of the eggs with Hoechst dye showed colocalization of the BrdU and DNA signals, suggesting mitochondrial DNA replication. This finding provides important support to our proposal that ectoplasmic mitochondria are actively proliferating.

Effect of drugs indicated that redistribution of mitochondria was mostly an actin-based process

Drug-treated eggs became very fragile and broke after dechorionation. Therefore, they could not be loaded with mitotracker to investigate the effect of drugs on mitochondrial segregation. However, these treated eggs can be fixed-cleared, procedure that turns the ectoplasm opaque (see Materials and Methods). Since the ectoplasm of mid first interphase eggs is very rich in mitochondria, the distribution of opacities mimics the distribution of mitochondria.

Normal furrowing and ectoplasm rearrangement were generally not prevented in eggs microinjected with colchicine shortly before initiation of furrowing (compare Figs. 15 and 16a). However, the fact that in some treated eggs ectoplasm did not accumulate along the entire length of the contraction bands suggested, that microtubules may have certain participation in the segregation of mitochondria. Deficient furrowing and abnormal redistribution of mitochondria were seen in eggs subjected to continuous incubation in cytochalasin B from 0 h of development. Thus, furrowing activity was mostly restricted to the vegetal pole region of the egg, where a contraction ring (Fig. 16d) and short shallow contraction bands formed. As expected, ectoplasm concentrated at the sites of furrowing and also at the vegetal pole itself (Fig. 16b). Actin filaments and microtubules were also found in the same places (Fig. 16c,e). From these results it may be concluded that: (a) microtubules do not appear to play a crucial role in ectoplasm (mitochondria) redistribution during the second step of teloplasm formation, (b) actin filaments remained in the vegetal pole region of the egg after drug treatment and (c) redistribution of ectoplasm (mitochondria) during mid first interphase is predominantly an actin-based process. However, it remains to be determined why cytochalasin B only affects anially-located actin filaments.

By the end of mid-first interphase the egg became bipolar

Furrowing was also accompanied by important changes in the external structure of the egg. First, membrane folds became concentrated at the polar rings, where they ran concentrically, and at the bands of contraction, where they extended along their main axis. Second, microvilli were now profuse at the surface of the polar rings. A moderate number of microvilli were found along the surface of the contraction bands and fewer in other regions of the egg that again became bumpy. In summary, plasmalemma appears concentrated at the surface of transient ectoplasmic domains created at sites of furrowing.

Third step: Furrow extinction and bipolar segregation of mitochondria

Dynamic of furrow extinction: rings and bands of contraction

During a first phase the polar rings moved poleward very rapidly and the bands of contraction stretched across the egg surface. During the second phase, rings moved slowly, the continuity of the bands of contraction was interrupted at the egg equator and the furrows began to shorten toward either of the rings (Fig. 17a-c). In this manner, dynamics of the polar rings involved formation,
translation and extinction of annular furrows, whereas dynamics of contraction bands involved formation, stretching and gradual extinction of their furrows. By the time furrows disappeared from the egg surface ectoplasm (mitochondria) was concentrated at the poles and teloplasma formation was considered to be completed.

**Mitochondria, as well as other organelles, comigrated in a bipolar fashion**

Displacement of the polar rings and shortening of the contraction bands were accompanied by bipolar segregation of mitochondria: organelles associated with animal furrows moved to the animal pole whereas those associated with vegetal furrows moved to the vegetal pole. Most mitochondria in the bands moved in clusters of different size that, although advanced separately, finally coalesced at the periphery of the rings where they formed large clumps (Figs. 18-20). Interestingly, clusters of mitochondria moved more rapidly than the furrows shortened. Weak mitotracker signals, detected toward the equatorial end of the shortening bands, could correspond to smaller clusters of mitochondria whose fate and dynamics is not yet understood. Lack of mitotracker signals between the bands indicated paucity of mitochondria in the rest of the ectoplasm (Fig. 18a,b), a conclusion supported by observations with the electron microscope. Time lapse video microscopy of mitotracker loaded eggs showed unambiguously the bipolar convergent translocation of mitochondrial clusters (Figs. 19 and 20). A last event in the formation of teloplasmas concerned with blending of the mitochondrial clumps with the ring material (Fig. 20f).

Besides numerous mitochondria, the ectoplasm moving along the contraction bands contained smooth endoplasmic reticulum, Golgi complexes, vesicles, 20 nm granules and microtubules (Fig. 21a-c). The close relationship between mitochondria and microtubules was marked by specializations between the two structures in the form of small periodical densities about 100 nm apart (Fig. 21d). The structure and relationships between mitochondria had not changed much. However, observation of closely apposed mitochondria of different size in tandem arrangement was more frequent now than before. Many of these rows of mitochondria were directed toward the egg poles. The ectoplasm between the contraction bands was thinner and enclosed fewer mitochondria as well as other organelles and microtubules.

**Proliferation of mitochondria continued during their poleward movement**

Eggs pulse labeled with BrdU during late first interphase showed incorporation of the precursor in the animal and vegetal teloplasms (Fig. 22). These findings strongly suggested that mitochondrial DNA was replicating in preparation for division of the organelle. Hence, the peculiar relationships among mitochondria of different size seen under the electron microscope is likely to be related to the manner the organelle proliferates.

**Poleward displacement of mitochondria is a microtubule-and actin-based process**

Eggs microinjected with colchicine at the peak of furrowing (termination of mid first interphase) were examined by the time controls had formed the teloplasmas (termination of late first interphase). Fixed-cleared drug-treated eggs were seen to form smaller teloplasmas than controls (compare Figs. 23 and 24a). That is, less ectoplasm was transported to the egg poles. For reasons given in

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**Fig. 10.** (a,b) Low power scanning electron micrographs to illustrate 2 types of furrowing patterns generated in mid first interphase eggs laid by leeches breeding at different temperatures. (a) Meridional furrowing pattern seen in eggs laid by leeches raised at room temperature. (b) Reticulated furrowing pattern seen in eggs laid by leeches bred at 14°C. (c) Animal polar ring; fu, furrows. Bar, 100 µm.

**Fig. 11.** (a-e) Digitally-processed (a, b) and routine fluorescence (c-e) images of whole-mounted mid first interphase live eggs previously loaded with rhodamine mitotracker. Note the progressive accumulation of mitochondria in the walls of the polar rings and contraction bands. (a) Clusters of mitochondria are visualized at the egg surface. (b,c) Mitochondria accumulate at the animal polar ring. (d) Concentration of mitochondria in the polar rings and in the walls of meridionally-oriented furrows. (e) Concentration of mitochondria in the polar rings and in the walls of a reticulated system of furrows. Bar, 100 µm.

**Fig. 12.** (a,b) Stained plastic sections of mid first interphase eggs that show the organization of the ectoplasm accumulated at the animal polar ring (a) and meridional bands of contraction (b), that were transversally sectioned. The ectoplasm consists of a deeply stained inner sector, or core, rich in mitochondria (mi) and a lightly stained outer sector (arrow heads) with fewer mitochondria. Bar, 50 µm.

**Fig. 13.** (a-c) Electron micrographs that illustrate the structure of the ectoplasm accumulated at the wall of contraction bands in mid first interphase eggs. (a) The fine structure of the outer (os) and inner (is) sectors of the ectoplasm are shown. Numerous 20 nm granules are present in both sectors, whereas mitochondria are particularly concentrated in the inner sector. (b,c) The inner sector encloses mitochondria of different size and shape. Presence of numerous figure of eight mitochondria (arrow heads) and of clusters of closely associated large and small mitochondria (arrow) are taken as indicative that the organelle is actively proliferating. Bar, 1 µm (a); 0.5 µm (b,c).}

**Fig. 14.** Whole-mounted egg that was pulse labeled with BrdU during mid first interphase (about 15 min). Notice incorporation of the precursor in the walls of the animal polar ring (arrow) and meridional bands of contraction (arrow heads). This result is taken as indicative that ectoplasmic mitochondria of mid first interphase eggs have replicated their DNA. Bar, 100 µm.

**Fig. 15.** Normal whole-mounted fixed-cleared mid first interphase egg that shows the accumulation of opaque ectoplasm in the walls of the animal polar ring (arrow) and meridional bands of contraction (arrow heads). This micrograph allows comparison of ectoplasmic distribution between normal and drug-treated eggs. Bar, 100 µm.

**Fig. 16.** (a-e) Whole-mounted mid first interphase eggs that show the effect of drugs on the distribution of the ectoplasm and its cytoskeleton. (a) Fixed-cleared egg pulsed with microinjected colchicine (5 mM) during mid first interphase (15 min). Ectoplasm accumulated in the walls of the animal polar ring (arrow) and meridional bands of contraction (arrow heads). (b-e) Eggs incubated in cytochalasin B from 0 h of development (total time of incubation in the drug was 3.30 h). (b) Fixed-cleared egg whose ectoplasm appears accumulated at the vegetal pole (arrow), in a ring (double arrow) and meridians (arrow heads) linking the first two structures. The opaque spot in the animal hemisphere corresponds to the perinuclear lam (pp), whose position has been altered by the drug. (c) Egg stained for β tubulin that shows the distribution of microtubules. Notice that microtubules are present in the same regions where actin and ectoplasm were accumulated (compare with figs. 16b, e). (d) Live cytochalasin B-treated egg that shows formation of a single ring of contraction (double arrow) at the vegetal hemisphere. (e) Same egg stained for actin filaments with rhodamine-phalloidin. It shows the presence of actin filaments at the vegetal pole (arrow) and in the walls of the ring (double arrows). Bar, 100 µm.
the Materials and Methods section, a similar experiment could not be performed with cytochalasin B. However, eggs incubated in cytochalasin B from 0 h of development only formed vegetal teloplasms and a neighbor ring of ectoplasm (Fig. 24b). That is, the egg hemisphere lacking actin filaments (see Fig. 16e) failed to transport mitochondria poleward. These results suggested that both microtubules and microfilaments were needed to ensure proper bipolar transportation of organelles.

Establishment of the teloplasms was accompanied by reorganization of the plasmalemma

Teloplasm formation led to another episode of plasmaleminal rearrangement. First, a substantial part of the egg surface regained a bumpy appearance as result of bulging out of yolk platelets against a thinned ectoplasmp. This phenomenon was sometimes accompanied by formation of microvilli at the equatorial region of the egg. Meanwhile, the number of microvilli at the top of both egg hemispheres remained very high (Fig. 25a,b).

Discussion

Results demonstrate that teloplasms formation in the leech egg is a stepwise process that involves simultaneous formation and segregation of mitochondria. Other components of the ectoplasmp, such as smooth endoplasmic reticulum, Golgi complexes and granules, probably behave similarly. Redistribution of ectoplasms is accompanied by gradual reorganization of the plasmalemma and relocation of the endoplasm (unpublished observations). Hence, teloplasms formation is the result of an orchestrated redistribution of egg components that gives rise to a highly organized bipolar cell suited to initiate the cleavage program. A diagram summarizing the relationship between furrowing pattern and distribution of mitochondria during the stepwise formation of teloplasms is presented in Figure 26.

Ectoplasms thickening is a microtubule-based process

The available evidence indicates that the first step of teloplasms formation relies on microtubules. Support for this conclusion comes from the observation that a microtubule inhibitor, colchicine, blocks ectoplasms thickening and that the monaster fibers of the early first interphase egg connect endo and ectoplasms with one another (Fernández and Olea, 1995). Mitochondria are then available to sustain transport of cell components between the two egg compartments. Since the microtubule organizing center of the first interphase egg lies at the egg center, the monaster microtubules probably have their plus ends directed toward the egg surface (Fernández and Olea, 1995). Hence, organelles and granules moving from endo to ectoplasms would travel from the (-) to the (+) end of the microtubules. The fact that ectoplasms thickening is accompanied by internalization of yolk platelets suggests that these egg components are also moving but in the opposite direction, that is from the (+) to the (-) end of the microtubules. This is not surprising because perinuclear plasm formation, by organelle accumulation around the centrally-located sperm centrosome, is a microtubule-dependent process that occurs in the (+) to (-) direction (Fernández et al., 1994; Fernández and Olea, 1995). Presence of organelle-rich pockets of cytoplasm at the endoplasm/ectoplasms boundary suggests that the ectoplasms grows by addition of organelles to its inner surface.

Ectoplasms thickening (formation of a subcortical layer) in preparation for pole plasm formation in Tubifex eggs also occurs as result of organelle transportation from the underlying endoplasm. However, this process is sensitive to cytochalasin B and thus relies on microfilaments (Shimizu, 1982b).

Centrifugal transportation of mitochondria probably concludes at the initiation of mid first interphase as result of functional disconnection between ecto and endoplasm due to microtubule depolymerization. Although this possibility needs to be tested, it is known that shortening of monaster fibers occurs by about this time (Fernández and Olea, 1995).

Furrowing and mitochrondial rearrangement across the ectoplasms are mostly microfilament based processes

Since furrowing and mitochondrial rearrangement appeared as sequential processes mostly based on actin filaments, the furrowing pattern can determine the manner these organelles become distributed across the egg surface. That furrowing may be modified by the temperature at which gravid leeches are maintained sug-

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**Fig. 17.** (a-c) Fluorescence micrographs of a live late first interphase egg that illustrate stretching and shortening of meridionally-oriented contraction bands (arrow heads). (a) Contraction bands extended between the two polar rings (arrows) that have already reached the upper third of the egg hemispheres. (b) 10 min later the contraction bands have begun shortening from the egg equator. (c) 20 min later the shortened contraction bands are only visible at the egg poles. Bar, 100 μm.

**Fig. 18.** (a,b) Fluorescent micrographs of live late first interphase eggs loaded with rhodamine 123 that show the gradual poleward displacement of mitochondria in eggs that generated a reticulated (a) or meridionally-oriented (b) furrowing patterns. Notice that mitochondria (m) in the contraction bands move in clusters that finally fuse with the constricting animal polar ring (ar). Bar, 100 μm (a); 50 μm (b).

**Fig. 19.** (a-d) Montage of digitally-processed images of a whole-mounted live late first interphase egg loaded 30 min before with rhodamine mitotracker, that shows poleward displacement of mitochondria. Images were captured every 5 minutes. Arrow heads point toward 4 clusters of mitochondria that gradually approach and fuse with the constricting animal polar ring (ar). Bar, 100 μm.

**Fig. 20.** (a-f) Montage of digitally-processed images of a whole-mounted live late first interphase egg loaded 30 min before with rhodamine mitotracker. Images were captured every 4 minutes. It shows that mitochondria in the animal polar ring (ar) and clusters of mitochondria (arrow heads) in the walls of a reticulated system of contraction bands move poleward, converge and fuse to form the animal teloplasms (at). Bar, 100 μm.

**Fig. 21.** (a-d) Electron micrographs of late first interphase eggs that illustrate the structure of the ectoplasms associated with the walls of shortening contraction bands. (a-c) The poleward moving ectoplasms includes numerous granules, mitochondria, Golgi complexes (g), vesicles (v), and smooth endoplasmic reticulum (sr). Mitochondria of different size and shape form rows suggesting orderly proliferation of the organelle and also that they probably move in tandem. The close relationship between large and small mitochondria (arrow heads), on the other hand, may be indicative that the former are producing the latter by a sort of budding process. (d) A mitochondrion appears associated with the surface of a microtubule (mt) by means of periodically arranged bridges (arrow heads). Bar, 0.1 μm (a, d); 0.5 μm (b, c).
Fig. 22. Whole-mounted egg that was pulse labeled with BrdU during late first interphase (about 1 h). The animal teloplasma (at) appears highly fluorescent, indicating heavy incorporation of the precursor. This result is taken as indicative that during the third step of teloplasma formation mitochondria are still replicating their DNA. Bar, 100 μm.

Fig. 23. Normal whole-mounted fixed-cleared late first interphase egg showing the accumulation of ectoplasm at both egg poles to form the animal (at) and vegetal (vt) teloplasms. This figure allows comparison between normal and drug-treated eggs. pp, perinuclear plasm. Bar, 100 μm.

Fig. 24. (a,b) Whole-mounted fixed cleared late first interphase eggs showing the effect of drugs on teloplasma formation. Compare with Fig. 23. (a) Egg pulsed with colchicine (5 mM) during late first interphase (1 h). Notice formation of smaller teloplasms and absence of perinuclear plasm. Colchicine disperses the perinuclear plasm as consequence of microtubule depolymerization (unpublished observation). Therefore, absence of the perinuclear plasm is a good indicator that the drug led to microtubule disassembly. (b) Egg incubated in cytochalasin B from 0 h of development (total time of incubation in the drug was 3.15 h). Notice that ectoplasm was finally accumulated in a vegetally situated annular structure (double arrow) and in the vegetal teloplasma (vt). at, animal teloplasma; pp, misplaced perinuclear plasm. Bar, 100 μm.

Fig. 25. (a,b) Low magnification scanning electron micrographs of an egg that has completed formation of the teloplasms. Short microvilli (mv) appear concentrated at the animal (a) and vegetal (b) poles, where the teloplasms have formed. Bar, 5 μm.

gests the idea that the furrowing pattern is determined during oogenesis. It is conceivable that furrowing geometry may be specified across the developing oocyte cortex by accumulation of cytoplasmic determinants involved in the regulation of actin polymerization and assembly.

Furrowing is particularly prominent in the annelid egg, in which sulci form in relation to discharge of the pole cells, accomplishment of ooplasmic segregation and cleavage (see Shimizu, 1982a, 1995; Jeffery and Wilson, 1983; Fernández et al., 1987). The mechanisms involved in different type of furrowing, however, are likely to be similar. Data derived from studies of dividing cells indicate that the cytokinesis ring of contraction forms by redistribution of the cortical actin meshwork (Wang, 1987; Cao and Wang, 1990a,b; Mabuchi, 1994; Sanger et al., 1994). A similar situation has been described to occur in leech (Fernández and Olea, 1995) and Tubifex (reviewed by Shimizu, 1995) eggs. However, the great increase in fluorescence of rhodamine phalloidin labeled actin filaments reported in this paper to take place at sites of furrowing suggests that de novo polymerization of actin may also occur.

Furrows appear as centers of ectoplasm accumulation. In order to fulfill this function actin filaments must have appropriate orientation and connections and also be able to translocate organelles and microtubules. The first condition is satisfied because the ectoplasmic actin meshwork looks continuous (Fernández and Olea, 1995) so that microfilaments in the furrows are connected with those present between the furrows. We can speculate that myosin-type of motors (reviewed by Mitchison and Cramer, 1996) might be available for translocation of organelles and microtubules toward rings and bands of contraction. Determination of actin filament polarity will help to understand the vectorial translocation processes discussed above.

Although our data indicate that microtubules are not the initiators of furrowing, long treatments with colchicine disturb the formation and extinction of sulci (unpublished observations). Fur-
thermore, the fact that colchicine may prevent accumulation of ectoplasm along the entire length of contraction bands suggests that microtubules would play some role in the redistribution of ectoplasmic organelles.

**Bipolar segregation of mitochondria is based on both microtubules and microfilaments**

Two lines of evidence indicate that microtubules and microfilaments are involved in poleward flow of mitochondria as well as other organelles. First, the disturbing effect of drugs such as colchicine and cytochalasin B that lead to formation of abnormal teloplasms. In the first case teloplasms are smaller and in the second case only vegetal teloplasms is formed. Second, the fact that mitochondria move with the rings and bands of contraction, regions having a highly structured cytoskeleton of microtubules and microfilaments. It is interesting that after formation of cytoplasmic domains (teloplasms) mitochondria and the cytoskeleton remain together (Fernández and Olea, 1995). Although the manner microtubules and microfilaments contribute to this process has not yet been ascertained, the evidence suggests that: (1) microtubules would be directly involved in organelle translocation along bands of contraction. This conclusion is supported by our unpublished observations indicating that labeled mitochondria and microtubules comigrate to the poles of live eggs (2) actin-based contractile machinery would ensure poleward displacement of the mitochondria enclosed in the polar rings and also of those accumulated at their periphery. It is likely, however, that microtubules and microfilaments work synergically in mitochondrial transportation. Use of live eggs having labeled microtubules, actin filaments and mitochondria will help to discern the role of cytoskeletal components in bipolar segregation.

Bipolar segregation of organelles in Tubifex eggs is sensitive to cytochalasin B and thus appears to depend on actin filaments only (Shimizu, 1982b, 1995). The same process in the egg of the leech Helobdella Isnerialis (Astrow et al., 1989) is sensitive to microtubule inhibitors and thus seems to depend on a different cytoskeletal component. It appears, then, that Theromyzon rude eggs differ from those of other leeches and Tubifex in that bipolar segregation of organelles relies on both microtubules and microfilaments.

**Mitochondria, and ectoplasm in general, move in clusters**

An interesting feature of mitochondrial transportation is that the organelles move in clusters which attain the largest size during the third step of teloplasms formation. Since mitochondrial proliferation is concurrent with ooplasmic segregation, clusters of moving mitochondria may correspond to "clones" of the dividing organelle. It seems likely that such clusters are associated with numerous bundles of microfilaments and/or microtubules engaged in organelle transportation. Clustering of mechanoenzymes along these transport pathways may also account for grouping of moving organelles. Coalescence of small moving clusters into larger moving clusters demonstrate that the first interphase cytoskeleton of the leech egg is able to transport large clumps of organelles.

**Proliferation of mitochondria occurs during the first interphase**

The fertilizing sperm makes a small contribution of organelles to the egg (see Longo, 1991; Lambert and Battaglia, 1993). Most organelles must, therefore, be provided by the oocyte. It is not surprising, then, that the egg is a cell endowed with numerous mitochondria of maternal origin. In fish (see reviews of Selman...
and Wallace, 1989: Wallace and Selman, 1990), amphibia (Heasman et al., 1984) and birds (Brambell, 1925) the starting pool of mitochondria for the developing egg seems to be largely established during oogenesis. In the leech egg this condition seems to be largely fulfilled in the uncleaved egg during the first interphase. A similar strategy for building up a stock of mitochondria is likely to take place in other invertebrate eggs as in Tubifex (Weber, 1958; Shimizu, 1982a). Evidence of mitochondrial proliferation in leech eggs may be considered likely from the following facts: (1) although no precise counts are available more mitochondria are seen after than before the first interphase, (2) profiles considered to correspond to dividing mitochondria (figure of eight, elongated or "budding" mitochondria) are present in regions where ooplasm is being accumulated, (3) incorporation of BrdU occurs in regions that are rich in mitochondria and that stain with the Hoechst dye. The fact that most mitochondria appear touching one another also suggests that they are proliferation products. If the dividing organelle is moving in a certain direction mitochondria will adopt a tandem arrangement, a phenomenon observed during all the steps of teloplasma formation. The origin of large mitochondria is ignored and the fact that they have not yet been seen in meiotic eggs suggests that small numbers of them are present in the early uncleaved egg. Large mitochondria may arise by a fusion of smaller mitochondria, a phenomenon reported to take place during leech spermatogenesis (see Fernández et al., 1992) or b) from the single large mitochondrion of the fertilizing sperm (Fernández et al., 1994). Marking sperm mitochondria with appropriate probes may help decide the possible paternal origin of large mitochondria.

During the first interphase a polarized postmeiotic cell becomes a bipolar egg

Both internally and externally the early first interphase egg corresponds to a polarized cell. Thus, it has a thickened ectoplasm and a developing female pronucleus at the animal pole (Fernández and Olea, 1995), perinuclear plasm with the developing male pronucleus at the center of the egg (Fernández et al., 1994) and large yolk platelets at the vegetal hemisphere. The concentration of microvilli is high at the animal pole and decreases gradually toward the vegetal pole. With the formation of the teloplasmas at the egg poles, the persistence of an enlarged centrally-located perinuclear plasm and the redistribution of microvilli, that become particularly abundant at the poles, the egg becomes a bipolar cell. Cleavage divides the egg unequally producing another bipolar cell, the blastomere CD. Descendants of this blastomere remain bipolar until formation of the polarized cells SM and SNOPQ, precursors of the meso and ectoderm respectively (see Fernández and Olea, 1982). Bipolarity of the cleaving egg has, then, important consequences in lineage formation during early leech development.

Materials and Methods

Eggs of the leech Theromyzon rude were used. Animals came from a breeding population maintained in the laboratory at 12-14 or 20-22°C. Fertilization is internal and eggs that have been laid are blocked at the metaphase of the first meiotic division. (Fernández and Olea, 1982). To secure synchronously developing eggs the ovisacs of pregnant mothers were removed and opened in filtered spring water, which constitutes a convenient culture medium. A total of 50 animals that provided about 3,000 eggs were utilized.

Preparation of fixed-cleared eggs.

Accumulation of ectoplasm at the wall of rings and bands of contraction, as well as at the egg poles, was studied in whole eggs fixed in ALFAC (ethanol, formaldehyde and acetic acid), dehydrated in absolute ethanol and cleared in methyl benzoate (see Fernández and Olea, 1995). This procedure turns the yolk transparent whereas ooplasm accumulations remain opaque.

Light, transmission and scanning electron microscopy

For light and transmission electron microscopy eggs were fixed at 4°C or at room temperature for about 2 h in 2% glutaraldehyde in 0.1 M caccodylate buffer pH 7.4 or in a 50% solution of Karnovsky fixative. After 2 h rinsing in the same buffer, eggs were postfixed for 1 h in 1% OsO4, dehydrated in graded ethanol and embedded under vacuum in Epon 812. For light microscopy thick sections were stained with toluidine blue and for electron microscopy thin sections were double stained with uranyl acetate and lead citrate. For scanning electron microscopy eggs were fixed as above in glutaraldehyde cacodylate buffer, dehydrated in acetone, critical-point dried from CO2 and coated with gold. A Philips EM300 electron microscope equipped with a scanning device was used.

Fluorescence microscopy

Staining of microtubules and microfilaments was performed in eggs permeabilized for about 30 min at room temperature in PHEM buffer (Schliwa, 1980; Schliwa and van Blerkom, 1981) containing 0.15% Triton X-100 and anti proteases. After 1-2 h fixation in 4% paraformaldehyde in PBS, eggs were rinsed in PBS for the same time. For tubulin staining eggs were incubated for 2 days at room temperature in monoclonal anti-J-tubulin (Amersham) 1:100 in a PBS solution containing 0.1% sodium azide, 0.15% Triton X-100 and 2% bovine serum albumin (PBS-Naz-Tr-Bsa). After 1 day rinsing in the above solution eggs were incubated for 2 days at room temperature in goat antimouse IgG (Cappel) conjugated with rhodamine or fluoresceine at a concentration of 100 μg/ml in PBS-Naz-Tr-Bsa. Eggs were whole-mounted between coverslips with glycerol/PBS (9:1). For actin staining, permeabilized eggs were incubated for 1-3 days in rhodamine phallolidin (Sigma) at a concentration of 1 μg/ml in PBS-Naz-Tr. After 1 day of rinsing in the same buffer, eggs were whole-mounted as above.

Mitochondrial DNA labeling

Dechonorated eggs were pulse labeled with BrdU dissolved in spring water (0.5 mg/ml). Pulses were given during the entire early, mid or late first interphase. After labeling eggs were rinsed in filtered spring water and then fixed overnight in 85% ethanol containing 10% formaldehyde and 1% DMSO. After rinsing in distilled water eggs were transferred to 2M HCl for 30 min and then to 0.1 M sodium borate for 1 min. Eggs were incubated for 30 min in PBS-Triton (0.1%) and then in anti-BrdU 1:20 in PBS-Triton-DMSO (1%) for 24 h under constant agitation. After rinsing in PBS-Triton for 2-3 h, eggs were incubated for 2 days under constant agitation in a 1:100 solution of rhodamine-labeled goat antimouse IgG in PBS-Triton-Bsa. After several rinses in PBS (total 2-3 h) eggs were whole-mounted with 9:1 glycerol- PBS. To achieve double labeling of mitochondrial DNA, the fixative solution as well as all of the other fluids contained the Hoechst dye 33258 at a concentration of 45 μg/ml. Colocalization of the Hoechst and BrdU signals was possible by changing filters in the fluorescence microscope.

Confocal microscopy

Live eggs loaded with mitochondrial probes were examined in a Zeiss Axiovert laser scanning microscope equipped with an argon laser. Optical
sections were taken every 0.7 μm, frame averaged and the gray level adjusted. Live images of eggs loaded with Mitotracker were acquired every 3-5 min.

**Video microscopy and image processing**

Live eggs loaded with microinjection probes were observed in an Axiovert 135 inverted Zeiss fluorescence microscope equipped with a CCD Sony camera (model DCC-C1). Recorded images were digitized by a LG-3 Scientific frame grabber (PCI version, Scion Corporation, Frederick, MD). Image analysis was performed on a Power Macintosh 8500/120 computer using the public domain NIH image program (written by Wayne Rasband at the U.S. National Institutes of Health). Images were frame averaged, contrast enhanced, the gray level properly adjusted and in some cases pseudocolor was assigned. Live images were recorded every 2-5 minutes.

**Drug treatment**

Colchicine (5-10 mM) was pressure-microinjected at the equatorial region of eggs that had initiated early, mid or late first interphase. Cytochalasin B could not be microinjected because the site of injection did not seal and the cytoplasm flowed back into the egg. Hence, the effect of Cytochalasin B (70-μg/ml) was judged after continuous incubation of the eggs in the drug, starting at 0-90 min of development. Incubation could not be initiated later because the drug did not enter or failed to reach a critical cytoplasmic concentration. To check the effect of either of the drugs whole eggs were inspected under the dissecting microscope, either live or fixed-cleared. Sectioned eggs were examined under the light microscope after fixation in 50% Karnovsky fluid, embedding in Epon and staining of thick sections with Toluidine blue.

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