Development 125, 3655-3666 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV6312

In vivo analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway

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Accepted 2 July; published on WWW 25 August 1998

SUMMARY

Anterior patterning of the *Drosophila* embryo depends on localization of *bicoid* (*bcd)* mRNA to the anterior pole of the developing oocyte, and *bcd* mRNA localization requires both the *exuperantia (exu)* gene and an intact microtubule cytoskeleton. To gain insight into the mechanism of anterior patterning, we have used time lapse laser scanning confocal microscopy to analyze transport of particles containing a Green Fluorescent Protein-Exu fusion (GFP-Exu), and to directly image microtubule organization in vivo. Our observations indicate that microtubules are required for three forms of particle movement within the nurse cells, while transport through the ring canals linking the nurse cells and oocyte appears to be independent of both microtubules and actin filaments. As particles enter the oocyte, a final microtubule-dependent step directs movement to the oocyte cortex. However, our observations and previous studies suggest that the polarity of the oocyte microtubule network is not in itself sufficient to generate anterior asymmetry, and that additional factors are required to restrict morphogens to the anterior pole. Based on these observations, we propose a multi-step anterior localization pathway.

Key words: Microtubule, Oogenesis, Ring canal, *Drosophila*, Cytoplasmic transport

INTRODUCTION

The axes of the *Drosophila* embryo are established during oogenesis, as key regulatory mRNAs and proteins are localized to the poles of the developing oocyte (reviewed by St Johnston and Nüsslein-Volhard, 1992). The *Drosophila* oocyte is transcriptionally inactive through much of oogenesis, and the majority of these mRNAs and proteins are synthesized in a cluster of 15 nurse cells that are linked to the oocyte by large cytoplasmic bridges called ring canals (reviewed by Mahajan-Miklos and Cooley, 1994). Axis specification in *Drosophila* depends on transport of these factors from the nurse cells to the oocyte, and subsequent localization and anchoring at the appropriate oocyte pole. The transport mechanisms that underlie axis specification are not well understood. We therefore initiated an analysis of cytoplasmic transport during *Drosophila* oogenesis, focusing on anterior localization of the *exuperantia* (*exu*) protein (Exu).

Anterior patterning of the *Drosophila* embryo requires anterior localization within the oocyte of a pool of mRNA encoding the Bicoid (Bcd) transcription factor (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988; reviewed by St Johnston, 1995; Lipshitz, 1995). After egg activation, translation of this localized mRNA produces an anterior to posterior transcription factor gradient that triggers the patterned expression of gap genes within the early embryo (reviewed by St Johnston and Nüsslein-Volhard, 1992). The *exu* gene is required during oogenesis for *bcd* mRNA localization to the anterior pole of the oocyte (Berleth et al., 1988; St Johnston et al., 1989; MacDonald et al., 1991; Marcey et al., 1991). A transgene producing a fusion of Exu and Green Fluorescent Protein (GFP-Exu) supports *bcd* mRNA localization and complements the embryonic anterior axis defects produced by *exu* mutations (Wang and Hazelrigg, 1994). During mid-oogenesis, the GFP-Exu fusion protein assembles into particles that are concentrated around the nurse cell nuclei, cluster at the ring canals that link the germline cells of the egg chamber, and accumulate at the anterior pole of the oocyte (Wang and Hazelrigg, 1994). At these stages, *bcd* mRNA also shows a perinuclear or apical distribution in nurse cells, and accumulates at the anterior cortex of the oocyte (St Johnston et al., 1989; Stephenson and Pokrywka, 1992). These observations suggest that the GFP-Exu particles are transport RNPs containing *bcd* mRNA, and that these particles are targeted to the anterior cortex of the developing oocyte (Wang and Hazelrigg, 1994). Although direct proof for an association of *bcd* mRNA with these particles has not yet been obtained, microtubule depolymerization disrupts both *bcd* mRNA and Exu protein localization (Pokrywka and Stephenson, 1991, 1995; Wang and Hazelrigg, 1994), suggesting that these two

components utilize a similar, if not identical, anterior localization pathway.

To gain insight into the cytoplasmic transport steps that lead to anterior axis specification in *Drosophila*, we have therefore analyzed GFP-Exu particle movements within living egg chambers. These in vivo analyses reveal a multi-step anterior localization pathway composed of both microtubule-dependent and microtubule-independent steps. In the nurse cells, microtubule-dependent processes lead to perinuclear particle clustering, particle accumulation at the ring canal junctions, and rapid particle movements within the bulk cytoplasm. In vivo analysis of microtubule organization reveal three populations of microtubules that appear to mediate these movements. A process that is resistant to microtubule and actin inhibitors then transports GFP-Exu particles through the ring canal junctions at the anterior pole of the oocyte. In a final microtubule-dependent localization step, GFP-Exu particles entering the oocyte are directed to the anterior cortex. Prevailing models hypothesize that transport along a polarized oocyte microtubule cytoskeleton leads to anterior-posterior axis specification. However, our analyses of GFP-Exu particle behavior and microtubule organization strongly suggest that the polarity of the oocyte microtubule cytoskeleton is not sufficient to specifically localize GFP-Exu to the poles. Based on these observations, and work from several other laboratories, we propose a modified anterior localization model.

MATERIALS AND METHODS

Imaging GFP-Exu and microtubules in living egg chambers

GFP-Exu particle movements were visualized as follows. Adult female flies carrying the GFP-Exu transgene (*w*[*1118*]; *cn exu*[*SC02*] *bw* ; *NG3*/+) were transferred to a pool of halocarbon oil on a 22 mm × 40 mm cover glass. Ovaries were dissected from these flies under oil, and individual egg chambers were then teased from the ovaries using no. 5 biological grade forceps. During dissection, the ovaries were held against the cover glass with one set of forceps, and egg chambers were removed with a second pair of forceps, taking care to keep the forceps in contact with the cover glass while pulling the egg chambers and ovarioles away from the ovary. This helps keep the egg chambers in contact with the surface of the cover glass, which is essential for high resolution imaging. The cover glass was then transferred to the stage of a Nikon Diaphot inverted microscope equipped with a BioRad MRC 600 laser scanning confocal attachment and time lapse imaging was performed using the BHS filter combination and 40× plan neofluor or 60× planapo objectives. Typically, full frame images (512×756 pixel) were captured at 10 second intervals and saved to the hard drive of the controlling computer. For analysis of particle rates, 250×756 pixel frames were captured at 2 second intervals. The increased temporal resolution of these sequences (relative to full frame) facilitated particle tracking and identification.

To analyze microtubule organization in vivo, egg chambers were dissected from adult females, microinjected with rhodamine-conjugated tubulin, and analyzed by time-lapse confocal microscopy as described previously (Theurkauf, 1994). For video-rate play back of GFP-Exu particle and microtubule sequences, time-lapse images were transferred to a Sony LVN 3000R optical disk recorder. Staging of egg chambers was according to King (1970).

Under the confocal imaging conditions used for these studies, the optical sections ranged from 0.5-1.0 μm. Because the particle movements were rapid relative to rate of image capture, we could not obtain images at different focal planes for each time point, and four dimensional data could not be generated. Our time-lapse analyses were therefore limited to particles moving within a single focal plane. Nonetheless, we were able to track large numbers of particles for multiple time points, and could therefore accurately determine the velocity and direction of movement for these particles. We cannot formally rule out the possibility that particles moving in other planes have different properties from the particles that we were able to analyze. However, we believe that it is more likely that the particle behavior we observed is representative of particle movement in general.

Inhibitor studies

To determine the effects of colcemid and cytochalasin D on particle mobility, egg chambers were dissected on cover slips and transferred to the inverted microscope stage as described above. Inhibitors were diluted to 20 μg/ml in Schneider's insect culture medium (Gibco BRL, Grand Island, NY). A 1 μl drop of inhibitor solution was then placed in the oil on the slide, and a micropipette held in a Narashigi micromanipulator was front-filled from this drop. The solution was then placed around the egg chamber using a micropipette. To

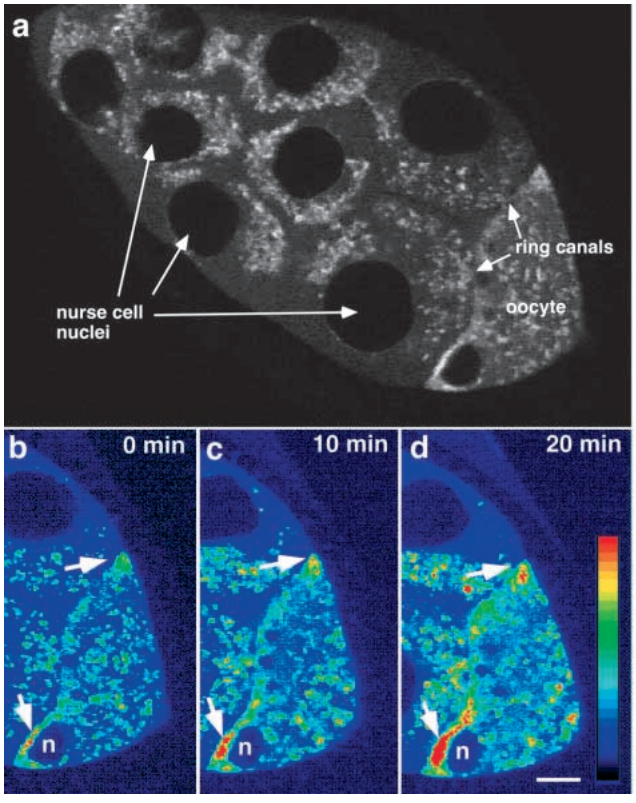

Fig. 1. In vivo analysis of GFP-Exu accumulation at the anterior of a stage 8 oocyte. A late stage 8 egg chamber, dissected from an adult female carrying the GFP-Exu transgene, was analyzed by time-lapse confocal microscopy. (a) A single full frame at the start of the recording. Particles are observed around the nurse cell nuclei, near the ring canal junctions, and at the anterior margin of the oocyte. (b-d) GFP-Exu accumulation at the anterior pole (arrows) of the oocyte at 0 minutes, 10 minutes, and 20 minutes after initiating the time lapse sequence. The false color in b-d reflects pixel intensity, as indicated by the key in d, 0=black, 254=red. Bar, 20 μm. A QuickTime movie of this data is available at http://www.ummed.edu/dept/pmm/wt.html.

inactivate colcemid, the egg chambers were exposed to UV light for 3-4 seconds as follows. The Nikon UV-1A epifluorescence filter cube was put in place, and the barrier to the 100 W mercury light source of the epifluorescence attachment was removed for the desired time. The barrier was then replaced, the filter cube was removed, and time lapse confocal imaging was resumed. Microtubule repolymerization was indicated by resumption of microtubule-dependent bulk ooplasmic movements (Theurkauf, 1994). In addition, egg chambers that had been microinjected with rhodamine-tubulin were treated with colcemid by the above procedure, and microtubule disruption was directly assayed. Under these conditions, colcemid blocked assembly of microtubules in both nurse cells and the oocyte (not shown), while numerous dynamic microtubules were observed in control egg chambers (see Results).

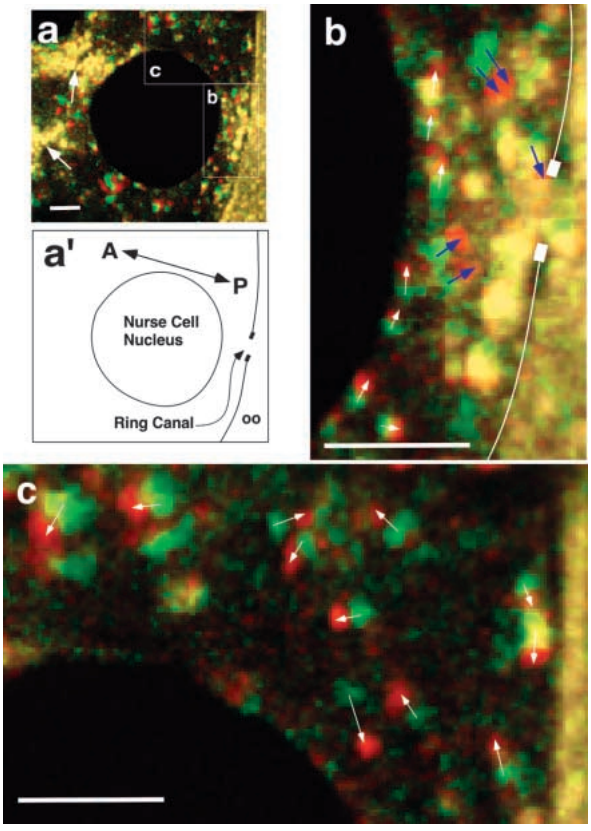

Fig. 2. The directionality of GFP-Exu particle movement varies as a function of position in a nurse cell cytoplasm. This figure shows an optical section through a nurse cell that is linked directly to the oocyte. (a) To reveal the direction of particle movements as a function of position within the nurse cell, two images captured 10 seconds apart were superimposed, with t=0 displayed in green and t=10 seconds displayed in red. Where particles have not moved they appear yellow (arrows). Individual particles at the two time points were identified by examining complete time lapse sequences, as described in Methods. (a′) A diagram of the cell in a. The axis of the egg chamber is indicated by the double-headed arrow, with anterior and posterior labeled A and P, respectively. (b). Enlargement of a region near the ring canals linking the oocyte and nurse cell. Particles in this region often move (blue arrows) toward the ring canal junction. (c) Enlargement of a region further from the ring canal. In this region, particles show no clear directionality (arrows) with respect to the egg chamber axis. Bars, 20 μm.

RESULTS

Transport within the nurse cells

Drosophila oocytes develop within egg chambers that are composed of 16 germline cells surrounded by a monolayer of somatic follicle cells. The 16 germline cells form a single oocyte and 15 nurse cells, which are interconnected by large cytoplasmic bridges, called ring canals (Mahajan-Miklos and Cooley, 1994). To characterize GFP-Exu particle movements within the germline cells, egg chambers from adult females carrying the GFP-Exu transgene were imaged using time lapse laser scanning confocal microscopy (Theurkauf, 1994; see Methods). Using these methods, Glotzer et al. (1997) have observed microinjected RNA localizing to the oocyte cortex over the course of several hours, and we find that rapid GFP-Exu particle movements and ooplasmic streaming continue for at least an hour. Nonetheless, in order to avoid possible artifacts associated with the culture method, we limited our analyses to the first half hour after dissection.

Consistent with earlier reports, our analysis of living egg chambers revealed GFP-Exu particles dispersed throughout the nurse cell cytoplasm, clustered around the ring canal junctions, surrounding the large polyploid nurse cell nuclei, and localized to the anterior margin of the oocyte (Fig. 1a; Wang and Hazelrigg, 1994). Exu is also transiently localized to the posterior pole of the oocyte, although a function for Exu in posterior patterning has not been demonstrated (Wang and Hazelrigg, 1994). We therefore focused our analysis on particle

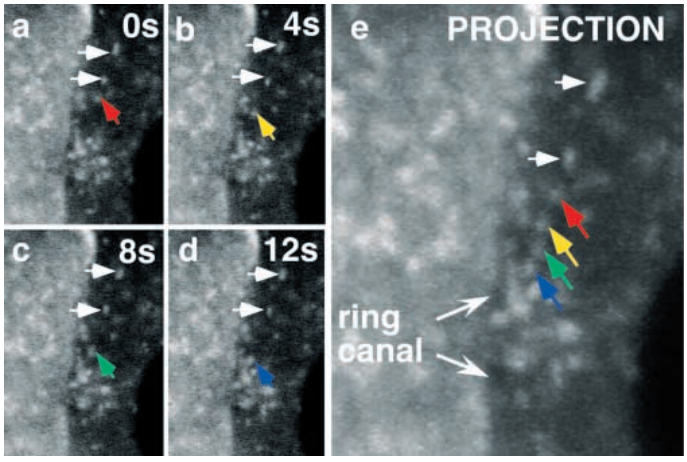

Fig. 3. GFP-Exu particle movement to the ring canal junction. Near the ring canals linking the nurse cells with the oocyte, a subset of particles move directly to a region near the ring canal entrance. (a-d) Four time lapse images, taken at four second intervals. In this sequence, a single particle is observed moving linearly toward the ring canal junction. The position of this particle is indicated by the red, yellow, green and blue arrows in a-d respectively. (e) A temporal projection formed by superimposition of these four time points. In this projection, the motile particle appears as a linear series of dots. The color coded arrows indicate particle position at the four time points. The small white arrows indicate particles that do not move over the 12 seconds covered in this recording. The position of the ring canal is indicated by the arrows in e. The oocyte is on the left, and the nurse cell is on the right. A QuickTime movie of this data is available at http://www.ummed.edu/dept/pmm/wt.html.

movements within the nurse cells and toward the anterior pole of the oocyte. Time lapse microscopy revealed rapid movement of the dispersed cytoplasmic particles, particle translocation through the ring canal junctions, and accumulation of GFP-Exu particles at the anterior of the oocyte (Fig. 1b-d; see QuickTime movies at http//www.ummed.edu/dept/pmm/tw.html). These initial observations demonstrated that GFP-Exu particle movements could be observed in isolated egg chambers, and that anterior localization continued in these egg chambers.

The first step in GFP-Exu anterior localization is transport within the nurse cell cluster. The three spatially distinct groups of GFP-Exu particles in these cells showed different dynamic properties. The single particles that were dispersed through most of the nurse cell cytoplasm moved rapidly over relatively short distances. The perinuclear clusters of particles, by contrast, showed relatively little movement. However, particles occasionally dissociated from the perinuclear clusters and began rapid movement within the cytoplasm. Particles clustered near the ring canals also showed relatively little movements, although a subset of these particles moved through the cytoplasmic bridges into the oocyte.

The clustering of particles at the ring canal junctions and accumulation of GFP-Exu at the anterior of the oocyte (Fig. 1) indicated that there is net transport of GFP-Exu from the nurse cells to the oocyte. We therefore analyzed the movement of the GFP-Exu particles for indications of directional transport. To display the direction of particle movement as a function of position in the nurse cell, images taken 10 seconds apart were superimposed, with the first time point displayed in green and the second in red. In the composite image, particles that have moved during the 10 sec. interval appear as red and green dots, while particles that have not moved are yellow, reflecting superimposition of the red and green signals. Clusters of particles that have not moved significantly over the 10 sec. interval are found at the ring canal junctions and near the nurse cell nuclei (Fig. 2a). In the bulk of the cytoplasm, however, numerous red and green particles are observed. By examining complete time lapse sequences, we identified the particles that had moved during the 10 sec. interval, and assigned arrows to these particles with ends defined by position at the two time points. The length of the resulting vectors reflect particle velocity, and the arrow indicate direction of movement (Fig. 2b,c). Particle movement within much of the nurse cell cytoplasm has no clear polarity with respect to the egg chamber axis, and particles that lie in close proximity to each other often move in opposite directions (Fig. 2c, arrows). By contrast, a significant fraction of the particles near the ring canals move toward the cell-cell junctions (Fig. 2b, blue arrows).

High temporal resolution analysis (see Methods) confirmed that particles near the ring canals linking the nurse cells with the oocyte often move continuously and over relatively long distances toward the ring canal junctions (Fig. 3). These studies also allowed accurate determinations of particle velocities throughout the cytoplasm. Because particle velocity was high relative to the rate of image capture, we were unable to obtain images at multiple focal planes in our time lapse studies, and four dimensional analyses could not be performed. Our quantitative analyses were therefore limited to particles moving within the plane of a single confocal optical section, although we assume that the behavior of these particles is representative of particle movement in general. These analyses show that particles within the nurse cell cytoplasm move at rates ranging from 15 to 145 μm/minute. The histogram of the rate data, shown in Fig. 4, could be composed of several distinct velocity peaks. The GFP-Exu particles vary significantly in size and direction of movement, but we have found no correlation between particle size or direction and velocity.

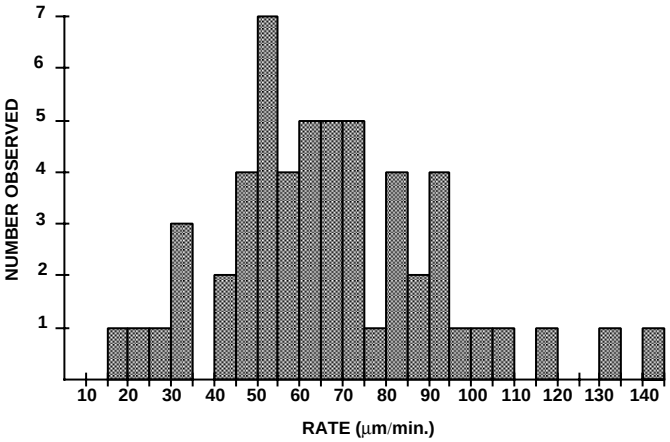

Fig. 4. Particle velocity in the nurse cell cytoplasm. The rate of particle movement was determined from time lapse sequences of stage 8 and 9 egg chambers (See Fig. 3). For these studies, only particles that could be tracked for three or more frames were scored. The histogram displays the number of particles showing velocities within 5 μm/minute ranges between 5 and 145 μm /min. Particle velocities varied from 15 to 145 μm/min.

Transport through the ring canal junctions

In addition to particle movements in the nurse cell cytoplasm, particles were observed passing through the ring canals linking the nurse cells with the oocyte (Fig. 5). In contrast to the random movements in most of the nurse cell cytoplasm, transport through these ring canals was consistently directed from the nurse cells to the oocyte, and was significantly slower and more uniform in velocity than particle movement in the bulk nurse cell cytoplasm (Fig. 6a). These observations suggested that distinct mechanisms drive GFP-Exu movement in the nurse cell cytoplasm and through the ring canal junctions.

The 15 nurse cells and the oocyte are interconnected by a series of ring canals, and the arrangement of the ring canal connections is determined by the pattern of the cystoblast divisions that produced these 16 germline cells (reviewed by Spradling, 1993). As a result of these patterned divisions, the oocyte is directly connected to only four of the nurse cells, while the remaining nurse cells are indirectly linked to the oocyte through three of these four oocyte-proximal cells. We have focused our analyses on movements within the four nurse cells immediately adjacent to the oocyte. However, transport of material from the more distant nurse cells almost certainly contributes to anterior patterning. We have qualitatively analyzed movement through the ring canals that connect nurse cells to each other. As discussed above, movement through the nurse cell-oocyte ring canals is unidirectional. In the set of ring canals linking the oocyte-proximal nurse cells with the remainder of the cyst, transport

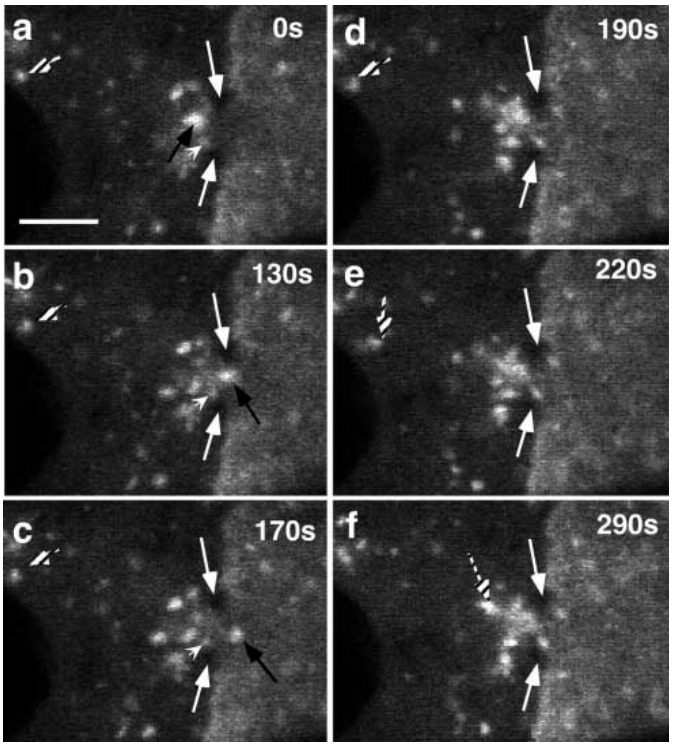

Fig. 5. GFP-Exu particle movement in the nurse cell cytoplasm is microtubule-dependent, while particle movement through the ring canals is not effected by microtubule assembly inhibitors. Egg chambers were dissected and treated with the reversible microtubule inhibitor colcemid (see Methods). Elapsed time is indicated in the upper right hand corner of each panel. The egg chamber was treated with inhibitor approximately 5 min. before the 0s time point. (a-c) Movement of particles in the nurse cell cytoplasm is blocked by colcemid (striped arrow), while transport through the ring canal junctions proceeds normally (black arrow). Note that a particle (arrowhead) at the ring canal entrance remains stationary while the particle indicated by the black arrows moves through the cytoplasmic bridge (white arrows). Transport through the ring canal thus does not appear to reflect non-selective bulk flow of cytoplasm. (d-f) At 180s, the egg chamber was irradiated with UV light to inactivate the colcemid, and recording was resumed. At 220s, 40 seconds after inhibitor inactivation, particles within the nurse cell cytoplasm are moving rapidly toward the ring canal (Striped arrow in e and f). Bar, 20μm. A QuickTime movie of this data is available at http://www.ummed.edu/dept/pmm/wt.html.

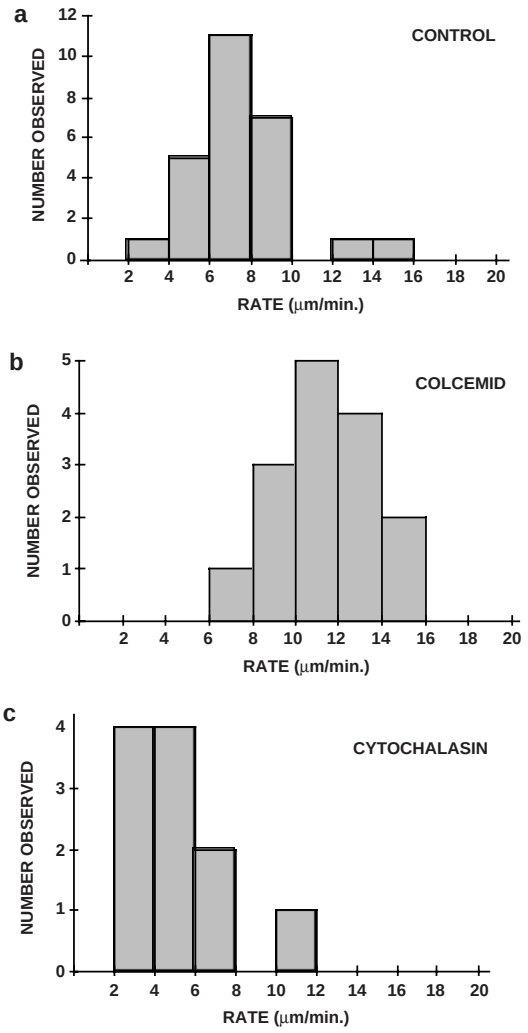

Fig. 6. Rate of particle movement through the ring canals linking the nurse cells and oocyte. (a) Particle rate in control egg chambers is relatively uniform, with the majority of particles moving between 6 and 8 μm/min. (b) Treatment with the microtubule depolymerizing drug colcemid caused an increase in average particle velocity, suggesting that microtubules interfere with particle transport through the ring canal junctions. (c) Treatment with the actin filament disrupting drug cytochalasin caused a slight decrease in average particle rate, but did not block these movements. Rate data are displayed as histograms, with particles grouped in 2 μm/minute ranges between 0 and 20 μm/minute.

was significantly slower than movement through the nurse cell-oocyte ring canals, and proved difficult to quantify (see sequence 1 at http//www.ummed.edu/dept/pmm/tw.html). These slower movements were generally directed toward the oocyte. By contrast, little net movement was observed though more distant ring canal junctions, and particles often slowly oscillated toward and away from the oocyte at these cytoplasmic bridges. Therefore, both the directionality and velocity of particle movement through the ring canals decreases as the distance from the oocyte increases.

Role of cytoskeletal elements in particle transport

Cytoplasmic transport is generally mediated by microtubules or actin filaments, and genetic and pharmacological studies have implicated both of these cytoskeletal systems in anterior-posterior axis specification (Pokrywka and Stephenson, 1991, 1995; Erdelyi et al., 1996). Particle behavior was therefore analyzed in the presence of specific cytoskeletal inhibitors (see Methods). To determine the role of microtubules in particle movement, the microtubule-depolymerizing drug colcemid was used. This inhibitor can be inactivated by UV light, so that the specificity of any defects observed can be confirmed by inducing microtubule repolymerization with UV irradiation. We found that colcemid rapidly blocked all GFP-Exu particle movement within the nurse cell cytoplasm (Fig. 5, 0s-170s, striped arrow). Significantly, brief irradiation of the egg chamber with UV light induced a rapid and dramatic resumption of particle movement in the nurse cells (Fig. 5,

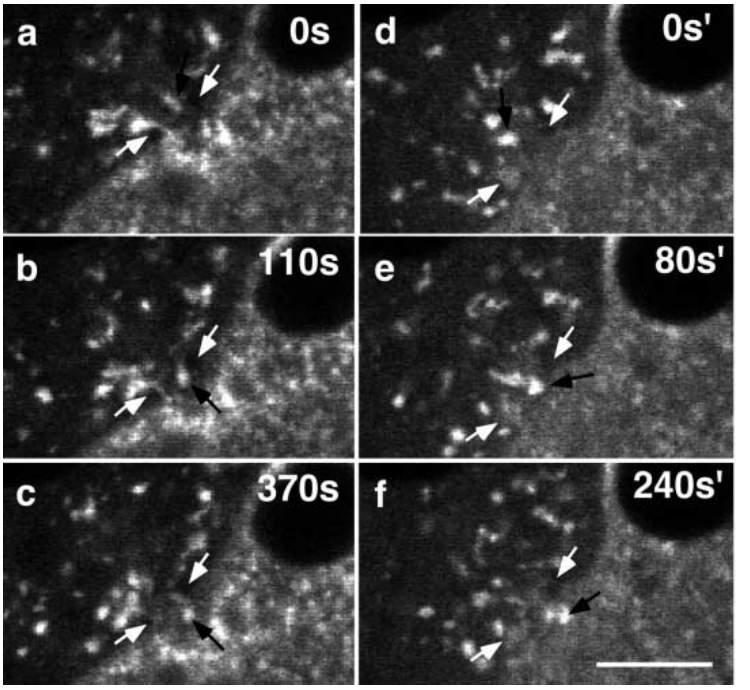

Fig. 7. GFP-Exu particle movement through the ring canals is not blocked by the actin-disrupting drug cytochalasin D. A stage 8 egg chamber was dissected and imaged as described above. Elapsed time is indicated in the upper left hand corner of each frame. (a-c) Particle movement (black arrow) through a ring canal (white arrows) prior to cytochalasin treatment. Cytochalasin was added shortly after the 370s time point. (d-f). 0s′ time point (shown in d) was approximately 13 minutes after cytochalasin D treatment. At this time, the inhibitor had caused gross changes in the shape of the nurse cell-oocyte boundary, consistent with disruption of the actin filament system. Nonetheless, particle movement through the ring canal junction continued. Bar, 25 μm. A QuickTime movie of this data is available at http://www.ummed.edu/dept/pmm/wt.html.

190s-290s, striped arrow). These observations indicate that essentially all particle transport within the nurse cell cytoplasm is microtubule-dependent.

In contrast to particle movements in the nurse cell cytoplasm, transport through the nurse cell-oocyte junctions was not blocked by colcemid treatment (Fig. 5, 0s-170s, black arrow). To determine if microtubule depolymerization caused a quantitative change in particle movement through the ring canals, we determined the rate of these movements in the presence of colcemid (Fig. 6b). Somewhat surprisingly, the average rate of particle transport though the ring canals increased slightly on treatment with colcemid, which could indicate that microtubules near the ring canals mechanically interfere with particle passage through the cell-cell junctions. To determine if movement through these ring canals was dependent on actin filaments, transport was assayed in the presence of cytochalasin D. This actin filament-inhibitor consistently led to rapid and dramatic changes in nurse cell and oocyte shape, indicating that the actin cytoskeleton had been disrupted (Fig. 7). However, particle transport through the ring canal junctions and rapid movement of particles in the nurse cell cytoplasm were not blocked (Fig. 7, 0s′-240s′, black arrow). Quantitative analysis demonstrated that cytochalasin produced a slight decrease in the average rate of particle movement through the ring canals (Fig. 6c), although the range of particle velocities overlapped the range observed in untreated chambers (compare Fig. 6a and c). These observations raise the possibility that GFP-Exu particle transport through the oocyte-nurse cell ring canals is independent of both actin filaments and microtubules. However, it is also possible that these movements are mediated by stable cytoskeletal elements that are not affected by colcemid or cytochalasin treatment. Nonetheless, these observations, combined with the rate data, strongly suggest that GFP-Exu particle transport through the nurse cell-oocyte ring canal junctions is mechanistically distinct from transport within the nurse cell cytoplasm.

Particle localization within the oocyte

The final step in Exu localization is accumulation at the anterior pole of the oocyte. Direct analysis of this final localization step proved technically difficult, however, because autofluorescent yolk granules within the ooplasm partially obscured the GFP-Exu signal, and because GFP-Exu particles appeared to spread out along the anterior cortex as soon as they entered the oocyte. Previous studies indicated that *bcd* mRNA and Exu protein localization to the anterior pole requires microtubules (Pokrwyka and Stephenson, 1991; Wang and Hazelrigg, 1994), while our studies show that transport of GFP-Exu particles through the ring canals linking the nurse cells to the oocyte is resistant to microtubule inhibitors (Figs 5 and 6). We therefore speculated that prolonged colcemid treatment would lead to continued transport of GFP-Exu particles to the oocyte, but a failure in particle localization to the anterior pole. Microtubule-dependent movement of these dispersed particles could then be observed after colcemid inactivation. We therefore raised adult female flies on food supplemented with colcemid for 8 to 12 hours (Theurkauf et al., 1993), and analyzed particle behavior in stage 8 and 9 egg chambers isolated from these females (Fig. 8). The oocytes in these egg chambers contained GFP-Exu particles dispersed throughout the ooplasm, as well as particles sparsely localized to the cortex in a non-polarized manner (Fig. 8a). In addition, these oocytes contained relatively little yolk, further facilitating particle visualization and indicating that yolk uptake is itself microtubule-dependent. These observations support the conclusion that normal anterior localization of GFP-Exu requires an intact microtubule cytoskeleton.

To analyze the behavior of the dispersed GFP-Exu particles after microtubule repolymerization, colcemid inactivation was

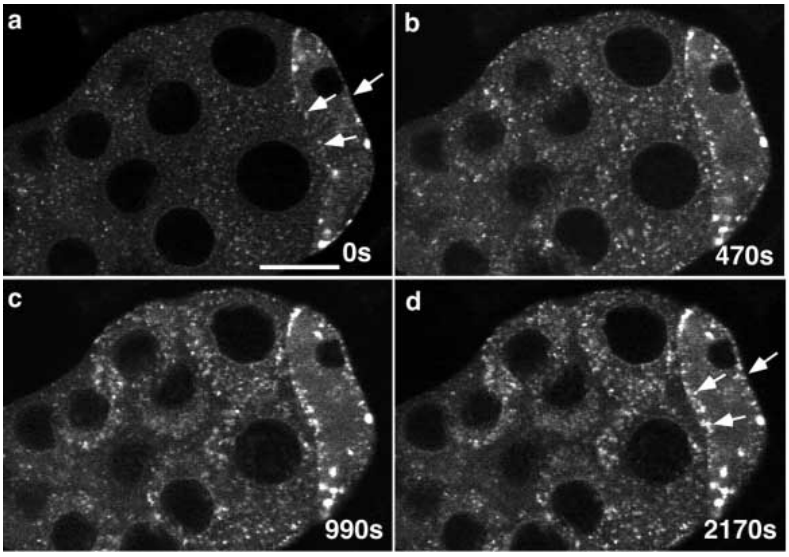

Fig. 8. Microtubule-dependent particle perinuclear clustering and movement to the oocyte cortex. To disrupt microtubules in egg chambers, adult females were raised for 8 hours on yeast paste containing the reversible microtubule inhibitor colcemid. Egg chambers were then dissected and time lapse images were recorded. (a) Microtubule disruption by colcemid blocked perinuclear accumulation and anterior localization of the GFP-Exu particles (arrows). In addition, this treatment blocked anterior/dorsal positioning of the oocyte nucleus. Colcemid was inactivated with UV light at 0s. (b-d) Perinuclear clustering of GFP-Exu particles was then reestablished, and particles moved from the ooplasm to the cortex. Significantly, particles moved to all surfaces of the oocyte, with only bias toward the anterior pole. This led to somewhat higher levels of GFP-Exu at the anterior cortex relative to other cortical regions (panel d, arrows).

induced by irradiation with UV light (Fig. 8). Approximately 30 seconds after egg chamber irradiation, a subset of particles within the ooplasm began to move directly to the oocyte cortex, and most of the GFP-Exu particles were eventually cleared from the ooplasm and relocalized to the cortex (Fig. 8b-d). These particle movements were biased towards the anterior pole, leading to somewhat greater accumulation of GFP-Exu at the anterior cortex (Fig. 8c,d). However, microtubule-dependent movements of GFP-Exu to essentially all other regions of the oocyte cortex were also observed. As a result, the wild-type pattern of GFP-Exu localization was not established, and a relatively non-polarized increase in cortical particle concentration was observed (Fig. 8d). Under these experimental conditions, therefore, microtubule dependent transport alone does not appear to be sufficient to restrict GFP-Exu particles to the anterior pole.

In addition to disrupting particle localization to the oocyte poles, colcemid treatment blocked perinuclear clustering of GFP-Exu particles within the nurse cells (Fig. 8a). On colcemid inactivation, particles within the nurse cell cytoplasm clustered around the large polyploid nuclei and increased in intensity and apparent size (Fig. 8). These observations indicate that the perinuclear clustering of GFP-Exu particles is also microtubule-dependent, and that assembly of larger particle complexes may also require a microtubule scaffold (see also Wang and Hazelrigg, 1994).

Microtubule organization in vivo

The behavior of GFP-Exu particles during mid-oogenesis provided indirect evidence for several distinct populations of microtubules within the nurse cells. To directly visualize these microtubules, nurse cells were microinjected with rhodamine-tubulin and analyzed by time lapse confocal microscopy. These studies reveal three populations of microtubules that appear to mediate movement in the nurse cells (Fig. 9). Microtubules were associated with the surface of the polyploid nuclei (Fig. 9c-e, red arrows), extended from the ring canal junctions (Fig. 9a,c-e, white arrows), and were dispersed throughout the cytoplasm (Fig. 9b). Time lapse analyses demonstrated that all of these populations of microtubules were dynamic, and reorganized dramatically over very short times (Fig. 9c-e; QuickTime movie at www.http//ummed.edu/dept/pmm/tw.html). This is diagrammed in Fig. 9f, in which the positions of microtubules at 0s, 10s, and 20s time points (Fig. 9c-e) are displayed in red, green, and yellow, respectively. There is almost no overlap between the microtubules at these time points, suggesting that most of the nurse cell microtubules turn over within 20 seconds. We conclude that the three dynamic populations of microtubules we observed in the nurse cells mediate essentially all of the cytoplasmic GFP-Exu particle movements we observe.

DISCUSSION

To gain insight into the cytoplasmic transport processes that underlie axis specification in *Drosophila*, we have analyzed the behavior of particles containing a GFP-Exu fusion protein, and the dynamics of microtubule organization in living egg chambers. These studies reveal a complex anterior localization pathway composed of both microtubule-dependent and microtubule-independent steps. In addition, our observations suggest that microtubule asymmetry within the oocyte is not, in itself, sufficient to localize GFP-Exu specifically to the anterior pole. Based on these observations, and a number of previous studies, we propose a revised anterior localization pathway.

Transport from the nurse cells to the oocyte

Exu protein and *bcd* mRNA are synthesized in a cluster of 15 nurse cells that are linked to the oocyte by ring canal bridges (reviewed by Cooley and Theurkauf, 1994). The first cytoplasmic transport steps in anterior patterning therefore take place within the nurse cells. Our analysis indicates that transport within the nurse cell cytoplasm is composed of at least three microtubule-dependent steps that produce a net movement of GFP-Exu particles toward the oocyte.

The majority of the individual GFP-Exu particles within the nurse cell cytoplasm move rapidly and with no apparent net directionality with respect to the egg chamber axis (Fig. 2c). These movements are reversibly inhibited by the microtubule-

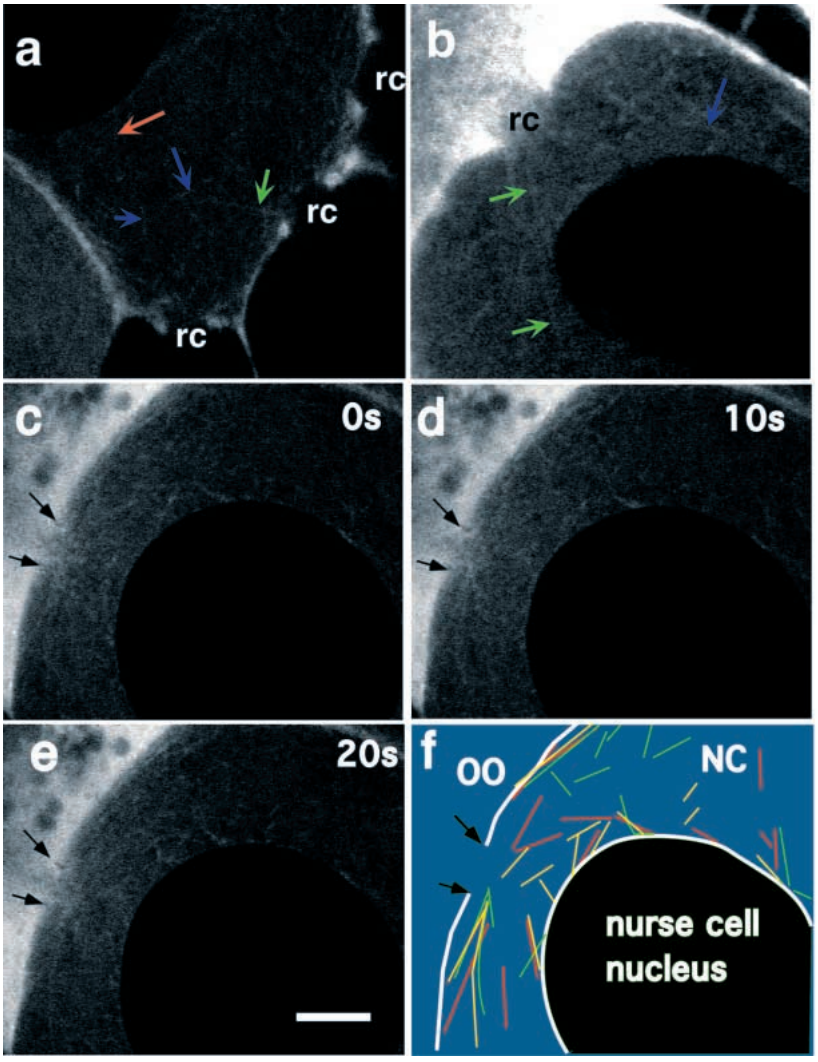

Fig. 9. Microtubule organization and dynamics in nurse cells. Early stage 9 egg chambers were microinjected with rhodamine-conjugated tubulin and microtubules were directly examined by time-lapse confocal microscopy. (a) Within the nurse cells, microtubules were observed in association with nurse cell-nurse cell ring canal junctions (green arrows) and the nurse cell nuclei (orange arrows). In addition, randomly oriented microtubules were observed throughout the cytoplasm (blue arrows). (b) Near the nurse cell-oocyte junctions, microtubules associated with the ring canal are more numerous, and these microtubules extend into the nurse cell cytoplasm (green arrows). (c-e) Microtubule reorganization near a nurse cell-oocyte ring canal (black arrows) during a 20-second interval. (f) Diagram of microtubule organization at the three time points shown in c-e. Identifiable microtubules at each time point were traced and are displayed in red (0s), green (10s) and yellow (20s) in this composite. All populations of microtubules in this cell change position and length over 20 seconds, although the ring canal microtubules appear somewhat more stable than the randomly oriented microtubules in the bulk cytoplasm. QuickTime sequences are available at http://www.ummed.edu/dept/pmm/wt.html. Bar, 10 μm.

disrupting drug colcemid, and we have directly observed microtubules throughout the nurse cell cytoplasm that lack clear orientation with respect to the egg chamber axis (Fig. 9). Based on these observations, we conclude that microtubules mediate random particle movements within the nurse cells. In the absence of microtubules, we have been unable to detect any movement or redistribution of GFP-Exu particles (Fig. 5; data not shown). Simple diffusion thus appears to be insufficient to efficiently disperse these large particles. We therefore speculate that the random microtubule-dependent particle movements are essential to dispersing GFP-Exu particles. As discussed below, we propose that this particle dispersal is required for efficient net particle transport through the nurse cells (Fig. 10).

Vectorial particle transport is observed in the region near the ring canals linking the nurse cells with the oocyte. In this region, particles tend to move directly to the cell-cell junctions (Figs 2, 3). These movements, like the random movements observed in bulk nurse cell cytoplasm, are reversibly inhibited by colcemid (Fig. 5). In addition, we have directly observed a dynamic population of microtubules associated with the nurse cell-oocyte ring canals (Fig. 9). We therefore propose that GFP-Exu particles approach the ring canal junctions by a microtubule-dependent random walk. The particles then associate with microtubules that are organized around the ring canals, and are transported to the nurse cell-oocyte junctions. It is this second step that imparts net directionality on particle transport through the nurse cell cytoplasm.

Previous ultrastructural analysis of Exu distribution failed to identify microtubules in direct association with Exu-containing structures, termed sponge bodies (Wilsch-Braüninger et al., 1997). However, our in vivo analysis indicates that at least some of the microtubules in the nurse cells turn over within 10 to 20 seconds (Fig. 9). These microtubules are therefore likely to be difficult to preserve by standard fixation procedures. The failure to identify microtubules directly associated with sponge bodies may reflect the dynamic nature of these filaments (Wilsch-Braüninger et al., 1997).

Perinuclear particle clustering in the nurse cells also appears to be microtubule-dependent. This process is reversibly disrupted by colcemid (Fig. 8), and microtubules are associated with the surface of the nurse cell nuclei (Fig. 9). The function of microtubule-dependent perinuclear clustering is not yet clear, although it seems unlikely that this process contributes directly to movement through the nurse cells. It has been

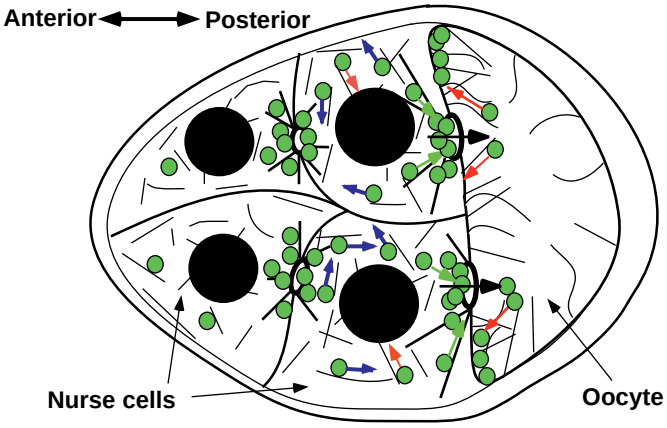

Fig. 10. A multi-step model for transport and anterior localization of Exu during stages 9 and 10 of oogenesis. We speculate that Exu protein assembles into particles within the nurse cell cytoplasm, perhaps at the nuclear periphery, where these particles are localized by a microtubule-dependent process (orange arrows). Particles dissociate from the perinuclear regions and random microtubule-dependent movements then distribute these large particles throughout the nurse cell cytoplasm (blue arrows). As particles approach the posterior of the nurse cell, they interact with microtubules originating near the nurse cell-oocyte ring canals, and are transported to the cell-cell junctions along these microtubules (green arrows). Particles are then transported through the ring canals in a second vectorial process that appears to be independent of both actin filaments and microtubules (black arrows). In the final transport step, particles entering the oocyte interact with microtubules originating at the anterior cortex of the oocyte, and are localized to the anterior in a microtubule-dependent step (red arrows). At the cortex, particle may associate with asymmetrically localized binding sites that stabilize the asymmetric distribution. To simplify the diagram, only 4 of 15 nurse cells are shown. The organization of microtubules in the oocyte is adapted from Theurkauf et al. (1991), Fig. 8c.

proposed that Exu particles are RNPs that contain *bcd* mRNA, as well as other proteins (Wang and Hazelrigg, 1994). If so, these particles could form in the perinuclear region as *bcd* mRNA exits the nurse cell nuclei, and microtubule-dependent transport could facilitate complex formation by concentrating cytoplasmic components of the particles in this region. Consistent with this suggestion, GFP-Exu particle size is decreased by microtubule depolymerization, and particles appear to increase in size and fluorescence intensity on microtubule repolymerization (Fig. 8).

Essentially all of the GFP-Exu particle movements in the nurse cell cytoplasm are microtubule-dependent, and these movements are presumably mediated by microtubule motor proteins. Microtubule motors are characterized by both direction of movement along the microtubule substrate, and the velocity of these movements (reviewed by Bloom, 1992). We have no direct data on the direction of Exu particle movement along the nurse cell microtubules. However, we have found that the velocity of microtubule-dependent movement within bulk cytoplasm varies over a wide range (Fig. 4a). We speculate that this reflects a function for several different microtubule motors in Exu particle motility. Alternatively, the variability in the rates and directionality of particle movements in the nurse cells could reflect complexities in the underlying microtubule cytoskeleton or particle-specific differences in the regulation of a single motor. However, the use of multiple motors for this transport process would serve to isolate axis specification from complete disruption by mutations in single motor protein genes, and mutations in known motor proteins have not yet been identified that disrupt these transport steps. Isolation and biochemical characterization of these transport particles and associated motors may be required to resolve this issue.

Once GFP-Exu particles are localized to the nurse cell-oocyte ring canals, a distinct transport process appears to drive movement through the cell-cell junctions. These movements are uniform in direction and velocity, raising the possibility that they reflect a very local flow of cytoplasm through the ring canal junctions. However, as shown in Fig. 5, some particles within the ring canal remain stationary (Fig. 5a-c, arrowhead) while other particles move continuously through the cytoplasmic bridge (Fig. 5a-c, black arrow). The apparent absence of bulk movement through the ring canal suggest that this step in the transport pathway is not due to cytoplasmic flow, but reflects the action of a more selective mechanism.

The best characterized specific transport processes require microtubules or actin filaments, yet movement through the ring canals is relatively insensitive to microtubule and actin assembly inhibitors (Fig. 4c). These observations raise the possibility that this transport step is independent of both actin filaments and microtubules. However, cytochalasin D and colcemid only affect dynamic filaments that are in equilibrium with subunits in the cytoplasm. It is therefore possible that stable actin filaments or microtubules mediate transport through the ring canals. The only cytochalasin-resistant actin filaments observed at this stage of oogenesis are associated with the rim of the ring canals, and these filaments are not oriented in a way that can easily account for vectorial transport (see Tilney et al., 1996). Similarly, colchicine-stable microtubules have not been observed within the ring canals, and inhibitor-resistant microtubules should be stable to fixation and therefore observable by electron or fluorescence microscopy. The inhibitor data reported here, combined with the published data, thus suggest that GFP-Exu transport through the nurse cell-oocyte ring canals is independent of both microtubules and actin filaments.

The movement of vesicles and mitochondria through the ring canals linking the nurse cells and oocyte has been characterized by contrast enhanced video microscopy (Bohrmann and Biber, 1993; Bohrmann, 1997). These authors showed that a subset of vesicles and mitochondria near the ring canals move uniformly toward the oocyte. In contrast to movement of GFP-Exu particles, these movements are inhibited by cytochalasin, suggesting that they are dependent on dynamic actin filaments. Both actin-dependent and actin-independent processes may therefore drive vectorial transport though the ring canals linking the nurse cells to the oocyte. If GFP-Exu transport through the ring canals is independent of actin and microtubules, how is this transport process driven? A voltage across the nurse cell-oocyte ring canals has been reported, and this electrical potential has been hypothesized to mediate macromolecular movement into the oocyte (Woodruff and Telfer, 1980). However, this hypothesis has not been supported by other studies (Sun and Wyman, 1993; Bohrmann and Schill, 1997), and other mechanisms are possible. Further analysis will be needed to resolve this issue.

Anterior morphogen localization reconsidered

Several observations have contributed to the formulation of a model for anterior-posterior axis specification in the *Drosophila* oocyte based on microtubule-dependent mRNA transport (see Wilhem and Vale, 1993; Theurkauf, 1994b, Clark et al., 1994; St Johnston, 1995). During mid-oogenesis, there is a dramatic reorganization of the microtubule cytoskeleton in the oocyte, such that a major microtubule organizing center (MTOC) located in the posterior of the oocyte is disassembled, and replaced by an anterior-posterior gradient of cortical microtubules, with a more dense concentration of microtubules at the anterior pole (Theurkauf et al., 1992; Clark et al., 1994). This view of the oocyte microtubule cytoskeleton, combined with the posterior localization of a kinesin β-galactosidase fusion, suggests that a preponderance of microtubule minus ends are at the anterior oocyte cortex, and that the plus ends of these microtubules extend towards the posterior pole. Since microtubules are required for the localization of RNAs to the anterior and posterior poles (Pokrwyka and Stephenson, 1991, 1995; Clark et al., 1994), and also for transient accumulation of Exu at the anterior pole (Wang and Hazelrigg, 1994; this report), a simple model has emerged in which *bcd* mRNA and Exu protein are specifically localized to the anterior pole through transport along microtubules with the aid of minus-end directed motor proteins, and posteriorly localized factors, such as *osk* mRNA, are directed to the posterior pole by plus-end directed microtubule transport. In this model, the positional information required for anteroposterior patterning is thus contained in the polarized oocyte microtubule cytoskeleton alone, and Exu protein or *bcd* mRNA present at any position in the oocyte is therefore predicted to associate with the microtubule scaffold and localize specifically to the anterior cortex.

This simple axis specification model is only partly consistent with the analysis of GFP-Exu particle behavior reported here, and does not explain several earlier observations on *bcd* mRNA localization (Pokrwyka and Stephenson, 1991; Glotzer et al., 1997). Consistent with this model and earlier experimental data, we find that GFP-Exu particles are dispersed in the ooplasm by microtubule depolymerization (Wang and Hazelrigg, 1995; this report). However, we also observe that these dispersed particles move to all regions of the oocyte cortex when microtubules repolymerize, with only a slight bias toward movement to the anterior pole (Fig. 8). It is possible that the relatively long 8-hour colcemid treatments used in our studies disrupt normal axial polarization of the oocyte microtubules, and that the non-polarized microtubule-dependent movement of GFP-Exu on colcemid inactivation thus reflects a colcemid-induced defect in cytoskeletal reorganization. However, other studies on *bcd* mRNA localization and microtubule organization suggest that this is not the case, and indicate that anterior localization of mRNAs and Exu protein in the oocyte is not as simple as earlier models suggest.

Pokrwyka and Stephenson (1991) first showed that microtubule depolymerizing drugs disrupt *bcd* mRNA localization to the anterior pole and lead to accumulation of this transcript throughout the ooplasm. Significantly, these authors also found that removal of the microtubule inhibitor and microtubule repolymerization led to a non-polarized localization of the dispersed transcript to all regions of the oocyte cortex. In addition, Glotzer et al. (1997) found that *bcd* mRNA, introduced into the center of the oocyte by microinjection, localized to the cortex in a non-polarized manner. Significantly, immunocytochemical and in vivo studies of microtubule organization show that microtubules associate with most of the oocyte cortex, with only a bias toward the anterior pole (see Theurkauf et al., 1991, Fig. 2). In addition, microtubules extending from the anterior to the posterior pole of the oocyte have not been identified. Thus, the movement of GFP-Exu and *bcd* mRNA or microtubule depolymerization and repolymerization, and direct cytological analyses of oocyte microtubule organization, suggest that microtubule-based transport alone is not sufficient to direct specific anterior localization.

The most compelling data supporting a simple model in which microtubule asymmetry alone is sufficient for anterior and posterior localization comes from analyses of a kinesin β-galactosidase fusion protein in the oocyte (see Clark et al., 1994). This fusion protein accumulates at the posterior pole of the oocyte during stages 8 and 9 of oogenesis, and this localization is disrupted in females raised on microtubule inhibitors. In addition, a number of mutations that lead to *bcd* mRNA localization at both the anterior and posterior poles of the oocyte also disrupt posterior localization of this fusion (Clark et al., 1994; Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995). Because kinesin is a plus-end directed motor, these observations support the hypothesis that oocyte microtubules, at these stages, are organized such that their minus ends are at the anterior pole and their plus ends extend to the posterior pole. However, the minus-end directed motor cytoplasmic dynein also localizes to the posterior of the *Drosophila* oocyte during stages 9 and 10 of oogenesis (Li et al., 1994). Thus, localization to the posterior pole and motor directionality do not strictly correlate. In addition, recent analyses show that mutations in the *mago nashi* gene can lead to posterior localization of *bcd* mRNA and transient persistence of a prominent microtubule organizing center at the posterior pole of the oocyte (Micklem et al., 1997). Nonetheless, these mutations do not disrupt kinesin β-galactosidase accumulation at the posterior pole. Microtubule organization and kinesin fusion protein distribution can therefore be uncoupled, at least in this specific genetic background. Finally, as discussed above, direct cytological analyses have not revealed microtubules originating at the anterior pole and terminating near the site of kinesin β-galactosidase accumulation at the posterior pole (see Theurkauf et al., 1992; Theurkauf, 1994; Emmons et al. 1995, Micklem et al., 1997). We believe that posterior localization of kinesin β-galactosidase reflects some aspect of oocyte axial asymmetry, and that this asymmetry depends on microtubule integrity. However, based on all of the available data, we conclude that the site of motor accumulation in the oocyte depends on factors in addition to microtubule polarity, and is not always a reliable indicator of microtubule organization.

We therefore favor the following revised model for anterior localization in the oocyte, which is similar to a passive trapping model first proposed by Berleth et al. (1988). As anteriorly-targeted particles pass through the ring canals and enter the anterior of the oocyte, they immediately encounter a dense network of cortical microtubules. In this model, microtubules are nucleated over the entire cortex of the oocyte, with only a bias in nucleation at the anterior pole. However, because

particle entry is restricted to the anterior pole, the vast majority of the microtubules first encountered by the transport particles originate at the anterior cortex. Thus, minus end-directed microtubule motors associated with the particles direct transport specifically to the anterior pole.

A number of mutations lead to ectopic accumulation of *bcd* mRNA at the posterior pole of the oocyte, and these mutations do not affect the geometry of transcript delivery (Ruohola et al, 1991; Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995; Micklem et al., 1997). We therefore speculate that particle trapping is somewhat inefficient, and that some of the anterior transport complexes escape initial capture and anterior localization. In wild-type oocytes, we speculate that these complexes are directed to essentially all regions of the cortex. This low level non-specific localization may not be developmentally significant, and would likely be difficult to detect by standard in situ hybridization techniques. By contrast, the persistence of a prominent MTOC at the posterior pole, which occurs in *mago nashi* and *PKA* mutations (Lane and Kalderon, 1994; Micklem et al., 1997), is predicted to focus these 'escaper' transcripts at the posterior pole.

Several observations indicate that factors in addition to egg chamber geometry and microtubule-dependent transport play a role in anterior axis specification. For example, *bcd* mRNA that is ectopically localized to the posterior of *mago nashi* and *PKA* mutant oocytes is dispersed as ooplasmic streaming begins at stage 10b, while the anteriorly localized transcripts in these oocytes are stable to streaming. Stable association of *bcd* mRNA with the cortex thus appears to be restricted to the anterior pole. In addition, several mRNAs are localized with *bcd* to the oocyte anterior during stages 9 and 10 of wild-type oogenesis (see Suter et al., 1989; Lantz et al., 1992; Pokrwyka and Stephenson, 1995). However, unlike *bcd* mRNA, these transcripts are dispersed upon ooplasmic streaming at stage 10b (Suter et al., 1989; Lantz et al., 1992; Pokrwyka and Stephenson, 1995). These observations indicate that anterior transcript binding sites are specific for *bcd* mRNA, or a complex containing this mRNA. We therefore favor a modification of the model first suggested by Pokrwyka and Stephenson (1995), in which localization of *bcd* mRNA depends on both microtubule-dependent movement to the cortex and transcript stabilization by a microtubule-independent mechanism. In this modified model, *bcd* mRNA binding activity is restricted to the anterior cortex, where it mediates pole-specific stabilization of transcript accumulation.

The molecular nature of the hypothesized asymmetric *biciod* mRNA binding sites remains to be determined. Exu is unlikely to anchor *bcd* mRNA at the anterior cortex, because this protein is only present during stages of oogenesis when *bcd* mRNA is in the process of being transported and localized to the anterior, and disappears late in oogenesis (MacDonald et al, 1991; Marcey et al., 1991; Wang and Hazelrigg, 1994). In addition, some Exu protein is transiently localized to the posterior oocyte pole during stage 9 of oogenesis (Wang and Hazelrigg, 1994), and *bcd* mRNA does not co-localize with Exu at this site. The product of the *swallow* gene (*sww*) is required to maintain *bcd* mRNA at the anterior oocyte cortex after stage 10b (Berleth et al., 1988; Stephenson et al., 1988; St Johnston et al., 1989), suggesting that it could be a component of this anchor. However, Sww protein is not specifically localized to the anterior cortex in mature oocytes and young embryos, indicating that it is not the asymmetric component of this anchor (Hegde and Stephenson, 1993). Staufen, a double-stranded RNA binding protein that can recognize *bcd* mRNA with some specificity (St Johnston et al., 1992; Ferrandon et al., 1994, 1997), is required for maintaining *bcd* mRNA at the anterior pole late in oogenesis, but does not appear to play an essential role in anchoring this transcript during earlier stages of oogenesis (St Johnston et al., 1989, 1991). The anterior anchor is therefore likely to be composed of asymmetrically localized factors that have not yet been identified, or to require anterior-specific modifications of known components that are not exclusively localized to the anterior pole.

A full understanding of anterior axis specification will require identification of the microtubule motors and other proteins required for transport within the nurse cells and oocyte, as well as characterization of the factors responsible for stable anchoring at the anterior oocyte cortex. If *bcd* mRNA is transported in the particles visualized with GFP-Exu, biochemical analysis of the components of these particles could lead to the identification of a number of the missing components in this essential patterning process.

W. E. T. was supported by grants for the National Institutes of Health (GM50898) and the Human Frontier Science Program (RG 0375-1997M). T. I. H. was supported by a grant from the National Institutes of Health (RO1GM48060).

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