

Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport

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Summary

Inhibitor studies have implicated microtubules in at least three important developmental processes during *Drosophila* oogenesis: oocyte determination and growth during stages 1 through 6, positioning of the anterior determinant *bicoid* mRNA during stages 9 through 12, and ooplasmic streaming during stages 10b through 12. We have used fluorescence cytochemistry together with laser scanning confocal microscopy to identify distinct microtubule structures at each of the above three periods that are likely to be involved in these processes. During stages 1 through 7, maternal components synthesized in nurse cells are transported through cytoplasmic bridges to the oocyte. At this time, microtubules that appear to originate in the oocyte pass through these cytoplasmic bridges into the adjacent nurse cells; these microtubules are likely to serve as a polarized scaffold on which maternal RNAs and proteins are transported. During stages 7 and 8, microtubules in the oocyte cortex

reorganize to form an anterior-to-posterior gradient, suggesting a role for microtubules in the localization of morphogenetic determinants. Finally, when ooplasmic streaming begins during stage 10 b, it is accompanied by the assembly of subsurface microtubule arrays that spiral around the oocyte; these arrays disassemble as the oocyte matures and streaming stops. During ooplasmic streaming, many vesicles are closely associated with the subsurface microtubules, suggesting that streaming is driven by vesicle translocation along microtubules. We believe that actin plays a secondary role in each of these morphogenetic events, based on our parallel studies of actin organization during each of the above stages of oogenesis.

Key words: microtubules, actin filaments, mRNA localization, ooplasmic streaming.

Introduction

Understanding the mechanisms that generate and maintain cell asymmetry are central problems in developmental biology. In *Drosophila*, developmentally significant asymmetry is first established at the cytoplasmic level, during oogenesis. Oogenesis in *Drosophila* begins with four divisions of a stem cell. As a result of incomplete cytokinesis at each division, the resulting 16 germline cells remain linked by cytoplasmic bridges. Despite sharing a common cytoplasm, these 16 syncytial cells give rise to a single oocyte and 15 nurse cells. Although the oocyte is translationally active, it is transcriptionally inactive through most of oogenesis (King and Burnett, 1959). During stages 1 through 6, the oocyte nonetheless grows at the same rate as the associated nurse cells and accumulates several specific mRNAs that appear to be synthesized only in the nurse cells (for examples, see Ephrussi et al., 1991; Kim-Ha et al., 1991; Suter et al., 1989). The interconnected oocyte-nurse cell cytoplasm is therefore functionally asymmetric, with the oocyte defining a special compartment. Among the maternal RNAs that are transferred to the oocyte are the morphogenetic determinants that specify anterior and posterior structures

in the embryo (for a review, see Nusslein-Volhard and Roth, 1989). These determinants become localized to the cortex at the anterior and posterior poles during stages 7 through 10 of oogenesis, demonstrating that the cortex of the oocyte itself develops asymmetry.

How is asymmetry maintained in the oocyte-nurse cell cysts, and within the oocyte itself? The effects of inhibitors indicate that cytoskeletal elements play important roles. Inhibitors of microtubule assembly disrupt oocyte determination and growth (Koch and Spitzer, 1983), ooplasmic streaming (Gutzeit, 1986b), and anterior localization of *bicoid* message (Pokrywka and Stephenson, 1991). Cytochalasins, which inhibit actin assembly, block bulk transfer of cytoplasm from the nurse cells to the oocyte (Gutzeit, 1986a). The organization of cytoskeletal elements during oogenesis is therefore of particular interest both early in oocyte differentiation, as its maternally-supplied components begin to accumulate, and later, as molecular asymmetry is first detected in the oocyte itself.

The major filaments in the cytoskeleton of most cells include microtubules, actin filaments, and intermediate filaments. Although proteins immunologically related to intermediate filament proteins have been identified in

Drosophila (Walter and Biessmann, 1984), intermediate filaments have not been identified at either the light or electron microscopic level, and it has been suggested that all arthropods lack these filaments (Bartnik et al., 1985). Analysis of the cytoskeleton during *Drosophila* oogenesis, therefore, is presently focused on the study of microtubules and actin filaments.

Immunofluorescence labeling has been widely used to examine the organization of cytoskeletal elements in cells. This technique presents technical challenges in *Drosophila* oocytes: the oocyte is surrounded by follicle and nurse cells through most of oogenesis, and immunolabeled cytoskeletal elements in these surrounding cells complicate conventional microscopic analysis of the oocyte. In addition, during the later stages of oogenesis the vitelline membrane and chorion block antibody penetration into the oocyte. To overcome these difficulties, we have utilized laser scanning confocal microscopy to optically section oocytes and eliminate out-of-focus signal from the follicle and nurse cells. We have also developed a simple method for the manual removal of the follicle cells, chorion, and vitelline membranes from later stage oocytes, thereby facilitating their immunolabeling. A combination of these two techniques has allowed us to determine the organization of actin filaments and microtubules throughout the entire period of *Drosophila* oogenesis.

Materials and methods

Egg chamber isolation and culture

Oocytes were isolated by a modification of the procedure of Mahowald et al. (1983). 3 to 5-day old Oregon R flies were anesthetized with CO₂ and transferred to a blender containing 200 to 300 ml of modified Robb's medium (55 mM potassium acetate, 40 mM sodium acetate, 100 mM sucrose, 10 mM glucose, 1.2 mM magnesium chloride, 1.0 mM calcium chloride, 100 mM HEPES, pH 7.4). The blender was pulsed 3 times for 2 seconds at low speed and the resulting disaggregated fly parts were passed through a loose mesh with approximately 500 µm diameter pores. The material retained on the mesh was returned to the blender with 200 ml of Robb's medium and the blender treatment was repeated. This material was passed through the same screen and pooled with the previously collected filtrate.

The pooled filtrate was left undisturbed for 5 minutes to allow egg chambers to settle to the bottom of a 1 liter beaker. The supernatant was then aspirated off and the settled egg chambers were resuspended in 200 ml of fresh Robb's medium. The mixture was passed through a screen with 250 µm diameter pores that removes most of the larger contaminants. Egg chambers in this final filtrate were allowed to settle for 2 to 3 minutes in a 250 ml beaker before the supernatant was aspirated off. This procedure yields stage 1 through 14 egg chambers, as well as germaria. We estimate that egg chambers represent from 60 to 80% of the material recovered. With additional rounds of resuspension and gravity settling, preparations that are almost exclusively egg chamber can be obtained. The isolated egg chambers were either fixed immediately or cultured at 23°C in Robb's medium.

Egg chambers, isolated as described above, were treated with cytoskeletal inhibitors as follows: egg chambers were transferred to 50 ml glass beakers and 10 ml of Robb's medium, containing either 20 µM colcemid, 20 µM colchicine, or carrier dimethyl sulfoxide only, was added. Cultures were incubated at room temperature with gentle rocking for 10 to 60 minutes, and they were

then fixed and prepared for immunofluorescence as described below.

Fixation, immunofluorescence labeling, and electron microscopy

Egg chambers were transferred to 10 × 75 mm test tubes and allowed to settle. The Robb's medium was removed and replaced by 5 ml of 100 mM potassium cacodylate, pH 7.2, 100 mM sucrose, 40 mM potassium acetate, 10 mM sodium acetate, 10 mM Na₃EGTA, 8% formaldehyde (E.M. grade) and incubated for 5 to 10 minutes. Fixed egg chambers were rinsed 3 times in phosphate-buffered saline (PBS, Karr and Alberts, 1986), extracted with 1% Triton X-100 in PBS for 2 hours, then rinsed twice in 0.05% Triton X-100 in PBS (PBST). For electron microscopy, 2% glutaraldehyde was added to the formaldehyde fixation solution, incubation time was increased to 30 minutes, and egg chambers were stained with 2% uranyl acetate overnight prior to embedding in Epon 812. Thin sections were post-stained for 30 minutes in 1% uranyl acetate, followed by a 2 minute treatment with 0.02% lead citrate.

Follicle cells, chorion and vitelline membranes were removed prior to whole mount antibody labeling as follows: fixed egg chambers, rinsed in PBS as described above, were transferred to the frosted surface of a glass slide. Most of the PBS was removed and a 22 mm × 50 mm cover glass was placed over the egg chambers, which were then rolled between the two surfaces (the edge of the cover glass must be drawn over these egg chambers to disrupt efficiently the chorion and vitelline membranes). The density of egg chambers on the slide is critical to efficient removal of vitelline membranes: at low density the oocytes are easily destroyed, whereas at high density the egg chambers do not roll, and membranes are not efficiently removed. "Rolled" egg chambers were rinsed into a 10 × 75 mm test tube with PBST and treated with 1% Triton X-100 in PBS for 1 to 2 hours. The egg chambers were then rinsed twice in PBST before staining for microscopy.

Microtubules were stained either with a monoclonal anti-tubulin antibody that had been directly conjugated with rhodamine (see below) or with an affinity-purified, rabbit polyclonal anti-maternal -tubulin antibody, followed by a fluorescent secondary antibody (Theurkauf, 1992). Nuclei were visualized either with 4,6-diamino-2-phenylindole (DAPI), or immunolabeled using a monoclonal anti-histone antibody (Chemicon Inc., Temecula, CA), followed by a fluorescent secondary antibody. Actin filaments were labeled with rhodamine- or fluorescein-conjugated phalloidin (Sigma, St. Louis, MO).

For antibody staining, the egg chambers were transferred to 0.5 ml Eppendorf tubes and resuspended in 0.5 ml PBST containing one of the above anti-tubulin antibodies (final concentration of 5 µg/ml), or monoclonal anti-histone (1:500 dilution). After incubation overnight at 4°C with slow rotation, the stained egg chambers were rinsed four times for 15 minutes in PBST at room temperature. Anti-histone and anti-maternal -tubulin labeled egg chambers were further incubated (either for 2 hours at room temperature or overnight at 4°C) in a 1:500 dilution of rhodamine-labeled goat anti-mouse or goat anti-rabbit secondary antibody (Cappel). These secondary antibodies had been preabsorbed against fixed *Drosophila* embryos, as previously described (Karr and Alberts, 1986). The fluorescently labeled egg chambers were then rinsed four times for 15 minutes in PBST.

To further stain any of the above preparations, PBST was removed and PBST containing fluorescein-labeled phalloidin (1 µg/ml) and DAPI (1 µg/ml) was added. After 10 minutes, egg chambers were rinsed four times for 15 minutes in PBST, followed by two rinses in PBS. The labeled egg chambers were resuspended in mounting medium (1 mg/ml p-phenylene diamine, 90% glycerol, in PBS), transferred to slides, and sealed under a cover

glass with nail polish. Alternatively, labeled egg chambers were dehydrated in three changes of 100% methanol (5 minutes each) and transferred to a 2:1 mixture of benzyl benzoate: benzyl alcohol. This mixture matches the index of refraction of the yolk, clearing the egg chambers. Cleared egg chambers were also transferred to glass slides and sealed under a cover glass with nail polish.

Rhodamine conjugation of anti-tubulin antibodies

Rhodamine-conjugated anti- α -tubulin antibody was prepared as follows: 3 mg of bovine brain tubulin, isolated as described previously (Mitchison and Kirschner, 1984), was coupled to 1 ml packed volume of cyanogen bromide-activated Sepharose (Sigma Chemical, St. Louis). 1 ml of the resulting tubulin-Sepharose was transferred to a 5 ml column and equilibrated with PBST. The column outlet was sealed and 500 μ g of monoclonal anti- α -tubulin antibody (Blöse et al., 1984) in 1 ml PBS was mixed with the tubulin-Sepharose. The inlet of the column was then sealed and the slurry in the sealed column was incubated for 1 hour at room temperature with constant end-over-end rotation. The Sepharose was allowed to settle and the column was washed with PBS until protein was no longer detectable in the eluate. The column outlet was again sealed and 1 ml of PBS containing 4 μ l of 50 mg/ml 5-(and -6)-carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes Inc.) was added. The Sepharose was resuspended in this mixture, the inlet sealed, and the reaction allowed to proceed for 30 minutes at room temperature with constant rotation. The slurry was again allowed to settle and was washed with PBS until rhodamine was no longer detectable in the eluate. Labeled antibodies were eluted from the column with 0.1 M glycine, pH 2.5. Fractions of 0.4 ml were collected into 0.1 ml of 1 M Tris, pH 8.0. Protein-containing fractions were pooled, dialyzed into PBS, and concentrated using a Centricon 30 centrifugal concentrator (Amicon, Danvers MA). Aliquots were frozen in liquid nitrogen and stored at -80°C .

Results

Polarization of the cytoskeleton in the early oocyte-nurse cell complex

The 16-cell cysts that give rise to the oocyte and nurse cells are formed in the germarium, a specialized structure at the anterior tip of the ovariole that contains pre-follicular cells, the stem cells, and early germ cell-follicle cell cysts. Once the germ cell cysts become completely surrounded by a layer of somatic follicle cells they then pinch off from the germarium, and the resulting germ cell-follicle cell complex is referred to as an egg chamber. Oocyte differentiation occurs within the egg chamber, which matures as it moves down the ovariole toward the oviduct (for a review, see Mahowald and Kambyzellis, 1980). Oogenesis has been divided into 14 stages based on egg chamber morphology (King, 1957; Cummings and King, 1969). During stages 1 through 6, the nurse cell nuclei are transcriptionally active and become polyploid, while the oocyte nucleus proceeds through meiotic prophase and is transcriptionally inactive (King and Burnett, 1959). Oocyte growth during these stages appears to depend on accumulation of RNA that is synthesized in the nurse cells, and is inhibited by the microtubule-depolymerizing drug colchicine (Koch and Spitzer, 1983).

During stages 1 through 6, the oocyte-nurse cell synctium is filled with a microtubule network that is dominated by a prominent microtubule-organizing center (MTOC) that is located in the oocyte (Fig. 1A and C, arrows). In stage 1 oocytes this MTOC is located near the ring canals at the anterior pole of the oocyte (Fig. 1A), and microtubules from this MTOC extend through the ring canals into the nurse cells. During this early stage it appears that the majority of the microtubules in the 16 cell cyst originate in the oocyte. During stages 2 through 6, the only clear MTOC is at the posterior pole of the oocyte, and microtubules still extend through the ring canals into the nurse cells (Fig. 1C). The nurse cells also contain microtubules that do not appear to originate in the oocyte during these stages, although MTOCs are not detected in these cells (Fig. 1C). Actin stains the ring canals that join adjacent cells in the cysts, and simultaneous staining of early egg chambers for actin and microtubules demonstrates that microtubules pass through the ring canals from the oocyte into the adjacent nurse cells (compare actin and microtubule distributions in Fig. 1A and A', C and C').

Actin filament organization during early oogenesis distinguishes the oocyte from the nurse cells, but does not readily suggest a role for actin in transport within the oocyte-nurse cell complex (or other specific functions). As previously described (Warn et al., 1985), the most striking actin-containing structures early in oogenesis are the ring canals (Fig. 1A', B', C'). Ring canal size is polarized within the oocyte-nurse cell complex: the largest canals are those associated with the oocyte, and canal size progressively decreases with increasing distance from the oocyte (Fig. 1C'). There is also a continuous network of actin filaments underlying the plasma membranes of both nurse cells and oocyte.

Anterior-posterior asymmetry in the oocyte cytoskeleton

During stages 7 through 10, morphogenetic determinants that specify the anterior and posterior poles of the embryo become asymmetrically localized within the oocyte (for reviews, see Nusslein-Volhard and Roth, 1989; Manseau and Schupbach, 1989). As observed in earlier egg chambers, actin is restricted to the ring canals and cortex of the oocyte and nurse cells during stages 7 through 10, and is not organized in a manner that suggests an obvious function in the localization of determinants. However, microtubule organization in the oocyte changes dramatically at this time. During stages 7 and 8 microtubule density at the posterior pole of the oocyte decreases, and microtubules accumulate at the anterior cortex of the oocyte (compare Fig. 2A, arrow, with 2B, arrows). These microtubules are initially concentrated at the anterior margin of the oocyte, where the oocyte, the nurse cells, and follicle cells meet (Fig. 2B, arrows). Later, by stage 9 and 10a, a broader anterior-to-posterior cortical gradient develops (Fig. 2C, D). Development of structural asymmetry within the oocyte cytoskeleton is therefore concomitant with the localization of determinants that specify the anterior-posterior axis of the embryo.

The anterior-to-posterior gradient in microtubule density that develops during stages 7 through 10 is most clearly

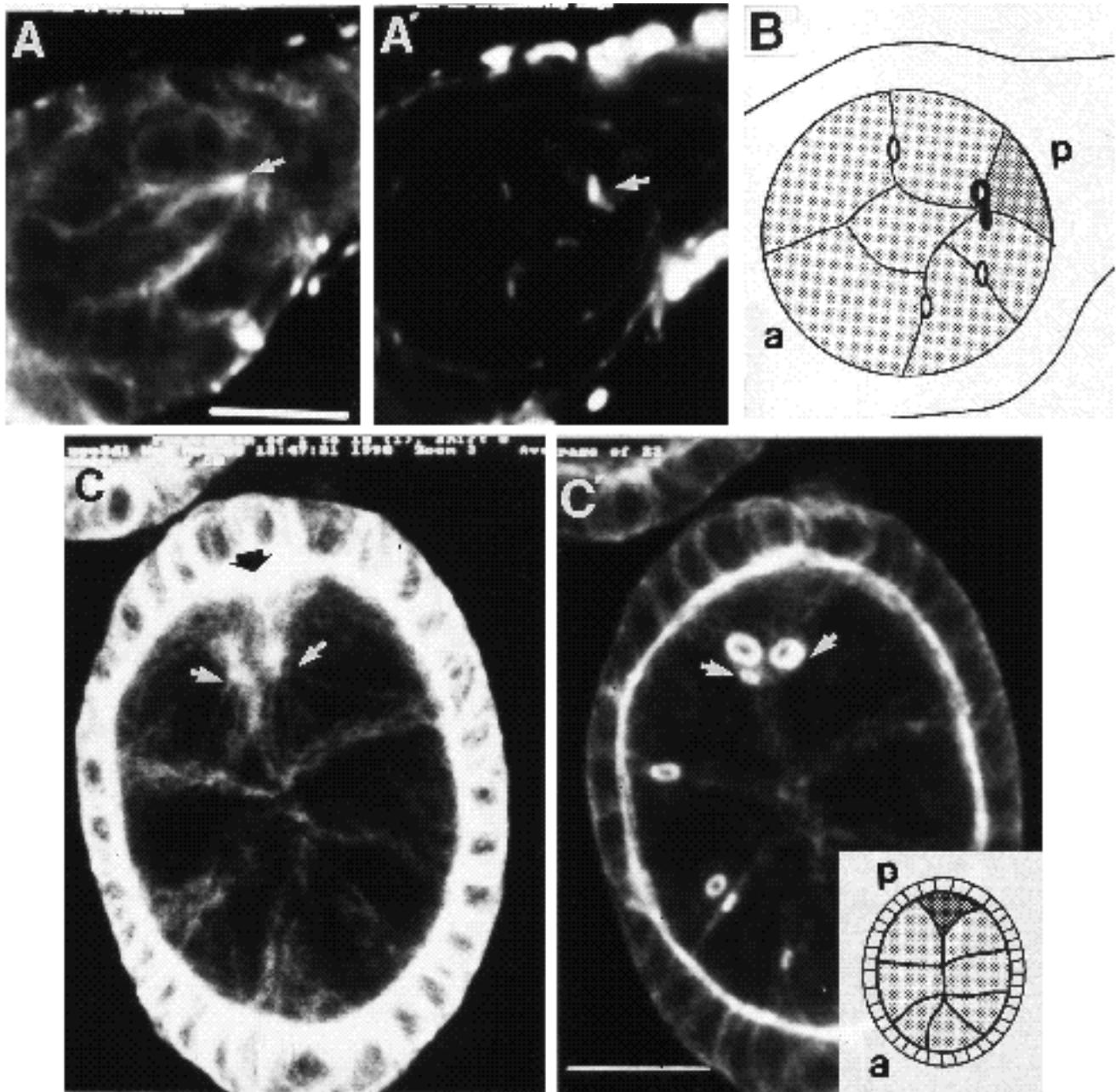


Fig. 1. Microtubule and actin filament organization in stage 1 through 5 egg chambers. (A and A') Microtubules (A) and actin (A') in a stage 1 egg chamber. At this early stage, microtubules are most concentrated at the anterior pole of the oocyte, near the ring canals (arrow), and extend through the entire oocyte-nurse cell complex. (B) Diagram showing the cellular organization of the egg chamber in A and A'. The follicle cells are unshaded, the nurse cells are light grey, and the oocyte is dark grey. The anterior (a) and posterior (p) poles are indicated. (C and C'). Microtubule (C) and actin (C') distributions in a stage 5 egg chamber. The inset in C diagrams the positions of follicle cells, nurse cells, and oocyte in this egg chamber, and is shaded as described above for B. Ring canals associated with the oocyte and microtubules that appear to pass from the oocyte into the nurse cells are indicated by the white arrows. Beginning at stage 2 to 3, microtubules are most concentrated at the posterior pole of the oocyte (black arrow). Microtubules were labeled with a rhodamine-conjugated monoclonal anti- β -tubulin antibody that stains follicle cell, nurse cell and oocyte microtubules. Actin filaments were labeled with fluorescein-conjugated phalloidin. The images are linear projections of 5 optical sections taken at 1 μ m intervals on the BioRad MRC 600 scanning confocal microscope. The "projection" program supplied with this microscope was used to generate the images. Bars, 10 μ m.

visualized with an anti-maternal β -tubulin antibody that recognizes a germ line-specific β -tubulin isotype. Because this antibody does not label the microtubules in the follicle

cells that overlie the oocyte, it allows clearer visualization of germline microtubules. In the germline, the maternal β -tubulin colocalizes with the constitutive β -tubulin isotype,

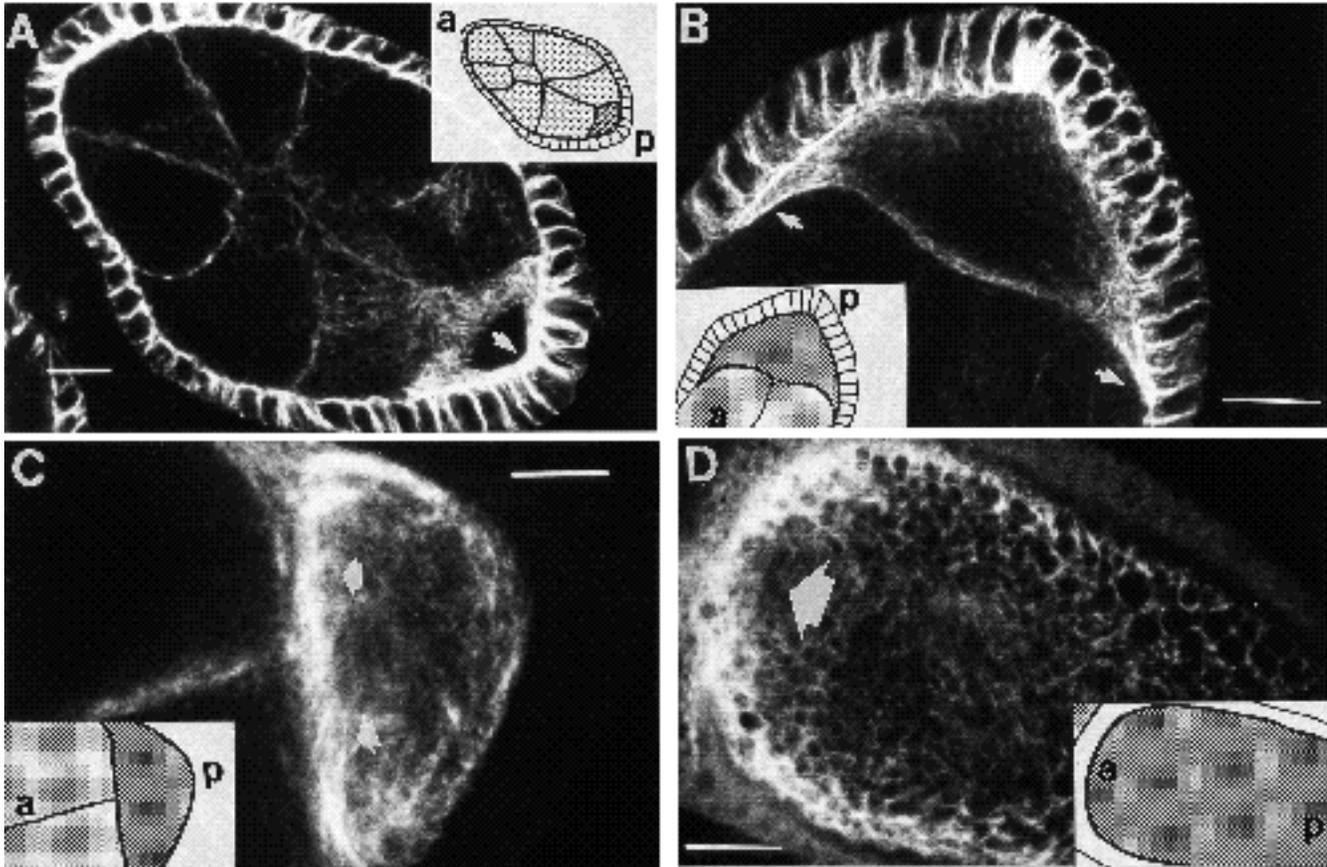


Fig. 2. The major center for microtubule organization shifts from the posterior to the anterior pole during stages 7 through 10a. (A) Stage 6 egg chamber labeled with an antibody that reacts with all α -tubulins shows microtubules concentrated at the posterior pole of the oocyte (arrow). (B) Stage 7 egg chamber labeled as in A shows loss of posterior microtubule concentration and accumulation of microtubules at the anterior margin of the oocyte, in the region where the oocyte, nurse cells, and follicle cells meet (arrows). (C) Microtubules in a stage 8 egg chamber labeled with an anti-maternal α -tubulin antibody. Microtubules are concentrated along the entire anterior surface of the oocyte (arrows). (D) Microtubules in a stage 10a egg chamber labeled with the anti-maternal α -tubulin antibody. Microtubules are most concentrated at the anterior cortex (arrow). The insets diagram the locations of follicle cells (unshaded), nurse cells (light grey) and the oocyte (dark grey) in each panel. The anterior (a) and posterior (p) poles are also indicated. Microtubules in A and B are labeled with rhodamine conjugated anti- α -tubulin. Microtubules in C and D were labeled with an anti-maternal α -tubulin antibody that stains the microtubules in the oocyte and nurse cells, but not the microtubules in the follicle cells. Confocal micrographs were obtained as described in Fig. 1; in C and D, the oocyte nucleus is out of the plane of focus. Bars, 10 μ m.

so that anti-maternal isotype labeling reveals total microtubule polymer (Theurkauf, 1992). This antibody reveals the anterior accumulation of cortical microtubules in stage 8 oocytes (Fig. 2C), and a gradient of cortical microtubules at stage 10a (Fig. 2D).

The first cytological indication of dorsal-ventral asymmetry develops during stages 8 and 9, as the oocyte nucleus migrates from a central position to the dorsal surface near the anterior pole (Mahowald and Kambyzellis, 1980). Colchicine treatment causes dissociation of the oocyte nucleus from the dorsal surface, suggesting that microtubules are required to maintain this asymmetric localization (Koch and Spitzer, 1983). In a subset of stage 8 oocytes, we observe microtubules extending from one side of the oocyte to surround the centrally located nucleus (Fig. 3B). After the nucleus has moved to the dorsal surface it is surrounded by a "cage" of microtubules (Fig. 3C). These two observations raise the possibility that microtubules that

originate at the future dorsal surface surround the centrally located nucleus and then mediate the movement of the nucleus to that site.

Cytoplasmic movements

Two independent forms of cytoplasmic movement occur during stages 10b through 12: a cytochalasin-sensitive transfer of nurse cell cytoplasm into the oocyte and a colchicine-sensitive ooplasmic streaming (Gutzeit, 1986a,b). Cytoplasmic actin fibers that link nurse cell nuclei to adjacent plasma membranes assemble at the time that a bulk flow of cytoplasm to the oocyte begins (Gutzeit, 1986a). The distribution of actin filaments and cytoplasmic myosin in nurse cells during cytoplasmic transfer, described elsewhere (Theurkauf, unpublished data), suggests that contraction of the cortical actin filament network beneath the nurse cell plasma membrane drives the transfer of nurse cell cytoplasm, with the cytoplasmic actin fibers holding

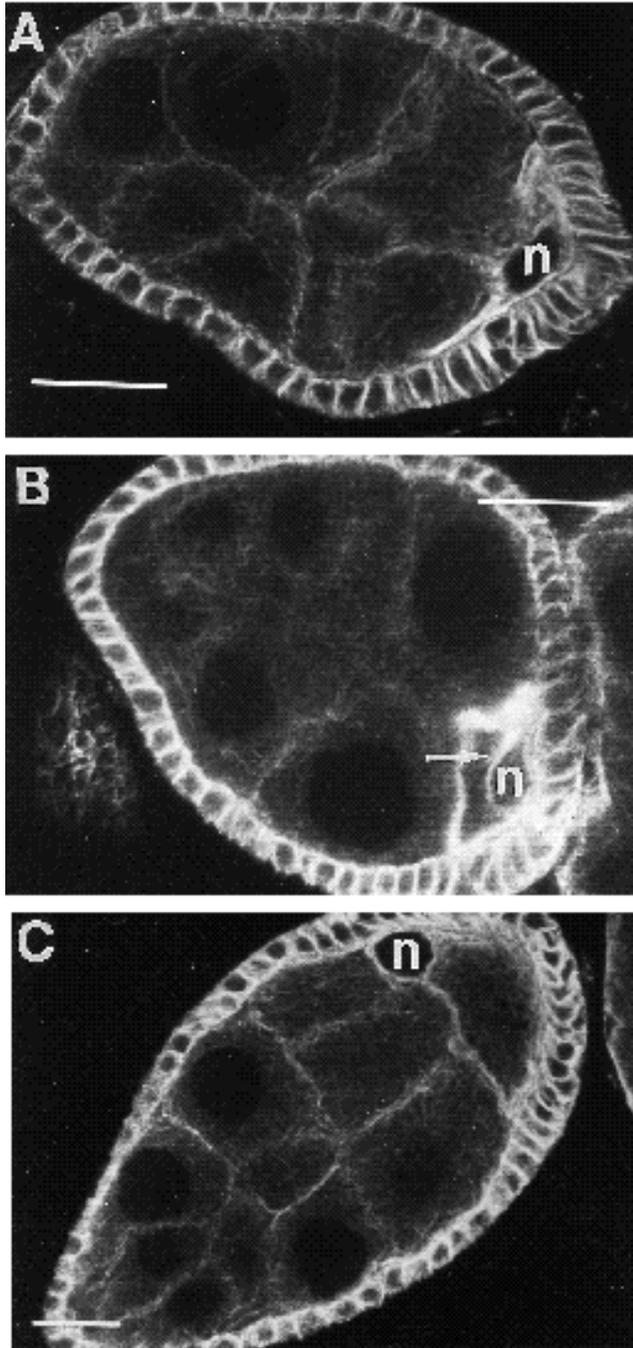


Fig. 3. Microtubule organization during movement of the oocyte nucleus to the dorsal surface. The oocyte nucleus (n) is positioned in the center of the oocyte from stage 1 through stage 7. During stage 8 the nucleus moves to the dorsal surface at the anterior of the oocyte. (A) Microtubule organization in a stage 7/early stage 8 egg chamber: the nucleus is centrally located. (B) A stage 7 or early stage 8 egg chamber, just prior to dorsal repositioning. Microtubules from one side of the oocyte surround the centrally located nucleus in this egg chamber (arrow). (C) A stage 8 egg chamber in which the nucleus is positioned at the dorsal surface, where it is surrounded by microtubules. Microtubules were labelled as described in the legend to Fig. 1. Bars, 10 μm .

the nurse cell nuclei in place and preventing them from entering and blocking the ring canals.

The polarized cortical microtubule network first established in the oocyte during stage 7 is maintained until stage 10a (Fig. 4A). Microtubules in the oocyte reorganize dramatically as ooplasmic streaming begins during stage 10b, however, when parallel arrays of microtubules appear 5 to 10 μm below the surface (Figure 4B, C). Optical sectioning of a stage 10b oocyte (Fig. 4C) demonstrates that these microtubule arrays are present under most of the oocyte surface. The subsurface microtubules appear to arise from the anterior cortex (Fig. 4B, arrow, also see inhibitor studies below), although some microtubules originating over the remainder of the cortex also appear to join the subsurface arrays (Fig. 4B). As described below, these subcortical microtubule arrays will later disassemble as streaming stops and the oocyte matures.

The temporal correlation between the presence of subcortical microtubule arrays and ooplasmic streaming, plus the colchicine sensitivity of this process (Gutzeit, 1986b), strongly suggest that the subcortical microtubules play a direct role in this cytoplasmic movement. To gain further insights into the streaming mechanism, we have examined stage 10b and 11 oocytes at higher magnification, using the electron microscope (Fig. 5). This analysis reveals a population of nonstaining vesicles that are specifically excluded from the region between the subcortical microtubules and the oocyte surface (Fig. 5A). Some of vesicles are closely associated with the subcortical microtubules (Fig. 5B and C). Exclusion of the nonstaining vesicles from the cortical cytoplasm is particularly noticeable at the anterior pole (Fig. 5A), which contains the highest concentration of microtubules extending from the surface into the cytoplasm (Fig. 4B, and see below for additional examples). Mitochondria and yolk granules are not excluded from the cortical region and are not found closely opposed to microtubules. Exclusion from the cortical cytoplasm and close association with microtubules, therefore, appear to be properties specific to the nonstaining vesicles.

Oocyte maturation

During stages 13 and 14, the oocyte matures and arrests in metaphase of the first meiotic division. A number of biochemical and structural changes accompany maturation: the oocyte shrinks as it dehydrates, the nuclear envelope breaks down and the spindle assembles, and protein synthesis slows (reviewed by Mahowald and Kambyzellis, 1980).

Cytoplasmic actin and microtubule organization also undergo dramatic changes as the oocyte matures. During stages 10b through 12, subcortical microtubules are present under most of the surface (Fig. 4B; Fig. 6A). After maturation the subcortical microtubules are replaced by short, randomly oriented filaments (Fig. 6B). These short microtubules are present throughout the cytoplasm, although their density appears somewhat higher near the cortex (Fig. 6F). Meiotic spindle assembly occurs during maturation by an unusual process described in detail elsewhere (Theurkauf and Hawley, 1992). Actin also reorganizes during maturation. In oocytes that have not matured, a dense actin network is present at the cortex, with punctate phalloidin-staining material deeper in the ooplasm (Fig. 6C). Mature stage

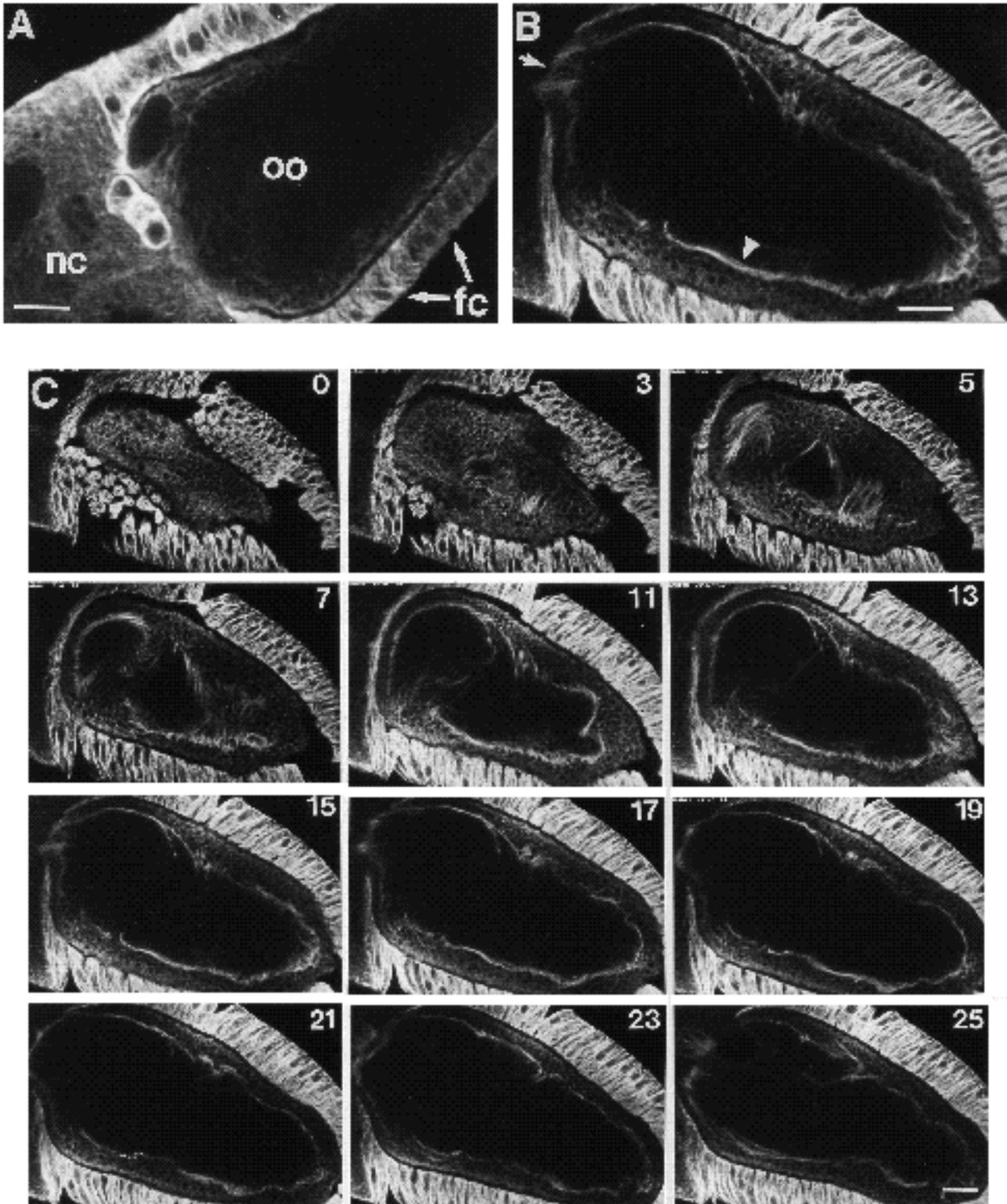


Fig. 4. Subcortical microtubule arrays assemble during ooplasmic streaming. (A) Microtubules in a stage 10a oocyte (oo), prior to the onset of ooplasmic streaming; follicle cells (fc) are marked with arrows. The nurse cell (nc) complex is at the lower left in this micrograph. (B) Subcortical microtubule arrays (arrowhead) in a stage 10b oocyte. Microtubules appear to interact directly with the oocyte surface at the anterior pole (arrow). (C) Optical sections of the stage 10b oocyte shown in (B). The distance below the oocyte surface, in microns, is indicated in the upper right hand corner of each frame. Large numbers of microtubules interact with the cortex at the anterior pole. Bars, 10 μ m. Microtubules were labelled as described in Fig. 1; thus, both follicle cell and germ line cell microtubules are stained.

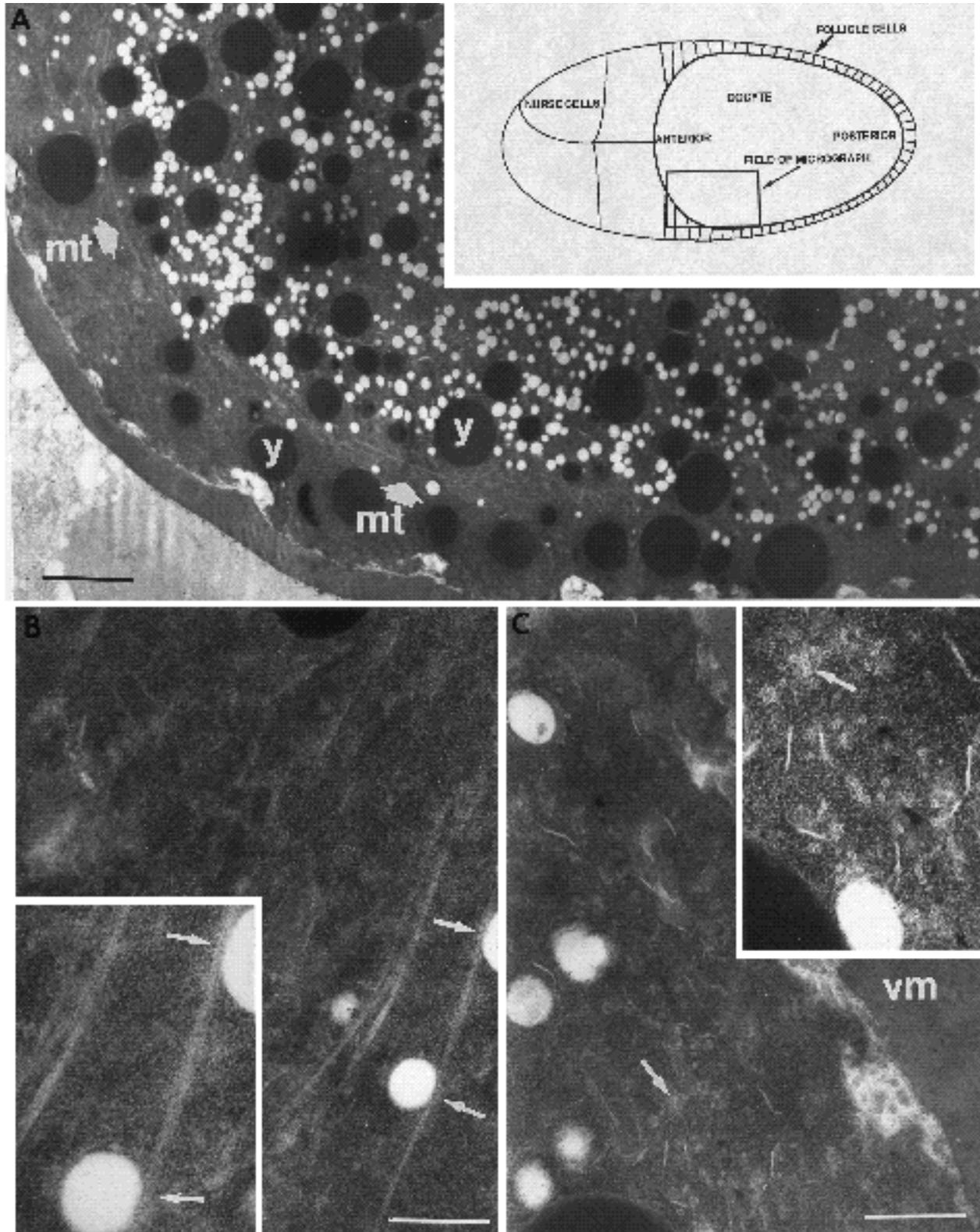


Fig. 5. Electron microscopic analysis of a stage 10b egg chamber. (A) Low magnification view. Microtubules run parallel to the oocyte surface (mt, arrows). Abundant nonstaining vesicles are excluded from the region of cytoplasm between the surface and the subcortical cortical microtubule bundles. Large yolk granules (y), in contrast, are found on both sides of the subcortical microtubules. (B) High magnification views showing the lateral association of vesicles with microtubules (arrows). (C) Cross section showing that the subcortical microtubules are restricted to a narrow band and are often tightly packed together (arrow); the vitelline membrane is also marked (vm). The inserts in B and C are higher magnification views of regions of each panel, showing microtubules associated with vesicles (B) and bundles of microtubules (C). Bars: A, 1 μ m; B,C, 300 nm.

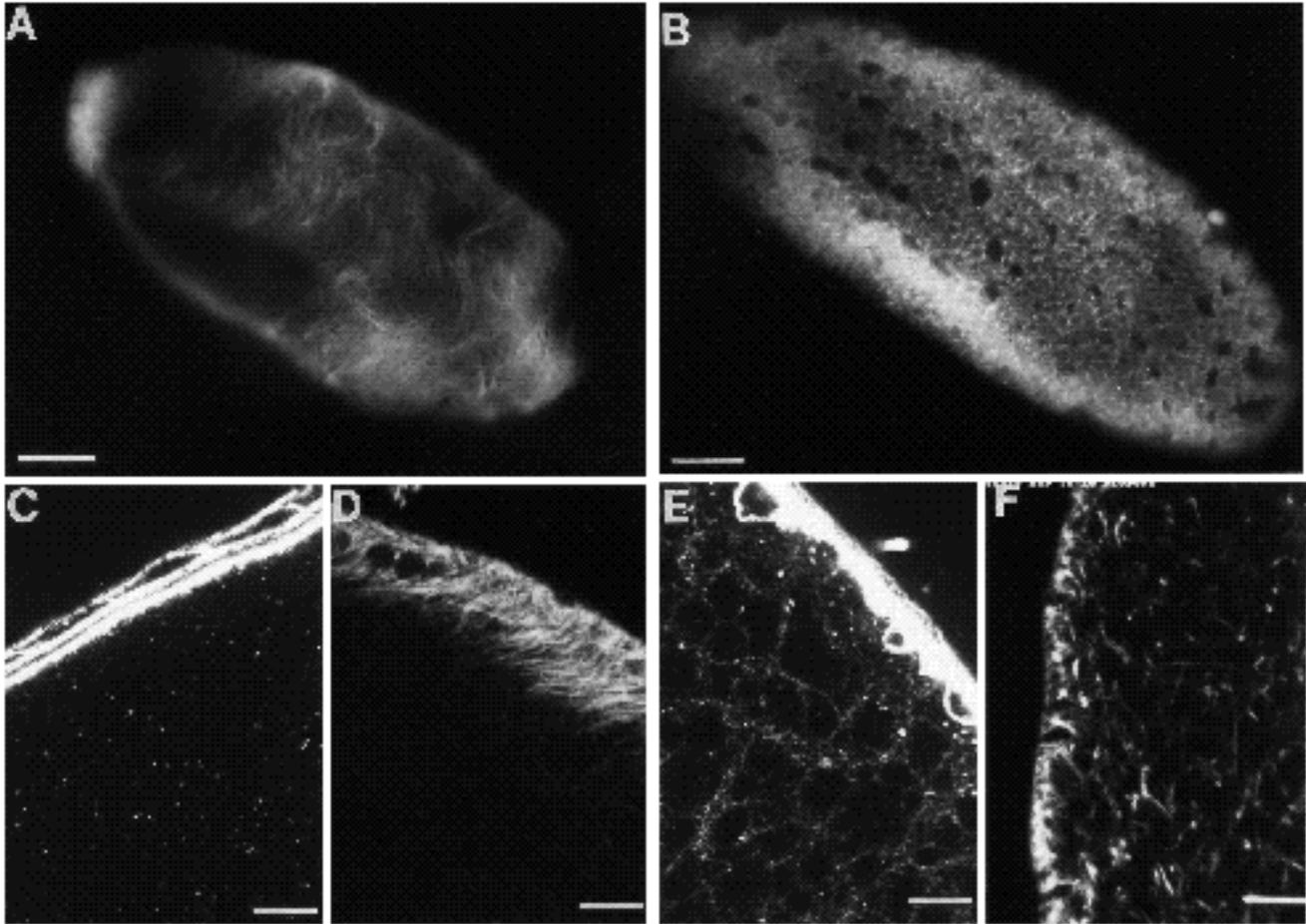


Fig. 6. Microtubule reorganization during oocyte maturation. (A) Microtubules in a stage 12/13 oocyte with follicle cells and vitelline membranes removed; long subcortical microtubules spiral around the oocyte. (B) Microtubules in a mature stage 14 oocyte arrested in metaphase of the first meiotic division; short microtubules are present throughout the oocyte. (C) Optical section of a stage 13 oocyte showing a dense layer of cortical actin filaments and punctate phalloidin-staining in the cytoplasm. (D) Optical section showing microtubules near the anterior cortex of a stage 13 oocyte. Microtubules interact end-on with the actin rich cortex. (E) Optical section showing actin filaments in a stage 14 oocyte; an internal mesh of cytoplasmic actin filaments is present in addition to the cortical actin layer present during earlier stages. (F) Optical section showing microtubules in a stage 14 oocyte. Microtubules are somewhat more concentrated near the surface, but extend deep into the ooplasm. A and B are conventional epifluorescence images. C through F are laser scanning confocal microscopic images. Microtubules and actin filaments were visualized as described in Fig. 1. Bars: A and B, 50 μm ; C-F, 10 μm .

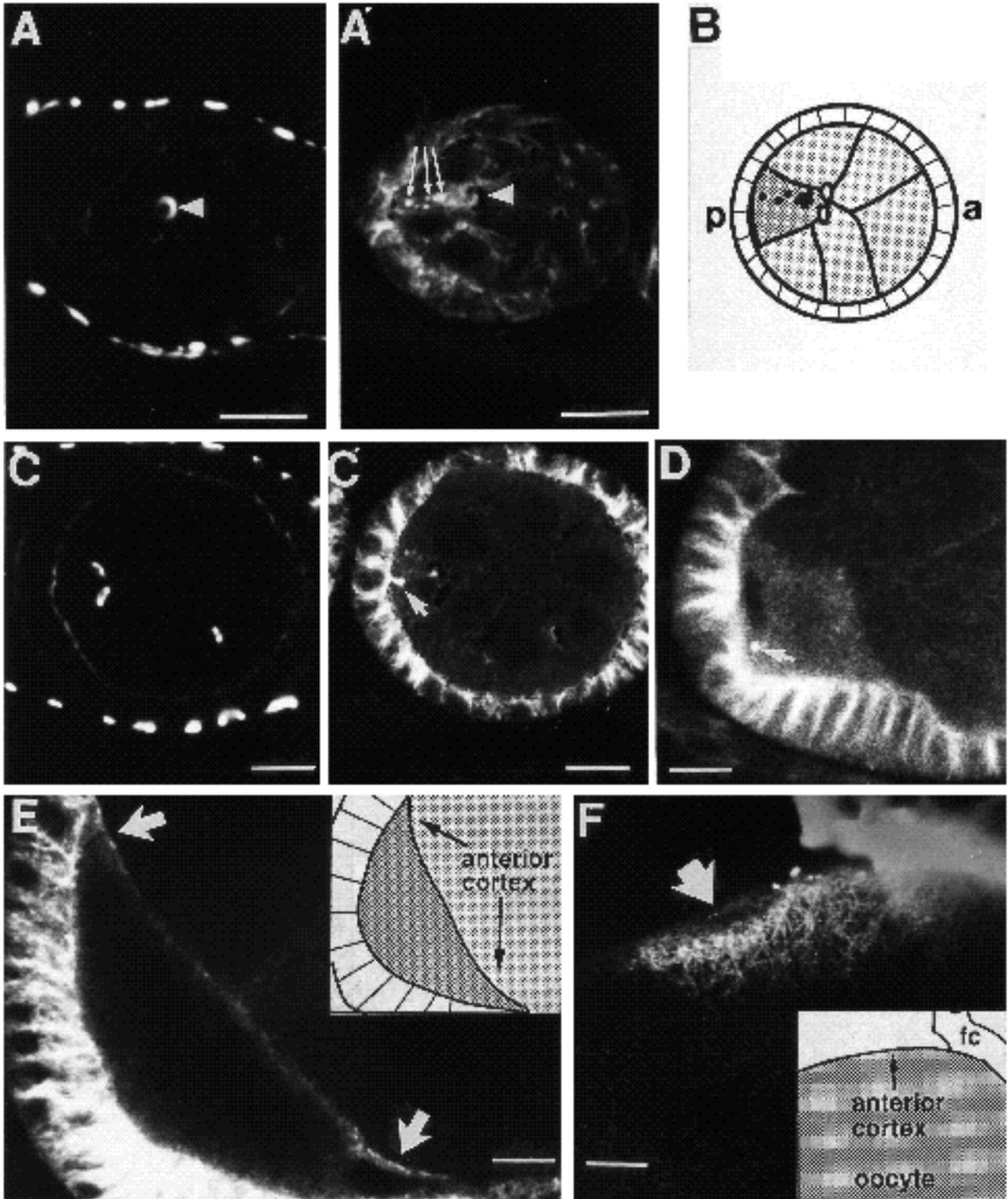
14 oocytes, in contrast, contain an extensive network of actin filaments deep in the cytoplasm in addition to the layer of cortical actin filaments (Fig. 6E).

Microtubule inhibitor studies

The cytological observations described here, combined with the biological effects of microtubule depolymerization, indicate that microtubules are involved in diverse functions during oogenesis. To examine these microtubules further, we have used brief treatments with microtubule-assembly inhibitors just prior to fixation and staining. Because microtubules are normally nucleated at their minus ends and polymerize and depolymerize from their plus ends, such a brief treatment with a depolymerizing drug can leave short microtubules associated with the nucleation site. These experiments, therefore, provide information on microtubule nucleation sites in the oocyte, as well as probing for the

possible existence of a population of unusually stable microtubules.

In the experiment shown in Fig. 7, egg chambers were isolated and cultured *in vitro* in the presence or absence of the microtubule inhibitor colcemid, for 10 to 30 minutes, prior to being fixed and stained with anti-tubulin antibodies. The somatic follicle cells are rich in colcemid-resistant microtubules. In addition, a few microtubules are present in the oocyte-nurse cell complex of stage 1 oocytes treated with colcemid for 10 minutes (Fig. 7A). The most striking feature of inhibitor-treated stage 1 cysts, however, are clusters of tubulin-staining particles in the oocyte. These particles are most abundant near the ring canals at the anterior pole in stage 1 egg chambers, but they are also often observed between the anterior and posterior poles during this stage (Fig. 7A, arrows; diagrammed in Fig. 7B). In stage 2 through 6 egg chambers, similar tubulin-containing



particles are invariably located at the posterior pole (Fig. 7C, D). In stage 7 through 12 egg chambers these particles are no longer present (Fig. 7E); however, short microtubules are detectable at the anterior cortex after inhibitor treatment during these stages (Fig. 7E, F). Similar cortical microtubules are not observed in earlier egg chambers

treated in the same manner (Fig. 7A, C, D). A longer colcemid or colchicine treatment completely abolishes the anti-tubulin staining of the oocyte, eliminating the particles present during stages 1 through 6, as well as the short microtubules associated with the anterior cortex during stages 7 through 11 (data not shown).

Fig. 7. Microtubules in inhibitor-treated egg chambers. Egg chambers were isolated and cultured for 10 to 20 minutes in the presence of colcemid (20 $\mu\text{g/ml}$), colchicine (20 $\mu\text{g/ml}$), or with carrier dimethyl sulfoxide alone, and then fixed and labeled to display the location of actin filaments and microtubules. Control incubation of egg chambers without inhibitors did not alter microtubule or actin filament organization relative to samples fixed immediately after isolation (not shown). (A) Actin and (A) microtubule organization in a stage 1 egg chamber treated with colcemid for 10 minutes prior to fixation. Some microtubules originating near the anterior pole of the oocyte extend into the nurse cells. A number of anti-tubulin staining particles, which appear to be centrosomes, are also present in the oocyte (arrows). The position of one of the four large ring canals at the anterior pole of the oocyte is indicated by the arrowhead. (B) Diagrammatic representation of the egg chamber shown in A and A. Cell types are indicated by shading as described in the legend to Fig. 1, with apparent MTOCs indicated by black circles. The anterior (a) and posterior (p) poles are indicated. (C) Actin and (C) microtubule organization in a stage 4 to 5 egg chamber treated with colcemid for 10 minutes before fixation. Tubulin-staining particles are concentrated at the posterior pole of the oocyte (arrow). (D) Microtubules in a stage 6 egg chamber treated with colcemid for 10 minutes. Tubulin-staining particles are present at the posterior pole, but are somewhat removed from the cortex (arrow). (E) Microtubules in a stage 7 egg chamber treated with colcemid for 10 minutes before fixation. Short microtubules are associated with the anterior cortex (arrow), and the colcemid-stable particles present during earlier stages are no longer detected. (F) Microtubules associated with the anterior cortex of a stage 11 oocyte treated with colchicine for 20 minutes before fixation (arrow). The bright staining in the upper right corner of the micrograph is from a cluster of follicle cells (fc) that remained attached to the oocyte after a mechanical treatment removed the developing chorion and vitelline membranes. The inset diagrams in E and F show cellular organization and orientation of the egg chamber in these panels, and are shaded to indicate cell type as in Fig. 1. Microtubules were labeled and optical sections were obtained using a confocal microscope as described in Fig. 1.

In an attempt to examine the regrowth of microtubules after inhibitor treatment, and thus gain further information on microtubule polarity in the egg chamber, colcemid-treated egg chambers were washed and incubated in fresh medium prior to fixation. No microtubule repolymerization was observed (data not shown). The viability of these early egg chambers may be compromised during culture, however, making regrowth experiments difficult.

Discussion

Previous studies utilizing specific cytoskeletal inhibitors have implicated microtubules or actin filaments in a variety of developmentally important processes during *Drosophila* oogenesis. By this criterion, actin filaments are required for transfer of nurse cell cytoplasm to the oocyte (Gutzeit, 1986a), and microtubules play an important role in oocyte determination, oocyte growth, asymmetric positioning of the *bicoid* morphogen, and ooplasmic streaming (Koch and Spitzer, 1983; Pokrywka and Stephenson 1991; Gutzeit, 1986b). Understanding the precise role of the cytoskeleton in these processes requires high resolution

cytological data on the organization of the major cytoskeletal elements during oogenesis. We have therefore attempted to examine the actin filaments and microtubules in a systematic way. The present report deals primarily with microtubule organization and function during oogenesis. The role of actin filaments and cytoplasmic myosin in the transfer of nurse cell cytoplasm to the oocyte will be considered elsewhere (Theurkauf, in preparation).

Microtubules and oocyte growth

During the previtellogenic stages of oogenesis there is little detectable RNA synthesis in the oocyte, yet the oocyte grows and is translationally active (King and Burnett, 1959). Oocyte growth, therefore, appears to depend on the transport of maternal RNAs from the nurse cells to the oocyte. The mechanism of RNA transport during these

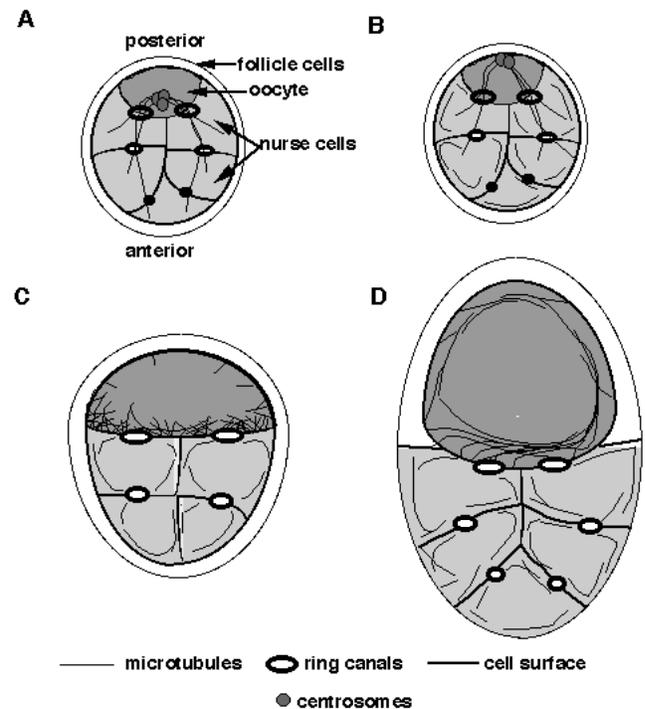


Fig. 8. Diagrammatic representation of the microtubule reorganization observed during *Drosophila* oogenesis. (A) Stage 1. Microtubules within the oocyte-nurse cell syncytium appear to be organized around a cluster of centrosomes (MTOC) located near the ring canals at the anterior pole of the oocyte. Microtubules extend from the oocyte into the nurse cell complex. (B) Stages 2 through 6. The centrosomes in the oocyte have shifted to the posterior pole. Microtubules extend from the oocyte into adjacent nurse cells, as in A. During these and subsequent stages the nurse cells also contain microtubules that do not appear to originate in the oocyte, and which do not focus on well-defined MTOCs. (C) Stages 7 through 10a. Centrosomes are no longer detectable at the posterior of the oocyte, and an anterior-to-posterior microtubule density gradient forms. The oocyte cortex appears to act as a MTOC and to organize microtubules, with the highest concentration of microtubule-nucleating activity associated with a broad region of the anterior pole. (D) Stages 10b through 12. Subcortical microtubule bundles assemble and ooplasmic streaming begins. The oocytes in A through D are not drawn to scale.

stages is not understood. However, the microtubule depolymerizing drug colchicine inhibits oocyte growth, suggesting that microtubules are involved (Koch and Spitzer, 1983). We have confirmed that colchicine inhibits oocyte growth, and found that the related inhibitor colcemid, as well as two pharmacologically distinct anti-microtubule drugs, vinblastine and podophylotoxin, also produce this phenotype (Theurkauf, unpublished data). It is therefore unlikely that a toxic side effect of colchicine causes growth inhibition. Rather, microtubule depolymerization appears to be specifically involved.

The organization of microtubules during stages 1 through 7 is consistent with a role for microtubules in vectorial transport within the oocyte-nurse cell cysts, as microtubules organized by a prominent MTOC in the oocyte extend through the ring canals into the adjacent nurse cells (Fig. 1, diagrammed in Fig. 8A and B). Because microtubules are intrinsically polar structures, this organization suggests that microtubules form a polarized scaffold that mediates intercellular transport. We speculate that maternal RNAs associate with microtubule motors and translocate along microtubules to the oocyte.

This speculation requires that the intercellular microtubules be of uniform polarity. Because microtubules are generally nucleated at their minus ends and polymerize and depolymerize from their plus end, forced microtubule depolymerization with specific inhibitors often leaves short microtubule remnants associated with the nucleation site. We have therefore used this technique to examine microtubule polarity in egg chambers. Short treatment of stage 1 through 6 egg chambers with the microtubule-depolymerizing drug colcemid leaves tubulin-staining particles in the oocyte that are in the same position as the MTOC in untreated egg chambers (compare Fig. 1 and Fig. 7). In addition, longer inhibitor treatments completely eliminate anti-tubulin staining in the germ line. These observations strongly suggest that the tubulin-staining structures present after short colcemid treatment are short microtubules associated with nucleating sites, so that the plus ends of microtubules would be directed away from the oocyte, and extend into the nurse cells during stages 1 through 6. If this proves to be the case, a minus end-directed microtubule motor, such as cytoplasmic dynein (Pascal et al., 1987), would be expected to mediate transport from the nurse cells to the oocyte.

Axis specification and reorganization of the oocyte cytoskeleton

Morphogenetic determinants accumulate at both the anterior and posterior poles of the oocyte during stages 7 through 9 (Ephrussi et al., 1991; Kim-Ha et al., 1991; Berleth et al., 1988; Lasko and Ashburner, 1990; Hay et al., 1990). Do microtubules mediate this process? Prior to stage 7, microtubules are concentrated around the posterior pole of the oocyte and extend through the ooplasm and into the adjacent nurse cells (Fig. 1 and Fig. 8B). During stages 7 and 8, however, the posterior organizing center is lost and microtubules associate instead with the cortex, with the highest concentration of cortical microtubules at the anterior pole (Fig. 2, and Fig. 8). Therefore, microtubule organization in the oocyte becomes polarized along the anterior-

posterior axis during the same stages in which molecular asymmetry is first detectable, consistent with a role for microtubules in establishing this asymmetry in the oocyte.

Inhibitor studies indicate that microtubules play a role in localizing the anterior morphogen *bicoid* mRNA (Pokrywka and Stephenson, 1991). Two alternative mechanisms could give rise to anterior localization of *bicoid* mRNA in the oocyte. Because *bicoid* mRNA is synthesized in the nurse cells and transferred to the oocyte through cytoplasmic bridges at the anterior pole, passive trapping of this mRNA by a cytoskeletal network as it enters the oocyte would lead to anterior localization. Alternatively, the cortex of the oocyte could be functionally polarized, and localization of *bicoid* mRNA could be mediated by a polar cortical structure. Two recent observations indicate that *bicoid* mRNA localization reflects functional cortex polarity. First, *bicoid* mRNA is localized to both the anterior and posterior poles of oocytes from females carrying the temperature-sensitive allele of *Notch* that are raised at the restrictive temperature (Ruohola et al., 1991). Posterior localization of *bicoid* mRNA in these oocytes cannot be explained by the trapping mechanism. Second, *bicoid* mRNA released into the ooplasm upon microtubule depolymerization returns to the cortex when the depolymerizing drug is removed (Pokrywka and Stephenson, 1991). These observations, combined with the cytological data presented here, strongly suggest that microtubules are actively involved in the movement of *bicoid* mRNA to the anterior pole.

The anterior-posterior asymmetry in cortical microtubule organization in stage 7 through 10 oocytes suggests several possible functions for microtubules in localization of morphogens. The gradient in microtubule density during stage 8 through 10 closely mirrors the distribution of *bicoid* mRNA in the oocyte (Berleth et al., 1989); thus, microtubule binding alone could be the basis for *bicoid* localization. Alternatively, *bicoid* mRNA could be translocated along microtubules to the cortex. The polarity of the cortical microtubules will determine the type of motor activity that such a translocation process would utilize. Inhibitor studies show that short microtubules remain associated with the anterior cortex after brief colcemid treatment only during stages when cortical microtubules are concentrated at the anterior (Fig. 8D), suggesting that the anterior cortex nucleates microtubule assembly, and that the minus ends of microtubules therefore associate with the anterior cortex. If this is the case, minus end-directed movement of *bicoid* mRNA along microtubules could lead to anterior localization.

The mechanism by which posterior morphogens accumulate at the posterior pole of the oocyte is not known. The pattern of *oskar* mRNA localization, however, shares two features with the pattern of microtubule reorganization. First, *oskar* mRNA localization is first detectable during stage 7 or 8, the same stages in which microtubules reorganize to form an anterior-to-posterior gradient (Fig. 2). Second, *oskar* message initially accumulates at the anterior margin of the oocyte, before being found exclusively at the posterior pole (see Kim-Ha et al., 1991, Fig. 3). Early microtubule accumulation at the anterior pole of the oocyte is also first detected at the anterior margin (Fig. 2B, arrows). Based on these similarities, we speculate that *oskar* mRNA

binds to the minus ends of microtubules at the anterior cortex, and then translocates toward the posterior along microtubules. This would account for the initial accumulation of *oskar* message at the anterior pole, followed by its accumulation at the posterior pole.

A possible mechanism for microtubule reorganization during stages 7 through 10 is suggested by the results of inhibitor studies. These studies reveal that the tubulin-staining particles at the posterior pole of stage 1 through 6 colcemid-treated oocytes, which are likely to represent the microtubule nucleating sites in these oocytes, are absent during stages 7 through 11 (Fig. 7). Short microtubules are associated with the anterior cortex in stage 7 through 11 egg chambers treated with colcemid, however, suggesting that microtubules are nucleated at the anterior cortex during these stages. We suggest that the microtubule reorientation during stages 7 through 10 is driven by a redistribution of microtubule-nucleating activity from the posterior MTOC to the anterior cortex.

The role of microtubules in ooplasmic streaming

Toward the end of oogenesis the bulk of the nurse cell cytoplasm is transferred to the oocyte and the ooplasm streams (Gutzeit and Koppa, 1982). Ooplasmic streaming, which appears to be required to mix the incoming cytoplasm with the pre-existing ooplasm, is inhibited by microtubule depolymerizing drugs (Gutzeit, 1986b). The microtubules that mediate this process have not been previously identified. During stage 10, as ooplasmic streaming begins, we observe assembly of subcortical microtubules below most of the oocyte surface (Fig. 4). These microtubules disassemble as streaming stops and the oocyte matures (Fig. 6). Formation and disassembly of subcortical microtubules are therefore temporally coordinated with the onset and cessation of streaming. In addition to this temporal correlation, real time analysis of ooplasmic streaming demonstrates that a region of the cytoplasm approximately 5 μm below the oocyte surface does not stream (Gutzeit and Koppa, 1982). The subcortical microtubules are located from 5 to 10 μm below the surface (Fig. 4), and are therefore present at the boundary between the streaming cytoplasm and the stationary subcortical cytoplasm. Finally, the pattern of streaming varies from egg chamber to egg chamber, and changes within individual egg chambers as streaming proceeds (Gutzeit and Koppa, 1982). We find that the pattern of subcortical microtubules also varies from oocyte to oocyte. In the example shown in Fig. 6A, the microtubules appear to spiral around the oocyte. In other examples, the microtubules appear to swirl, with regions of the oocyte containing microtubules running along the anterior-posterior axis. Based on these observations, we propose that the subcortical microtubules mediate ooplasmic streaming.

Ultrastructural analysis of stage 10b and 11 oocytes suggests that microtubules may serve as a scaffold that supports vesicle movement. The oocyte is filled with non-staining vesicles of an unknown nature, and these vesicles are excluded from the region between the oocyte surface and the subcortical microtubule arrays during stages 10b and 11 (Fig. 6). Yolk granules and mitochondria, in contrast, are not excluded from this cortical zone, suggesting that these organelles may be specifically transported away

from the cortex. These non-staining vesicles are often intimately associated with subcortical microtubules (Fig. 6), suggesting that they interact with microtubules at the molecular level. These observations raise the possibility that vesicles translocate away from the plasma membrane along microtubules originating at the cortex, and that ooplasmic streaming is driven by vesicle movement parallel to the surface along the long subcortical microtubules.

Maturation induces reorganization of the cytoskeleton

Oocyte maturation in *Drosophila* involves a series of biochemical and cytological changes, including completion of the chorion, follicle cell degeneration, a decrease in protein synthesis, and arrest in metaphase of the first meiotic division. The organization of actin filaments and microtubules also changes dramatically on maturation. Stages 1 through 13 oocytes contain a dense layer of cortical actin filaments, but only punctate phalloidin staining material in the cytoplasm. Stage 14 oocytes, however, contain in addition an extensive network of actin filaments deep in the cytoplasm (Fig. 6). Microtubules also reorganize dramatically as the oocyte matures. The subcortical arrays present during cytoplasmic streaming are replaced by short microtubules that fill the cytoplasm (Fig. 6F), the meiotic spindle assembles (Theurkauf and Hawley, 1992), and the axial specialization of microtubule organization that is prominent in earlier oocytes is no longer apparent. Most microtubules in the mature oocyte do not interact with the cortex, and appear to begin and end within the cytoplasm.

The functional consequences of maturation-induced actin and microtubule reorganization are unclear. The loss of axial microtubule organization on maturation, however, indicates that the mechanisms described above for microtubule-mediated positioning of morphogens cannot account for the maintenance of molecular asymmetry in the mature oocyte. We therefore speculate that microtubules are involved in moving and transiently positioning morphogens within the oocyte, but that other structural elements are involved in long-term stabilization of the asymmetric distributions. Consistent with this hypothesis, a substantial fraction of *bicoid* mRNA remains associated with the anterior cortex after egg chambers are treated with colchicine (Theurkauf and T. Jongens, unpublished data; also see Pokrywka and Stephenson, 1991, Fig. 3A). A similar two step process appears to function in localization of the Vg1 mRNA to the vegetal hemisphere of *Xenopus* oocytes. In this system microtubules are required to move Vg1 mRNA to the vegetal pole, and actin filaments are required to maintain this asymmetric location (Yisraeli et al., 1990).

The temporally-regulated reorganization of microtubules and actin filaments during oogenesis reported here have suggested roles for the cytoskeleton in several different, developmentally significant processes. These observations should also help in future analyses of the many known mutations that affect oocyte growth, oocyte differentiation, and axis specification.

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