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Formation of the Male Pronucleus, Organization of the First Interphase Monaster, and Establishment of a Perinuclear Plasm Domain in the Egg of the Glossiphoniid Leech *Theromyzon rude*

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Whole-mounted or sectioned eggs of the glossiphoniid leech *Theromyzon rude* were studied under the dissecting, fluorescence, light, and electron microscope. The egg is often penetrated by a single sperm that enters the animal hemisphere and becomes subjected to migration block. The latter is released shortly before or after discharge of the first pole cell, when the sperm centrosome initiates aster formation, the nucleus begins to be untwisted, and its chromatin decondensed. Sperm centration occurs along one side of the egg and appears to follow an arc-like trajectory as a result of vegetal and inward movements affected by colchicine and cytochalasin B but not by taxol. Results indicate that growing microtubules are needed for both movements, whereas actin filaments are essential for the vegetalward movement only. The sperm centrosome becomes the main microtubule organizing center (MTOC) of the egg and concomitantly originates the elaborate first interphase monaster. Additional peripherally situated MTOC form cytaster-like bodies whose visualization is improved by taxol treatment. A voluminous centrosphere, formed around the sperm centrosome, becomes a center of organelle accumulation, giving rise to a perinuclear plasm domain. This process seems to involve both import and replication of organelles.

INTRODUCTION

The glossiphoniid leech egg and embryo constitute favorable materials for the study of developmental processes in relatively simple metazoa. First of all, under laboratory conditions glossiphoniids may breed all year round and lay large and numerous fertilized eggs arrested in meiosis I. These eggs can be cultured outside the cocoon in simple saline solutions and may be subjected to several experimental manipulations (Schleip, 1939; Weisblat *et al.*, 1978; Fernández and Olea, 1982; Astrow *et al.*, 1987). Moreover, completion of meiosis and establishment of ooplasmic domains (teloplasms and perinuclear plasm) are accompanied by striking stereotyped deformation movements (Whitman, 1878; Fernández *et al.*, 1987; Fernández *et al.*, 1990), suggesting that the uncleaved egg is endowed with a versatile and complex cytoskeleton. Cleavage is highly stereotyped (Fernández, 1980; Stent, 1985; Sandig and Dohle, 1988) and cytoplasmic inhomogeneities, such as the teloplasms, are transferred in an orderly fashion from blastomere to blastomere to be finally sequestered into five pairs of stem cells or teloblasts. The descendants of the teloblasts, called primary blast cells, are made of teloplasm and appear arranged in longitudinal rows according to their time of birth. They are the founders of the ectodermal and mesodermal tissues of the body segments (Fernández, 1980; Fernández and Stent, 1980; Weisblat *et al.*, 1984). Clonal analysis reveals two important features of the primary blast cells. First, that their proliferation gives rise to invariant lineages because the fate and distribution of the resulting cells are highly determined, and second, that each of the 32 segments is founded by a discrete number of the orderly arranged primary blast cells (Weisblat *et al.*, 1984; Bissen *et al.*, 1986). For this reason the glossiphoniid leech embryo has become a very convenient model for the study of segment formation (Zackson, 1984; Weisblat *et al.*, 1988; Shankland, 1991; Wedeen and Weisblat, 1991; Nardelli-Haefliger and Shankland, 1992).

Formation of ooplasmic domains involve profound cytoplasmic changes monitored by the first interphase cytoskeleton. This partly explains why the glossiphoniid leech egg remains uncleaved for a relatively long period of time. Thus, at 20°C the first cleavage furrow appears 6–12 hr after oviposition (Fernández and Olea, 1982; Fernández *et al.*, 1987). Development of the glossiphoniid uncleaved egg extends throughout three periods: (1) completion of meiosis (stage 1a–1b), (2) first interphase (stage 1c–early 1e), and (3) completion of the first cleavage division (late stage 1e–1f).

Several aspects of leech egg fertilization, such as location of the sperm entry site, mechanisms of sperm penetration and centration, formation of pronuclei, and or-

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igin of a microtubule organizing center (MTOC), remain virtually unexplored. Although formation of ooplasmic domains, such as the teloplasms (pole plasms), has been described in some detail in the past (see Whitman, 1878; Schleip, 1939; Fernández, 1987), formation of other ooplasmic domains such as the perinuclear plasm is largely ignored. Also, very little is known about the mechanisms involved in organelle translocation during ooplasm segregation in leech eggs. This information is urgently needed to understand the manner in which the laid egg becomes ready to initiate cleavage. In this paper we describe the mechanisms involved in sperm centration and male pronucleus formation, the organization of the first interphase monaster, and its role in perinuclear plasm formation in the glossiphoniid *T. rude*.

MATERIALS AND METHODS

Eggs were obtained from a breeding population of the glossiphoniid leech *T. rude*, maintained under laboratory conditions. To secure synchronously developing eggs, the ovisacs were surgically removed from pregnant mothers and transferred to millipore-filtered spring water. After opening the ovisacs, the eggs were rinsed in two changes of filtered spring water, which constitutes a suitable culture medium for eggs and embryos. Fertilization is internal (Fernández and Olea, 1982) and eggs are ready to be laid when they turn brown and become clustered within the ovisacs. Development, however, is not initiated until the eggs leave the ovisac and contact the cocoon fluid or the culture medium. Mature eggs are about 700 μm in diameter and enclose numerous yolk platelets. Live eggs are thus very opaque and unsuitable for direct observation of internal events such as pronuclear formation and migration. For this purpose one has to rely on the observation of developmental series of whole-mounted fixed cleared or permeabilized eggs, visualized by epifluorescence, transmitted or reflected light.

The first sign that egg development is underway is the separation of the fertilization membrane and the appearance of the perivitelline chamber. A total of about 750 eggs, from 30 gravid leeches, maintained at 20°C, were used in this study.

Examination of Live and Fixed-Cleared Eggs

Development of live eggs was studied under the dissecting microscope using conventional or dark-field illumination. Examination of whole-mounted cleared eggs fixed in ALFAC (ethanol–formaldehyde–glacial acetic acid; Fernández *et al.*, 1987) or in FAC (formaldehyde–glacial acetic acid; Fernández, 1980) allowed detection of the meiotic spindle and of sites of ooplasm concentration. For microphotography T-max 100 film (Kodak) was used.

Light and Transmission Electron Microscopy

Eggs were fixed for 1.5–2 hr in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4, containing 0.15% tannic acid (Merck), at room temperature or in 50% Karnovsky solution for 2–2.5 hr at 4°C. After postfixation and acetone dehydration eggs were infiltrated under vacuum in Spurr resin. For light microscopy 1-μm sections were stained with 1% toluidine blue in 1% sodium borate. For electron microscopy double-stained sections were viewed in a Philips EM 300 electron microscope. For more details, see Fernández *et al.* (1987).

Staining of Eggs with Antitubulin Antibody

Eggs were dechorionated with fine tweezers and treated with Schliwa extraction buffer (Schliwa, 1980; Schliwa and van Blerkom, 1981). Prolonged extraction (15–25 min) of eggs at room temperature allowed greater penetration of the antibodies and better labeling of deep microtubules. After paraformaldehyde fixation eggs were rinsed in buffer, incubated in the first antibody, monoclonal anti-α-tubulin (Amersham), rinsed again, and then incubated in the second antibody, antimouse IGg conjugated with rhodamine or fluorescein (Cappel). After several rinses in PBS, eggs were whole-mounted in glycerol–PBS or dehydrated in ethyl alcohol and embedded in glycol methacrylate. Five-micrometer-thick sections were mounted on slides with Gurr Fluoromount. Whole-mounted and sectioned eggs were examined under epifluorescence with appropriate filters. For microphotography T-max 400 and p-3200 film (Kodak) were used. For more details, see Fernández *et al.* (1990).

Staining of Whole-Mounted Eggs with Hoechst Dye

A combination of fixation, clearing, and DNA marking allowed chromatin visualization and pronuclei tracing. For this purpose, eggs fixed in FAC containing 22–44 μg/ml of Hoechst 33258 dye were whole-mounted with glycerol–PBS containing the same concentration of the dye and studied under the epifluorescence microscope using an appropriate filter.

Drug Treatment

Eggs were incubated or microinjected with freshly prepared solutions of taxol (kindly donated by the National Cancer Institute, Bethesda, MD), colchicine, or cytochalasin B (Sigma) in spring water. For incubation 80 μM taxol and 10 μM colchicine were used, and the first drug effects were noticed with a delay of 15–30 min. For microinjection 1 μM colchicine was pressure-injected us-

ing 1- to 2-μm-tip micropipets, and its effect was noticed very quickly. A stock solution of cytochalasin B (10 mg/ml in DMSO) was stored at −20°C. Working solutions of the drug were prepared by dissolving aliquots of the stock solution in filtered spring water. Eggs were incubated in solutions containing 75 μg/ml of cytochalasin B, and the first effects were also noticed after 15–30 min of incubation. Control eggs were incubated in a 0.75% solution of DMSO in spring water. We failed to microinject eggs with cytochalasin B because abundant yolk leaked out of the egg from the hole opened by the micropipet.

RESULTS

Structure and Position of the Fertilizing Sperm in the Early Uncleaved Egg

The early *T. rude* uncleaved egg consists of the meiotic spindle and two cytoplasmic domains: ectoplasm and endoplasm. The yolk-deficient ectoplasm encloses an actin-rich cortex and a thicker organelle-rich subcortex. The endoplasm (vitelloplasm) includes numerous yolk platelets (see Fernández *et al.*, 1987).

The fertilizing sperm is found along the periphery of the egg animal hemisphere, often close to the meiotic spindle. However, when more than one sperm is detected, they may also be found at the periphery of the vegetal hemisphere. Although polyspermy often affects several eggs of the same clutch, we have not yet determined either the frequency or viability of these eggs.

Early uncleaved eggs stained with Hoechst dye show that the helically arranged nucleus of the arrested sperm consists of 16–18 turns and its chromatin is highly condensed (Figs. 1 and 2). Tubulin staining, on the other hand, indicates that microtubules have not yet been nucleated by the sperm centrosome. Comparison of fertilizing and epididymal sperms revealed striking similarities in the morphology of their nuclei and degree of chromatin condensation (compare Figs. 1 and 3). Moreover, study of fertilized eggs that were forced to remain within the ovisacs, hours or days beyond the time they were ready to be laid, indicates that under these conditions the position and morphology at the sperm nucleus and centrosome are similar to those of normally laid eggs. Two conclusions may be drawn from these results. First, the structure of the sperm seems to remain greatly unchanged during the long period of time extending between copulation and oocyte penetration. This is because intercourse takes place between males at the beginning of the breeding season, and the exchanged sperms must wait weeks before the ovaries mature and oocytes are ready to be fertilized. Second, after penetration the fertilizing sperm is subjected to migration block, and under these conditions the sperm morphology seems to remain greatly unchanged. If the fertilizing sperm is subjected to migration block soon after penetration, the sperm entry site would be normally located at the upper half of the animal hemisphere. Consequently, polyspermy might be associated with the existence of eggs having more than one sperm entry site distributed in both egg hemispheres.

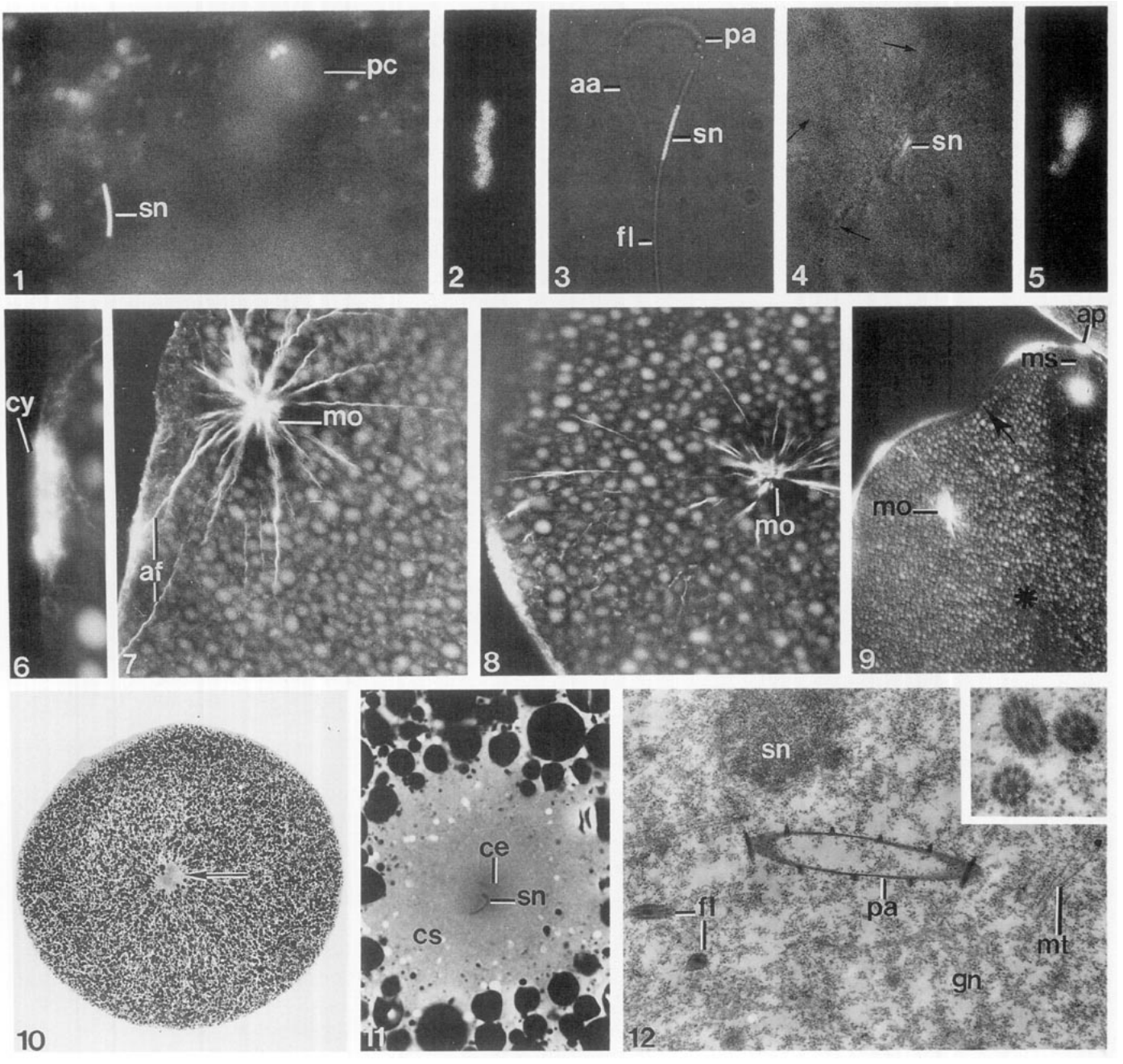
Tubulin staining also reveals that during completion of the first meiotic division highly fluorescent disk-shaped bodies, which may reach 30 μm in diameter, appear scattered across the egg ectoplasm. The fact that short bundles of microtubules appear connected to the surface of these bodies suggests that they may correspond to MTOC associated with the peripheral cytoplasm of the early uncleaved egg (Fig. 6). These fluorescent bodies have some resemblance to cytasters present in other eggs (see discussion) and for that reason they may be considered cytaster-like structures.

Migration of the Sperm and Early Development of the Monaster

The first sign that sperm migration block has been released is the detection of delicate bundles of microtubules growing out of its centrosome. This takes place shortly before or after discharge of the first pole cell, that is, at 60–90 min of development (stage 1a–1b transition). The tiny sperm centrosome together with the nascent astral fibers forms a small star-shaped body that constitutes the early monaster (Fig. 4). The route followed by the centralizing sperm has been mostly deduced by reconstructing the path of the growing monaster in tubulin-stained sectioned eggs during the second meiotic division (Figs. 7–9). The monaster descends along one side of the animal hemisphere, apparently following an arc-like trajectory resulting from a combination of inward and vegetalward displacements. Results of this study are summarized in fig. 18.

During centration the monaster increases considerably in size, not only by expansion of the astrosphere but also by appearance and enlargement of a pericentrosomal domain called the centrosphere. Sperm centration is accompanied by nuclear despiralization followed by chromatin decondensation. Despiralization is often initiated at one end of the nucleus by loosening of the outermost loops (Fig. 5). Chromatin decondensation in these loops gives rise to a mildly fluorescent growing mass of nuclear material. Despiralization of the sperm nucleus, but not chromatin decondensation, is completed during centration.

Centration concludes when the monaster centrosome reaches the animal/vegetal axis, at the center or slightly over the center of the egg. By this time the sperm components may be readily detected and studied by routine



FIGS. 1 AND 2. Whole-mounted early stage 1b (second meiotic division) eggs stained with Hoechst dye, which show the position and structure of the fertilizing sperm nucleus. (1) The sperm nucleus (sn) appears as a highly fluorescent rod located close to the animal pole, where the first pole cell (pc) has just been released. ×1100. (2) High-magnification micrograph showing part of the spiral-shaped sperm nucleus. ×200.

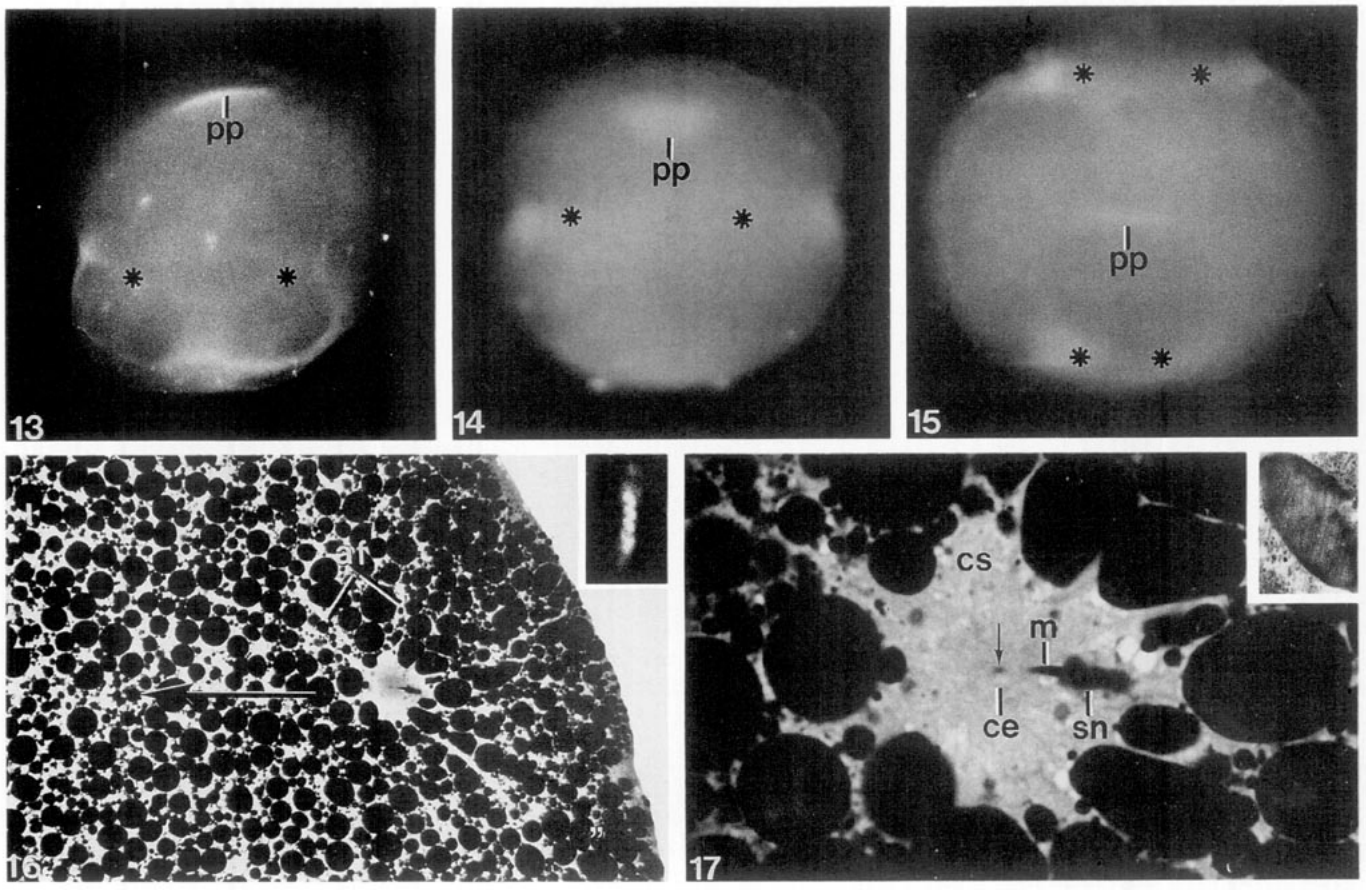
FIG. 3. Whole-mounted epydidimal sperm stained with Hoechst dye that shows the anterior (aa) and posterior (pa) achrosome, the spiral nucleus (sn), and the flagellum (fl). ×750.

FIG. 4. Whole-mounted stage 1b egg stained with Hoechst dye, showing part of the nucleus (sn) and growing aster (arrows) of a migrating sperm. Combined fluorescent and transmitted light illumination. ×500.

FIG. 5. High magnification of Fig. 4 that shows despiralization of the sperm nucleus. ×1800.

FIG. 6. Sectioned mid stage 1a egg (first meiotic division) stained for tubulin that shows a cytaster-like body (cy) embedded in the ectoplasm. ×1000.

FIGS. 7–9. Sectioned late stage 1a eggs stained for tubulin, which illustrate the structure and development of the sperm-derived monaster during centration. (7) The monaster (mo) is moving along one side of the egg. Notice that some astral fibers (af) have already made contact with the egg ectoplasm. ×480. (8) Further centration of the monaster (mo) is accompanied by considerable extension of its fibers. ×410. (9) Low magnification of an egg similar to that of Fig. 7 which shows the relative position of the monaster (mo) with respect to the animal pole (ap) and center of the egg (asterisk). Notice that the monaster is close to one side of the egg. The arrow marks the position of the contraction wave associated with release of the first pole cell. ms, first meiotic spindle. ×180.



FIGS. 13-15. Whole-mounted stage 1c (first interphase) eggs fixed in ALFAC, which were previously incubated in cytochalasin B from 0 (13), 1 (14), and 2 (15) hr of development, to show that misplaced sperms generate ectopic perinuclear plasms. Notice that the position of the perinuclear plasm (pp) depends on the time at which drug treatment was initiated. Since sperm centration is achieved at about 2.5 hr of development, ectopic perinuclear plasms were only formed in the earliest treated eggs of Figs. 13 and 14. The egg of Fig. 15 is indistinguishable from a normal egg. Ring-shaped opacities (asterisks), not described in this paper, correspond to regions where ooplasm is being accumulated. 13, ×60; 14, ×70; 15, ×80.

FIG. 16. Low-magnification micrograph of a stage 1b sectioned egg stained with toluidine blue which shows the position and structure of the monaster after incubation in cytochalasin B from 0 hr of development. Due to presence of the drug the growing monaster no longer moves vegetalward, but only inward (direction of arrow). Astral fibers (af) extend in all directions. The inset shows the sperm nucleus of a whole-mounted similar egg stained with Hoechst dye. Notice despiralization and chromatin decondensation. ×280; inset, ×2000.

FIG. 17. High-magnification micrograph of the MTOC of Fig. 16, showing that it consists of a centrosome (ce) and a expanding centrosphere (cs) that encloses other parts of the sperm such as its mitochondrion (m) and nucleus (sn). One of the sperm centrioles (arrow) is also visualized. The inset is an electron micrograph of the sperm mitochondrion. ×660; inset, ×21,000.

light or electron microscopy. Observation of toluidine blue-stained eggs shows that the centralized sperm consists of a mildly stained centrosome, which encloses the centrioles, and a lightly stained centrosphere. (Figs. 10 and 11). Observations with the electron microscope reveal that the centrosome and centrosphere consist of numerous granules, 20–25 nm in diameter, whose nature has not yet been disclosed. The centrosphere also includes different parts of the sperm and numerous radially oriented bundles of microtubules, which upon leav-

FIGS. 10 AND 11. Low- and high-magnification micrographs of stage 1b eggs stained with toluidine blue which illustrate the structure and development of the sperm-derived MTOC. The centrally located MTOC is marked by an arrow. Note the expansion of the centrosphere (cs) and presence of a centriole at the middle of the centrosome (ce). Part of the sperm nucleus (sn), which still remains condensed, is also visualized. 10, ×60; 11, ×750.

FIG. 12. Low-magnification electron micrograph of a stage 1b egg showing a centralized early centrosphere in which one finds bundles of microtubules (mt), granular material (gn), the decondensing sperm nucleus (sn), the empty posterior achrosome (pa), and profiles of the sperm flagellum (fl, see also inset). ×14,000; inset, ×34,000.

ing the centrosphere coalesce to form the monaster fibers. The walls of the posterior acrosome (outer plus inner cylinders) look normal but its axial rod has disappeared, presumably as result of enzyme release during the achrosome reaction. The nucleus lacks an envelope and its chromatin is still decondensing (Fig. 12). The large oval-shaped mitochondrion is about 1 μm in length and looks normal (Fig. 17). Many profiles of the axoneme are seen scattered throughout the centrosphere, suggesting that a long stretch of the sperm flagellum has also reached the center of the egg (Fig. 12). The centrioles have not yet been visualized with the electron microscope. Tubulin staining shows that upon centration of the sperm the monaster fibers reach the entire perimeter of the egg.

Effect of drugs. Treatment of early uncleaved eggs with taxol, colchicine, or cytochalasin B allowed us to determine the role of microtubules and actin filaments in sperm migration. Taxol treatment did not impair sperm centration, suggesting that this process does not involve cycles of tubulin depolymerization. Treatment of eggs with the other two drugs, on the other hand, indicates that integrity of microtubules and microfilaments is needed to successfully complete the centration process. A summary of these results is presented in Fig. 19. Continuous incubation in colchicine or cytochalasin B, from 0 to 2.5 hr of development, interrupts or modifies the centration process, causing sperm misplacement. However, it often does not make a difference if drug-incubation is initiated at 0 or 30 min of development (0 or 1 hr of development in the case of drug microinjection), confirming the observation that sperm centration is initiated at about 1 hr of development. Important differences in the location of the misplaced sperm are detected, however, when one compares eggs incubated in any of the two drugs from 1, 1.5, 2, or 2.5 hr of development. Thus, the earlier the drug treatment is initiated, the closer to the animal pole the location of the misplaced sperm is (Figs. 13–15), providing additional support to the observation that in most eggs the original location of the fertilizing sperm is close to the animal pole. According to the migration path, one might expect that the ectopic monaster of drug-treated eggs would be found closer to one side of the animal hemisphere. This turned out to be correct for colchicine-treated eggs because this drug provokes monaster disassembly followed by sperm arrest. In cytochalasin B-treated eggs, however, the monaster appears intact and invariably located along the animal/vegetal axis of the egg, indicating that this drug apparently interfered with only the vegetalward movement of the gamete. Further support to this conclusion comes from the observation that in cytochalasin B-treated eggs the sperm seems to move along a straight line perpendicular to the animal/vegetal axis (Fig. 16). From these data it may be concluded that whereas colchicine paralyzes the sperm centration, cytochalasin B blocks the vegetalward but not the inward centration movement.

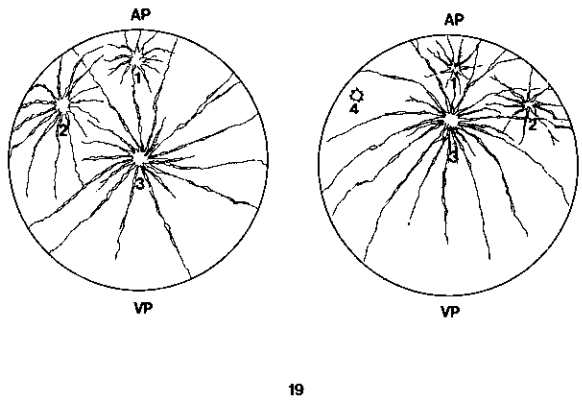


FIG. 18. Diagram that shows the normal trajectory (1–3) of the monaster during centration. 1, Early monaster near the animal pole (AP); 2, moving developing monaster; and 3, centrated monaster, VP, vegetal pole.

FIG. 19. Diagram that shows the effect of cytochalasin B (1–3) and colchicine (4) on monaster centration. 1, Early monaster near the animal pole (AP); 2, developing monaster in which the vegetalward movement has just been blocked by the drug; 3, monaster that moved inwards and became misplaced along the animal/vegetal axis; 4, arrested centrosome of a dismantled monaster produced by colchicine treatment. VP, vegetal pole.

Cytochalasin B-treated eggs lose rigidity, flatten on the culture dish, become very fragile, and fail to release the pole cells. The sperm nucleus appears to initiate despiralization and chromatin decondensation with some delay (Figs. 16 and 17), in some cases after 3 hr of development. Although disruption of the actin lattice (see also Fernández *et al.*, 1990) severely affects the egg motility, and consequently formation of ooplasmic domains (Figs. 13 and 14), development of the egg continues for several hours in presence of the drug.

Eggs incubated or microinjected with colchicine from 0 or 1 hr of development do not form a monaster, fail to discharge the pole cells, and development soon concludes. There is evidence, however, that nuclear despiralization and chromatin decondensation may be initiated in these drug-treated eggs. Colchicine treatments initiated at 2 or 3 hr of development not only provoke disassembly of the monaster, but also distorsion in the distribution of the ooplasmic domains.

Organization of the First-Interphase Monaster

Study of whole-mounted or sectioned eggs stained for tubulin reveals that the first interphase monaster consists of two types of fibers: long astral fibers associated with a single large and deeply situated MTOC founded

by the sperm centrosome (Figs. 20, 22, and 23) and short fibers associated with small disk-shaped MTOC scattered across the ectoplasm and peripheral endoplasm (Fig. 24). Figure 22 shows that long astral fibers may be subdivided into straight fibers, which extend directly to the animal or vegetal pole ectoplasm, and curved fibers, which extend to the animal or vegetal hemisphere ectoplasm and from there to the respective egg pole. Taxol induces MTOC of various sizes (10–40 μm in diameter) and shapes, at whose center one may detect a tubulin-free region (Figs. 25 and 26). These taxol-induced MTOC also produce short bundles of microtubules and hence may be considered in the category of cytaster-like structures. Curved astral fibers, together with those produced by the small MTOC, constitute the superficial or ectoplasmic cytoskeleton of microtubules of the first interphase (Figs. 21 and 22).

As a whole the normal monaster consists of two frameworks of microtubules of similar size, one for the animal and other for the vegetal egg hemisphere. Unequal frameworks of microtubules are produced by ectopic MTOC, such as those generated in cytochalasin B-treated eggs. In the majority of cases a misplaced sperm centrosome forms a small animal framework of microtubules and a large vegetal framework of microtubules (Figs. 19, 27, and 28).

Establishment of the Perinuclear Plasm Domain

Development of the centrated monaster involves further expansion of its centrosphere accompanied by organelle accumulation. Thus, numerous small mitochondria (0.2–0.3 μm in diameter), profiles of smooth endoplasmic reticulum, and Golgi complexes are accumulated among bundles of microtubules and 20- to 25-nm granules. Some yolk platelets are also present (Figs. 29–34). With the exception of the large sperm-derived mitochondrion, the original centrosphere lacks other membranous organelles. Hence, they may have invaded the centrosphere from the adjacent endoplasm (compare Figs. 11 and 17 with Figs. 29–31, and Fig. 12 with Fig. 33). Moreover, the fact that colchicine treatment prevents organelle accumulation, and concomitantly centrosphere expansion, suggests that microtubules are probably involved in transport of organelles toward the centrosphere. However, it can not be excluded that some of the mitochondria may have originated by fragmentation of the large sperm mitochondrion. Presence of partly divided and "eight-shaped" mitochondria in more developed centrospheres is taken as indicative that these organelles also increase in number by proliferation (Figs. 34 and 35).

The organelle-laden centrosphere constitutes a perinuclear plasm domain because it is destined to house the zygote nucleus. As shown in Figs. 30 and 33, segregation of organelles within the perinuclear plasm leads to formation of outer and inner sectors.

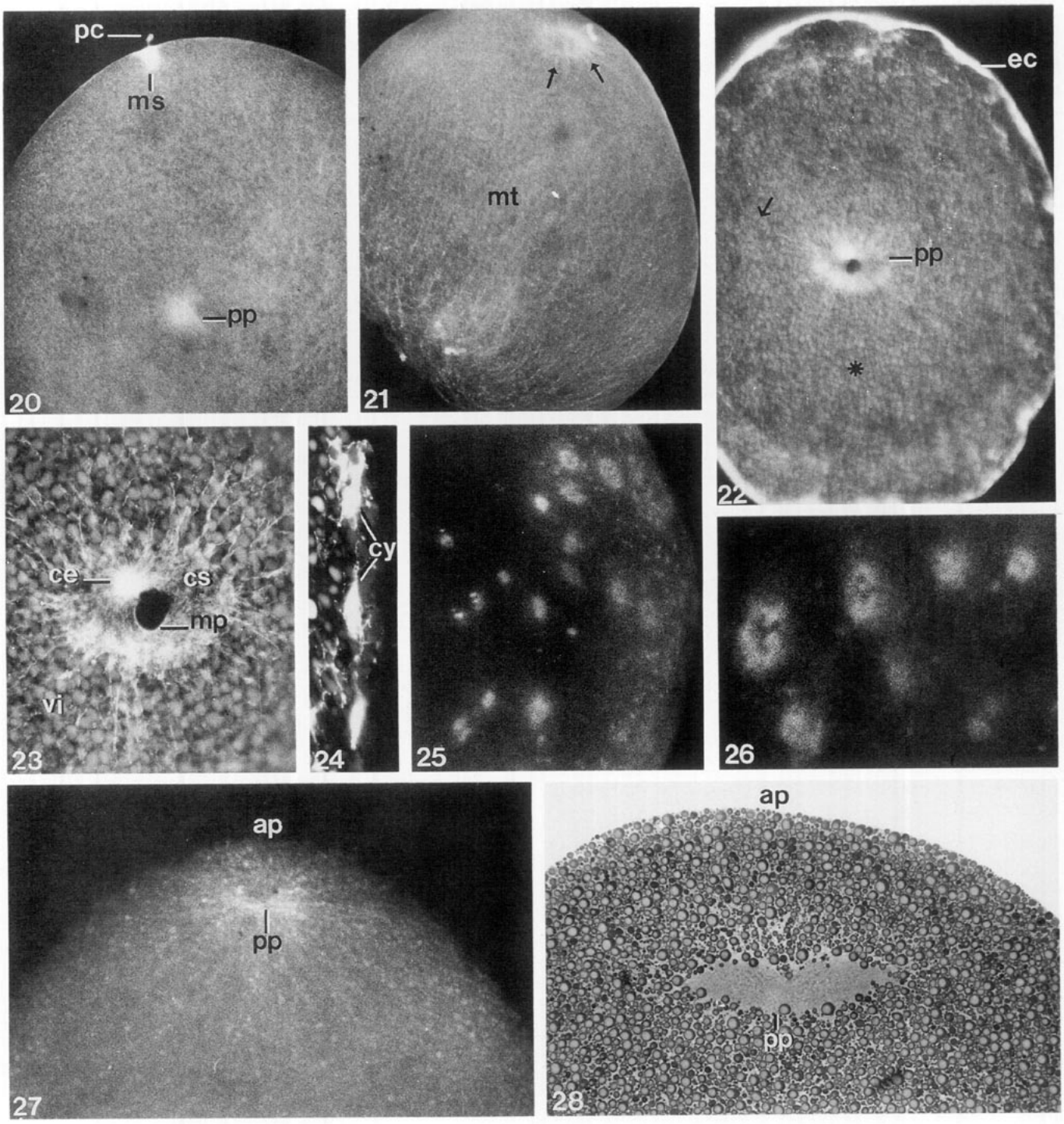
The perinuclear plasm domain normally forms and stays at the center of the egg (Fig. 15). However, ectopic perinuclear plasms (Figs. 13 and 14) may be produced in cytochalasin B-treated eggs, indicating that misplaced centrospheres are also able to develop this ooplasmic domain.

Formation of the Male Pronucleus

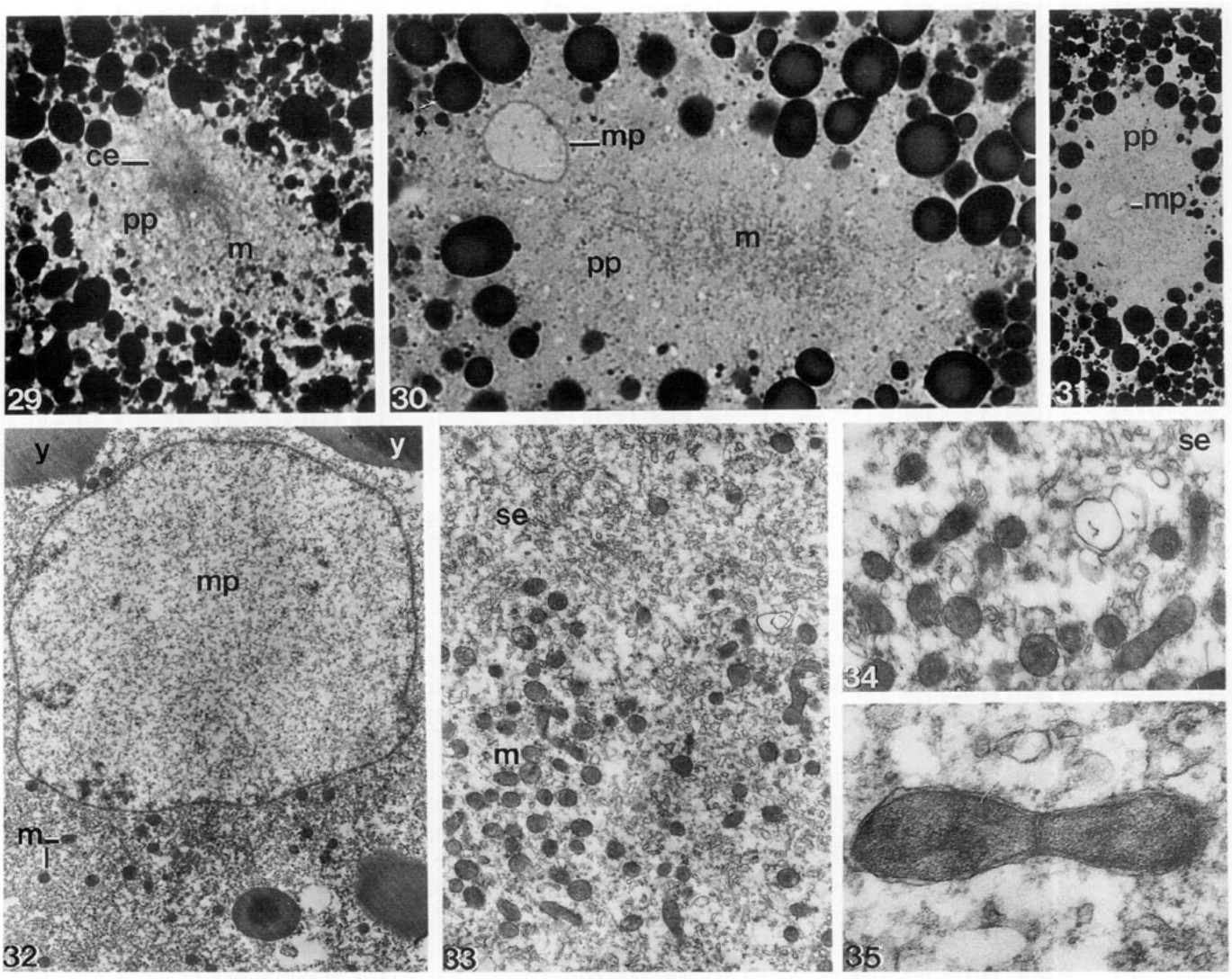
Formation of the male pronucleus concludes at the periphery of the perinuclear plasm domain when chromatin decondensation and assembly of the nuclear envelope are completed (Figs. 30 and 32). The male pronucleus appears as a rounded structure, 15–20 μm in diameter, that enters the perinuclear plasm (Fig. 31) and travels to the center of the mitochondrial cloud, where it waits for the arrival of the female pronucleus (Figs. 22 and 23). Centration of the male pronucleus concludes at about 2.5 hr of development (late stage 1b), when the second meiotic division is about to be completed.

DISCUSSION

Fertilization occurs in the ovisacs sometime before egg laying. However, eggs may be forced to remain within the ovisacs beyond the time they are ready to be laid. Under these circumstances fertilized eggs do not initiate development because they are subjected to meiosis I arrest (Fernández and Olea, 1982). Interestingly, this condition also affects the sperm movement because in meiosis-arrested eggs the fertilizing sperm does not initiate centration and remains at the periphery of the animal hemisphere. Moreover, the structure of the sperm seems to also remain greatly unchanged. Thus, the centrosome fails to grow an aster, the nucleus stays spiralized, and its chromatin stays condensed. In this manner, *T. rude*, and perhaps other leeches as well, differ from other invertebrates in which the male pronucleus and aster form at the egg cortex soon after sperm penetration. It is the case in sea urchins (Schatten, 1981), nematodes (Albertson, 1984), and many molluscs (Raven, 1966; Longo, 1983). Differences may be related to the fact that insemination is internal in leeches and takes place long before the eggs are laid and development is initiated. It is feasible that sperm movement arrest is due to the fact that its centrosome is prevented from nucleating microtubules. Egg laying, or removal of *ready* eggs from the ovisacs, releases meiotic and then sperm movement arrest, in the latter case probably because the sperm centrosome became an active MTOC. The fact that colchicine-arrested sperms may initiate



FIGS. 20–23. Stage 1c eggs stained for tubulin that illustrate the structure of the first interphase monaster. (20) Optical section passing across the center of a whole-mounted egg that shows the fluorescent perinuclear plasm (pp), the inner pole of the second meiotic spindle (ms), and the second pole cell (pc). Bundles of microtubules are seen leaving the perinuclear plasm. ×60. (21) Optical section passing across the surface of a whole-mounted egg that shows the peripheral (ectoplasmic) framework of microtubules (mt) of the monaster, which extends between the poles. Disassembling microtubules of the inner pole of the second meiotic spindle are marked by arrows. ×60. (22) Low-magnification micrograph of a sectioned egg showing the perinuclear plasm (pp) and the internal distribution of astral fibers. Notice the straight (asterisk) and curved (arrow) bundles of microtubules traveling toward the ectoplasm, where they form a fluorescent shell (ec). ×180. (23) High-magnification micrograph of the same egg showing the distribution of microtubules across the perinuclear plasm. Bundles of microtubules are seen coming out of the centrosome (ce), extending radially across the centrosphere (cs) and penetrate the vitelloplasm (vi) to travel between the yolk platelets. The centrated male pronucleus (mp) lies adjacent to the centrosome. ×400.



FIGS. 29–31. Light micrographs of stage 1b sectioned eggs stained with toluidine blue, which show centration of the male pronucleus (mp) and development of the perinuclear plasm domain (pp). (29) Microtubules are seen growing out of the sperm centrosome (ce), at whose center one sees a centriole. Mitochondria (m) have begun to accumulate across the centrosphere, thus marking the initiation of perinuclear plasm formation. ×1000. (30) The male pronucleus has just formed at the outer border of the centrosphere. Note that the perinuclear plasm consists of a granular inner sector, which is rich in mitochondria (m), and a less granular outer sector with fewer mitochondria. ×350. (31) The male pronucleus is about to reach the center of the perinuclear plasm. ×550.

FIGS. 32–35. Electron micrographs of stage 1b eggs. (32) Micrograph similar to that of Fig. 30, showing the male pronucleus (mp) entering the perinuclear plasm. m, mitochondria; y, yolk platelets. ×3200. (33) The perinuclear plasm consists of a mitochondria-rich core (m) and a smooth endoplasmic reticulum-rich periphery (se). ×12,000. (34) High-magnification micrograph that shows the structure of mitochondria and smooth endoplasmic reticulum (se). Elongated mitochondria are probably replicating. ×21,000. (35) Profile of a dividing mitochondrion. ×59,000.

FIG. 24. Sectioned stage 1b egg stained for tubulin that shows ectoplasmic cytaster-like bodies (cy). ×400.

FIGS. 25 AND 26. Surface view of whole-mounted stage 1c eggs treated with taxol, showing the structure and distribution of cytaster-like bodies. 25, ×130; 26, ×350.

FIG. 27. Whole-mounted stage 1c egg stained for tubulin, which shows the position and structure of the monaster after incubation in cytochalasin B from 0 hr of development. Note that the perinuclear plasm (pp) is very close to the animal pole (ap) and that the monaster is asymmetrical because it consists of a small animal and a large vegetal framework of microtubules. ×160.

FIG. 28. Stage 1c sectioned egg stained with toluidine blue, showing the misplaced perinuclear plasm (pp) of a cytochalasin B-treated egg. Incubation in the drug was initiated at 1 hr of development. The structure of this ectopic perinuclear plasm is nearly normal and the trajectory of the monaster fibers may be judged by the orderly arrangement of the yolk platelets. ap, approximate position of the animal pole. ×250.

despiralization and chromatin decondensation is taken as indicative that aster formation and sperm migration do not trigger nuclear changes.

There are good reasons to suspect that release of meiotic and sperm movement arrest are part of a cascade of processes leading to initiation of embryonic development. Release of meiotic arrest, perhaps one of the earliest events in the cascade, may be triggered by changes in the activity of substances such as maturation promoting factor (MPF), cytostatic factor (CSF), and cyclins, that regulate the cell cycle (Masui and Markert, 1971; Kishimoto *et al.*, 1982; Newport and Kirschner, 1984; Gerhart *et al.*, 1984; Standart *et al.*, 1987; Smith, 1989; Masui, 1991). If meiosis I arrest is produced by mechanisms that maintain MPF activity high, egg laying would release such arrest, perhaps by calcium entry from the cocoon fluid or culture medium (see Whitaker and Patel, 1990; Newport and Kirschner, 1984). Increase of cytoplasmic calcium may inhibit tubulin polymerization and hence nucleation of microtubules by the sperm centrosome. This would explain why development of a monaster in the laid egg is delayed by about an hour. Provided that the egg has a reasonable pool of tubulin, enzyme activation, synthesis of certain proteins and, of course, calcium sequestration after its initial rise would create favorable conditions for tubulin polymerization at the 1a–1b transition stage.

Centration of the sperm seems to follow an arc-like trajectory as result of two conjugated movements: an inward movement and a vegetalward movement, perhaps produced by differential elongation of the astral microtubules against the ectoplasm of one side of the egg. It is not known whether such elongation involves microtubule sliding. Positioning of the sperm centrosome at the egg center may be achieved as result of an equilibrium of forces generated by the monaster fibers that have reached the entire perimeter of the egg.

Sperm centration requires the integrity of both microtubules and microfilaments. Astral microtubules are generally considered to constitute the motor that drives the sperm toward the center of the egg (see also Zimmerman and Zimmerman, 1967; Manes and Barbieri, 1977; Schatten, 1981; Gerhart *et al.*, 1983; Longo, 1973, 1983, and 1991). It is not known, however, whether axoneme beating may also contribute to sperm centration. Microfilaments, which are present in large numbers in the ectoplasm of early uncleaved eggs (Fernández *et al.*, 1990), may play a rather passive role in sperm centration, by conferring the egg surface sufficient rigidity for the elongation of the astral microtubules. Thus, "softening" of the egg surface by cytochalasin B treatment might prevent astral fibers from performing mechanical work against the ectoplasm, and in these circumstances the sperm would fail to reach the egg center. This explains why the position attained by the misplaced sperm depends on the time at which incubation in the drug is initiated. This does not answer, however, why cytochalasin B-misplaced sperms are found at the level of the animal/vegetal axis and not, as expected, between this axis and one side of the egg. It is possible that inward and vegetalward movements rely on the elongation of different sets of microtubules, which in turn would present different requirements of ectoplasmic rigidity. Finally, it can not be excluded that the vegetalward movement relies on both microtubules and an actin-based contraction mechanism. The fact that microfilament inhibitors, such as cytochalasin B and E, do not interrupt centration of the male pronucleus in sea urchin eggs (Schatten, 1981) may be indicative that in this species actin is not involved in sperm centration or that their actin filaments are not affected by the drug.

During centration the structure and relationships of most sperm organelles remain greatly unchanged, indicating that the demembranated sperm moves coherently.

There are two lines of evidence that the sperm centrosome constitutes the main MTOC of the egg. First, the sperm centrosome initiates formation of the monaster when one can still detect the centrosome associated with the inner pole of the first meiotic spindle. Second, the monaster centrosome replicates to form the amphidiaster involved in the assembly of the cleavage spindle (our unpublished observations). Hence, leeches resemble most vertebrates and invertebrates in that the sperm furnish the microtubule organizing center of the egg (Wilson, 1925; Gerhart *et al.*, 1983; Albertson, 1984; Sluder *et al.*, 1989, 1993). Since the fate of the maternal centrosome has not been followed with appropriate markers, it can not be excluded that centrosomal material may be incorporated into the male centrosome or contribute to the formation of cytaster-like structures (Sluder *et al.*, 1989, 1993).

Cytasters or cytaster-like structures are normally present or may be induced by different procedures or agents, particularly taxol, in the egg of various invertebrates (see Harris *et al.*, 1980; Harris and Clason, 1992; Schatten *et al.*, 1992; Houliston *et al.*, 1993) and vertebrates (see Van Assel and Brachet, 1966; Maro *et al.*, 1988; Buendía *et al.*, 1990; Houliston and Elinson, 1991). Differences in the morphology of cytasters and cytaster-like structures may be related to the nature of the MTOC and the manner in which these organelles put together tubulin subunits.

An interesting feature of the developed sperm centrosome, which is also shared by the centrosomes of the egg meiotic spindle (Fernández *et al.*, 1990), is the development of a prominent centrosphere. Centrosomes and centrospheres differ in their structure and probably

also in their composition. However, they appear to form a functional complex involved not only in microtubule assembly but also in organelle accumulation. The majority of the curved and straight astral fibers appear to reach the egg poles and therefore their (−) ends are located at the level of the centrosome and their (+) ends at either the animal or vegetal pole. Therefore, assembly of both types of astral fibers is interrupted at the egg poles, may be by mechanisms that prevent further microtubule elongation. This might be achieved by capping proteins and/or local unfavorable conditions for further tubulin polymerization.

The fact that curved astral fibers of each egg hemisphere seem to travel only toward the corresponding egg pole suggests that animal and vegetal ectoplasms may have spatial cues that orient microtubule growth in the proper direction.

The nascent centrosphere has a giant mitochondrion provided by the sperm (see also Fernández *et al.*, 1992), microtubules, and numerous granules, some of which may be ribosomes. Therefore, accumulation of other organelles in the centrosphere is likely to start by importing them from the adjacent vitelloplasm. The fact that formation and growth of the perinuclear plasm are interrupted in colchicine-treated but not in cytochalasin B-treated eggs suggests that monaster microtubules are engaged in centripetal or (+) to (−) transfer of organelles. It would be of interest to determine whether cytoplasmic dynein is involved in such organelle movement. Previous work also indicates that the monaster microtubules are involved in poleward translocation of organelles during teloplasm formation (Fernández *et al.*, 1987). However, in the latter case organelles move toward the (+) end of the microtubules. The fact that replicating figures of mitochondria are common across the perinuclear plasm implies that organelle proliferation also contributes to the sustained growth of this ooplasmic domain. From these observations it may be concluded that the monaster astral fibers not only provide mechanical support to the interphase egg but also furnish orderly pathways for organelle transport during ooplasmic segregation. Accumulation and replication of mitochondria also take place at the centriolar region of *Xenopus* oocytes, where they form the so-called mitochondrial or Balbiani body (Tourte *et al.*, 1981).

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REFERENCES

Albertson, D. G. (1984). Formation of the first cleavage spindle in nematode embryos. *Dev. Biol.* **101,** 61–72.

Astrow, S., Holton, B., and Weisblat, D. (1987). Centrifugation redistributes factors determining cleavage patterns in leech embryos. *Dev. Biol.* **120,** 270–283.

Bissen, S. T., Ho, R. K., and Weisblat, D. A. (1986). Cell interactions in the developing leech embryo. *BioEssays* **4,** 152–157.

Buendía, B., Antony, C., Verde, F., Bornens, M., and Karsenti, E. (1990). A centrosomal antigen localized on intermediate filaments and mitotic spindle poles. *J. Cell Sci.* **97,** 259–271.

Fernández, J. (1980). Embryonic development of the glossiphoniid leech *Theromyzon rude:* Characterization of developmental stages. *Dev. Biol.* **76,** 245–262.

Fernández, J., and Stent, G. S. (1980). Embryonic development of the glossiphoniid leech *Theromyzon rude:* Structure and development of the germinal bands. *Dev. Biol.* **78,** 407–434.

Fernández, J., and Olea, N. (1982). Embryonic development of glossiphoniid leeches. *In* "Developmental Biology of Freshwater Invertebrates" (F. W. Harrison and R. R. Cowden, Eds.), pp. 317–361. A. R. Liss, New York.

Fernández, J., Olea, N., and Matte, C. (1987). Structure and development of the egg of the glossiphoniid leech *Theromyzon rude:* Characterization of developmental stages and structure of the early uncleaved egg. *Development* **100,** 211–225.

Fernández, J., Olea, N., Téllez, V., and Matte, C. (1990). Structure and development of the egg of the glossiphoniid leech *Theromyzon rude:* Reorganization of the fertilized egg during completion of the first meiotic division. *Dev. Biol.* **137,** 142–154.

Fernández, J., Téllez, V., and Olea, N. (1992). Hirudinea. *In* "Microscopic Anatomy of Invertebrates," Vol. 7, "Annelida," (F. W. Harrison and S. L. Gardiner, Eds.), pp. 323–394. Wiley-Liss, New York.

Gerhart, J., Black, S., and Scharf, S. (1983). Cellular and pancellular organization of the amphibian embryo. *In* "Modern Cell Biology" (J. R. McIntosh, Ed.), Vol. 2, pp. 483–507. A. R. Liss, New York.

Gerhart, J., Wu, M., and Kirschner, M. (1984). Cell cycle dynamics of an M phase specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* **98,** 1247–1255.

Harris, P., Osborn, N., and Weber, K. (1980). Distribution of tubulin-containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus* from fertilization through first cleavage. *J. Cell Biol.* **84,** 668–679.

Harris, P. J., and Clason, E. L. G. (1992). Conditions for assembly of tubulin-based structures in unfertilized sea urchin eggs: Spirals, monasters and cytasters. *J. Cell Sci.* **102,** 557–567.

Houliston, E., and Elinson, R. P. (1991). Patterns of microtubule polymerization relating to cortical rotation in *Xenopus laevis* eggs. *Development* **112,** 107–117.

Houliston, E., Carré, D., Johnston, J. A., and Sardet, C. (1993). Axis establishment and microtubule-mediated waves prior to first cleavage in *Beroe ovata. Development* **117,** 75–87.

Kishimoto, T., Kuriyama, R., Kondo, H., and Kanatani, H. (1982). Generality of the action of various maturation-promoting factors. *Exp. Cell Res.* **137,** 121–126.

Longo, F. J. (1973). Fertilization: A comparative ultrastructural review. *Biol. Reprod.* **9,** 149–215.

Longo, F. J. (1983). Meiotic maturation and fertilization. *In* "The Mollusca", Vol. 3, "Development," pp. 49–89. Academic Press, San Diego, CA.

Longo, F. J. (1991). Gamete interactions and the fate of sperm organelles in fertilized echinoderm eggs. *J. Electron Microsc. Technol.* **17,** 246–265.

Manes, M. E., and Barbieri, F. D. (1977). On the possibility of sperm aster involvement in dorso-ventral polarization and pronuclear migration in the amphibian egg. *J. Embryol. Exp. Morphol.* **40,** 187–197.

Maro, B., Houliston, E., and Paintrand, M. (1988). Purification of mei-

otic spindles and cytoplasmic asters from mouse oocytes. *Dev. Biol.* **129,** 275–282.

Masui, Y. (1991). The role of "cytostatic factor (CSF)" in the control of oocyte cell cycles: A summary of 20 years of study. *Dev. Growth Differ.* **33(6),** 543–551.

Masui, Y., and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177,** 129–145.

Nardelli-Haefliger, D., and Shankland, M. (1992). Lox2, a putative leech segment identity gene, is expressed in the same segmental domain in different stem cell lineages. *Development* **16,** 697–710.

Newport, J. W., and Kirschner, M. W. (1984). Regulation of the cell cycle during early *Xenopus* development. *Cell* **37,** 731–742.

Raven, C. P. (1966). "Morphogenesis: The Analysis of Molluscan Development." Pergamon. Oxford.

Sandig, M., and Dohle, W. (1988). The cleavage pattern in the leech *Theromyzon tessulatum* (Hirudinea, Glossiphoniidae). *J. Morphol.* **196,** 217–252.

Schatten, G. (1981). The movements of the nuclei during fertilization. *In* "Mitosis/Cytokinesis" (A. M. Zimmerman and A. Forer, Eds.), pp. 59–82. Academic Press. New York.

Schatten, H., Walter, M., Biessmann, H., and Schatten, G. (1992). Activation of maternal centrosomes in unfertilized sea urchin eggs. *Cell Motil. Cytoskel.* **23,** 61–70.

Schleip, W. (1939). Ontogenie der Hirudineen. *In* "Klassen und Ordnungen des Tierreichs" (H. G. Bronn, Ed.), Vol. 4, Book 4, Part 2, pp. 1–121. Akad. Verlagsgesellschaft, Leipzig.

Schliwa, M. (1980). Structural organization of detergent-extracted cells. *In* "Proceedings of the 38th Meeting of Electron Microscopy Society of America" (G. W. Bailey, Ed.), pp. 814–817. Claitors, Baton Rouge, LA.

Schliwa, M., and van Blerkom, J. (1981). Structural interaction of cytoskeletal components. *J. Cell Biol.* **90,** 222–235.

Shankland, M. (1991). Leech segmentation: Cell lineage and the formation of complex body patterns. *Dev. Biol.* **144,** 221–231.

Sluder, G., Miller, F. J., Lewis, K., Davison, E. D., and Rieder, C. L. (1989). Centrosome inheritance in starfish oocytes: Selective loss of the maternal centrosome after fertilization. *Dev. Biol.* **131,** 567–579.

Sluder, G., Miller, F. J., and Lewis, K. (1993). Centrosome inheritance in starfish zygotes II: Selective suppression of the maternal centrosome during meiosis. *Dev. Biol.* **155,** 58–67.

Smith, L. D. (1989). The induction of oocyte maturation: transmembrane signaling events and regulation of the cell cycle. *Development* **107,** 685–699.

Standart, N., Minshull, J., Pines, J., and Hunt, T. (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. *Dev. Biol.* **124,** 248–258.

Stent, G. S. (1985). The role of cell lineage in development. *Phil. Trans. R. Soc. London Ser. B* **312,** 3–19.

Tourte, M., Mignotte, F., and Mounolou, J. C. (1981). Organization and replication activity of the mitochondrial mass of oogonia and previtellogenic oocytes in *Xenopus laevis*. *Dev. Growth Differ.* **23**(1), 9–21.

Van Assel, P. S., and Brachet, J. (1966). Formation de cytasters dans les ouefs de batraciens sous l'action de l'eau lourde. *J. Embryol. Exp. Morphol.* **15,** 143–151.

Wedeen, C. J., and Weisblat, D. A. (1991). Segmental expression of an engrailed-class gene during early development and neurogenesis in an annelid. *Development* **113,** 805–814.

Weisblat, D. A., Sawyer, R. T., and Stent, G. S. (1978). Cell lineage analysis by intracellular injection of a tracer enzyme. *Science* **202,** 1295–1298.

Weisblat, D. A., Kim, S. Y., and Stent, G. S. (1984). Embryonic origins of cells in the leech *Helobdella triserialis*. *Dev. Biol.* **104,** 65–85.

Weisblat, D. A., Price, D. J., and Wedeen, C. J. (1988). Segmentation in leech development. *Development* **104**(Suppl.), 161–168.

Whitaker, M., and Patel, R. (1990). Calcium and cell cycle control. *Development* **108,** 525–542.

Whitman, C. O. (1878). The embryology of *Clepsine*. *Q. J. Microsc. Sci.* **192,** 421–429.

Wilson, E. B. (1925). "The Cell in Development and Heredity." MacMillan Co., New York.

Zackson, S. L. (1984). Cell lineage, cell–cell interaction, and segment formation in the ectoderm of a glossiphoniid leech embryo. *Dev. Biol.* **104,** 143–160.

Zimmerman, A. M., and Zimmerman, S. (1967). Action of colcemide in sea urchin eggs. *J. Cell Biol.* **34,** 483–488.