Cell, Vol. 35, 15–25, November 1983, Copyright © 1983 by MIT

0092-8674/83/110015-11 $02.00/0

Generation of Asymmetry and Segregation of Germ-Line Granules in Early C. elegans Embryos

Susan Strome and William B. Wood
Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80309

Summary

Germ-line granules in C. elegans embryos (P granules) can be visualized by immunofluorescence microscopy using a monoclonal antibody. In mutant zygotes with abnormal spindle orientations and in wild-type zygotes treated with the microtubule inhibitors nocodazole, colcemid, vinblastine, and griseofulvin, both P-granule segregation to the posterior pole and the concomitant pseudocleavage occur apparently normally, but the normally concurrent migration of the pronuclei is inhibited. Conversely, treatment of wild-type embryos with the microfilament inhibitors cytochalasins D and B inhibits P-granule segregation and pseudocleavage, as well as other manifestations of polarity, without preventing pronuclear migration. The results suggest that P-granule segregation does not require either the spindle or cytoplasmic microtubules, but that this process as well as generation of other asymmetries does require cytoskeletal functions that depend on microfilaments.

Introduction

Fertilization of an animal egg initiates several directed movements of cellular components, leading to fusion of the pronuclei and subsequent cleavage. In a mosaic embryo such as that of Caenorhabditis elegans, some of these movements generate asymmetries, which are later manifested as cellular differences in different parts of the embryo. Cleavage in C. elegans includes a series of asymmetric cell divisions, giving rise to blastomeres that differ in size and developmental potential. By the 16-cell stage, the five major somatic lineages and the germ line are established. The pattern of asymmetric divisions and developmental fates is invariant among wild-type embryos, and the complete lineage of the organism, from fertilized egg to mature adult worm, has been determined (Sulston et al., 1983). However, the mechanisms for establishing asymmetry and for distributing different developmental potential to different regions or cells of the embryo remain unknown.

In C. elegans embryos, at least some developmental fates seem to be specified early by cell-autonomous, internally segregating determinants (Laufer et al., 1980). Such determinants in invertebrate embryos are often assumed to be cytoplasmic (reviewed in Wilson, 1925), although direct evidence for this proposal has remained limited. In its support, recent experiments involving the redistribution of cytoplasm in ascidian (Whittaker, 1982) and C. elegans embryos (E. Schierenberg and W. B. Wood, unpublished results) suggest that cytoplasmic factors can affect determination of somatic nuclei.

Perhaps the most likely candidates for cytoplasmic determinants so far identified are found in the special egg cytoplasm that is partitioned into the germ line. Distinctive cytoplasmic structures, referred to as nuage, germ plasm, or polar granules, are observed by electron microscopy in the germ-line cells of many organisms (for reviews see Beams and Kessel, 1974; Eddy, 1975; and Mahowald, 1977). In Drosophila, there is evidence that the cytoplasm containing these structures acts to determine germ-line cells during embryogenesis (Illmensee and Mahowald, 1974, 1976). In C. elegans embryos, cytoplasmic granules termed P granules can be visualized by immunofluorescence microscopy. During the early embryonic divisions they are segregated to the germ line or P lineage (Strome and Wood, 1982) and are probably the same as granules morphologically identified as germ plasm that have been observed in C. elegans by electron microscopy (Wolf et al., 1983). A determinative role for these granules in the germ line has not been established. Nevertheless, they serve as an excellent marker for studying the mechanism by which a lineage-specific cytoplasmic component is asymmetrically partitioned into specific cells during early cleavage stages and for examining the relationship of this process to the establishment of embryonic asymmetries after fertilization.

We describe production of a monoclonal antibody that stains P granules and appears to be directed against a ~40,000 dalton polypeptide. Using Nomarski microscopy of living embryos, followed by fixation and immunofluorescence staining, we have examined the progression of several early events—pseudocleavage, pronuclear migration, spindle formation, and P-granule segregation—in various mutant embryos and in wild-type embryos treated with microtubule and microfilament inhibitors. Our results provide evidence that different directed movements in early embryonic topogenesis are mediated by different cytoskeletal elements, which appear to act concurrently but independently after fertilization.

Results

Production of a Monoclonal Antibody Directed against P Granules

Our initial studies of P granules were done with a commercial preparation of fluorescein-conjugated rabbit anti-mouse IgG serum that contains antibodies directed against these granules (Strome and Wood, 1982). We subsequently obtained sera with similar properties from 2 of 26 rabbits tested at a local rabbit farm. These sera, in addition to staining P granules in the germ line at all stages, react with nongonadal tissues in later-stage larvae and adults. These results suggest that the antibodies in these sera that react with C. elegans antigens were produced in

response to naturally occurring nematode infections, and that P granules might be quite immunogenic.

To obtain more specific immunologic probes for P granules, we utilized the hybridoma technique of Kohler and Milstein (1975) to generate monoclonal antibodies against individual components in a heterogeneous immunogen. We immunized mice with a crude C. elegans egg homogenate, fused the mouse spleen cells to mouse myeloma cells to establish hybridoma cell lines, and screened individual hybridoma culture fluid samples by immunofluorescence microscopy. As described in Experimental Procedures, 96 samples were tested, from which we identified and cloned one hybridoma cell line (K76) that secretes IgM directed against P granules.

The K76 antibody reacts specifically with granules in the germ-line cells of embryos (see Figure 2) and around the germ nuclei in the developing gonads of larvae (Strome and Wood, 1982). In adult hermaphrodites, in addition to staining germ-line granules in the gonad, the K76 antibody stains the spermatheca. Similar spermathecal staining has been observed using several monoclonal antibodies with different specificities (J. Culotti, personal communication, and our unpublished results) and thus may result from nonspecific association rather than antigenic cross-reactivity. In contrast to the rabbit serum used earlier (Strome and Wood, 1982), the monoclonal antibody does not stain sperm from either hermaphrodites or males.

Both immunoprecipitation and gel blotting experiments to be described elsewhere indicate that the K76 antibody reacts with a ~40,000 dalton polypeptide. Moreover, this antigen is found in embryos as well as adult worms, arguing against the possibility that it is a spermathecal component. K76 antibody was used to follow segregation of P granules in the experiments described below.

Review of Early Embryonic Topogenesis and P-Granule Segregation

Development of the early C. elegans embryo has been characterized by light microscopy (von Ehrenstein and Schierenberg, 1980), by electron microscopy (Wolf et al., 1983), and by immunofluorescence microscopy (Strome and Wood, 1982; Albertson, 1983). A brief review of these observations is necessary to provide background for the experiments we describe here. At the time of fertilization, the only visible manifestation of asymmetry in the egg is the acentric location of the egg pronucleus near the future anterior pole of the embryo. The sperm normally penetrates the egg at the opposite pole. It is not known whether developmental polarity is established before fertilization, or whether the site of sperm entry defines the posterior pole. After fertilization the egg pronucleus resumes meiosis, and two polar bodies are extruded at the anterior pole. The egg then undergoes contractions of the anterior membrane and pseudocleavage, involving formation and then regression of a cleavage furrow (Figures 1a–1c). Concurrently with pseudocleavage, the egg pronucleus migrates toward the sperm pronucleus, and the sperm pronucleus begins to move centripetally (Figure 1b). The two meet in the posterior half of the embryo (Figure 1c). On the basis of staining of C. elegans zygotes with anti-tubulin antibodies and anti-centrosome antibodies, the centripetal movement of the sperm pronucleus appears to be mediated by growth of astral microtubules from the centriolar regions adjacent to the sperm pronucleus (Albertson, 1983, and our unpublished results). Growth of the asters accompanies the movement of the two pronuclei to the center of the embryo, where the growing spindle rotates 90° to its final position along the anterior–posterior axis (Figures 1d, 1e). First cleavage is asymmetric, giving rise to a smaller posterior germ-line cell (P1) and a larger anterior somatic blast cell (AB) (Figures 1g, 1h).

The P granules at the time of fertilization are small, numerous, and dispersed apparently randomly throughout the cytoplasm (Figure 2a). They begin to coalesce and become localized in the posterior cytoplasm during pseudocleavage and pronuclear migration. By the time the pronuclei meet, the granules are around the periphery of the zygote at the posterior pole (Figure 2b), so that they are segregated to the P1 cell at the first cleavage (Figure 2c). This process of coalescence and asymmetric localization occurs at each of the subsequent unequal cleavages that give rise to a smaller germ-line (P) cell and a larger somatic blast cell; consequently, P granules are progressively segregated to cells P1, P2, P3, and finally P4, the exclusive precursor of the germ line (Strome and Wood, 1982).

The granules become localized in the cytoplasm destined for the next P-cell daughter as early as prophase of each P-cell cycle (Figure 2c). During this localization, the granules are at the periphery of the cell. However, the segregation of granules is not always complete; sometimes granules are observed in the somatic sister of a P cell (Figure 2d). The fact that granules are rarely observed in the somatic cells of later-stage embryos suggests that P granules degenerate in the somatic cytoplasm. Therefore, the presence of detectable P granules in only the germ-line cells of later embryos probably results from two processes: movement of the granules into the cytoplasm destined for the next P-cell daughter and disappearance of granules remaining in the somatic blastomeres. The experiments described below were carried out to study the mechanism of the apparent P-granule movement and its relationship to other events that occur during the establishment of early embryonic polarity.

Requirements for Zygote Topogenesis and P-Granule Segregation

By analyzing mutant embryos and drug-treated wild-type embryos, we have examined both the requirement for fertilization and the involvement of the mitotic spindle, cytoplasmic microtubules, and microfilaments in the directed movement of cellular components.

Fertilization Is Required for the Asymmetric Localization of P Granules

P-granule behavior was observed in oocytes that had passed through the spermatheca in mutant worms defec-

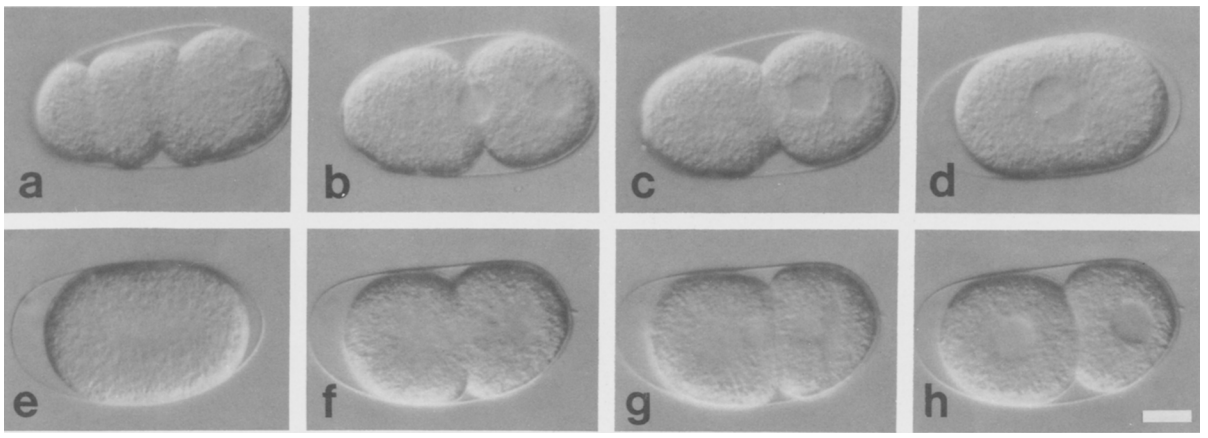
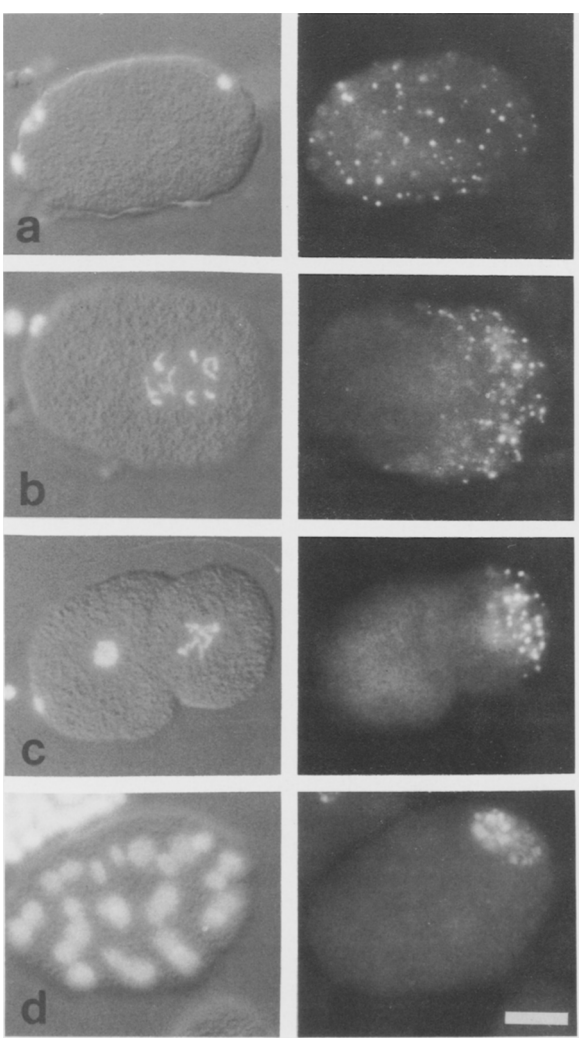


Figure 1. Nomarski Images of a Living Wild-type C. elegans Zygote Showing the Events Preceding First Cleavage

In this and all subsequent figures, embryos are oriented anterior-left, posterior-right. (a) Formation of pronuclei and contractions of the anterior membrane (approx. 27 min after fertilization at 25°C). The egg pronucleus at the anterior pole is not visible in this focal plane. (b) Pseudocleavage and pronuclear migration. (c) Meeting of the two pronuclei in the posterior half of the zygote. (d) Movement to the center and rotation of the pronuclei. (e) Formation of the first mitotic spindle. (f) Anaphase and the beginning of cleavage. The asters are visible as round, granule-free regions; their positions show the asymmetric location of the spindle along the anterior–posterior axis. (g) Telophase. Note the smaller disc-shaped aster in the posterior (P1) cell, as compared to the larger, spherical aster in the anterior (AB) cell. (h) Two-cell embryo. Bar: 10 μm.



tive in either sperm production or sperm function. The temperature sensitive (*ts*) allele *b245* at the *fem-2* locus prevents sperm production and converts hermaphrodite worms into "true females" at the restrictive temperature (L. Edgar, Ph.D. thesis, University of Colorado, 1982; Kimble et al., submitted). Two other *ts* alleles, *hc1* and *hc24* at the *fer-1* locus, result in production of defective sperm at the restrictive temperature (Ward and Carrel, 1979). In these mutant animals the oocytes are not fertilized; they undergo endoreplication of egg DNA, but no mitosis or cleavage. However, oocytes that come into contact with defective *fer-1(hc1)* sperm do show the increased Brownian and saltatory motion of cytoplasmic granules, termed "cytoplasmic activation," that is seen after normal fertilization; this cytoplasmic movement is not seen in oocytes produced by either mutant hermaphrodites that make no sperm or wild-type hermaphrodites that have exhausted their sperm supply (Ward and Carrel, 1979). In both *fem-2(b245)* and *fer-1(hc1* or *hc24)* oocytes, coalescence of P granules does occur after passage through the spermathecae, but segregation of granules to one end of the egg does not (Figure 3). Thus the oocyte apparently can initiate P-granule coalescence, perhaps triggered by ger-

Figure 2. Localization of P Granules after Fertilization

Embryos were cut out of hermaphrodites, fixed in cold methanol and acetone, and stained with K76 antibody and F-GAM as described in Experimental Procedures. The left panels show Nomarski images with DAPI-stained chromosomes, and the right panels show immunofluorescence images. (a) Zygote after meiosis II and prior to pronuclear migration. The egg pronucleus and both polar bodies are anterior (left), and the sperm pronucleus is posterior (right). Granules are dispersed throughout the cytoplasm. (b) Zygote at pronuclear meeting, showing localization of P granules at the posterior periphery. (c) Two-cell embryo in which the P1 cell (posterior, right) is in prophase and P granules are prelocalized in the region of cytoplasm destined for the next P-cell daughter. (d) 26-cell embryo in which some P granules are detected in the D cell as well as in the more intensely stained P4 cell above and to the left. Bar: 10 μm.

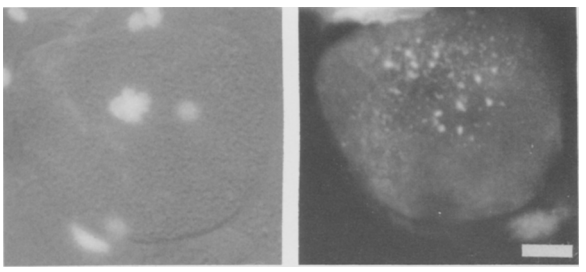


Figure 3. Immunofluorescence Staining of P Granules in a *fem-2(b245)* Oocyte

Oocytes were obtained from mutant hermaphrodites reared at the restrictive temperature (25°C). The left panel shows a Nomarski image with the DAPI-stained chromosomes of the oocyte (in the center and in focus), and the right panel shows P granules coalesced in the central region of the oocyte. Bar: 10 μm.

minal vesicle breakdown. However, fertilization appears to be requisite for P granules to become asymmetrically localized.

The Mitotic Spindle Does Not Mediate P-Granule Segregation

Mediation of P-granule segregation by the spindle, for example by selective attachment of the granules to the aster destined for the P cell, can be ruled out by three types of observations. First, as determined by staining zygotes with both anti-P-granule antibody and anti-tubulin antibody, P granules become localized in the posterior cytoplasm prior to rotation of the growing spindle to its final orientation along the anterior–posterior axis (Figure 4). Second, localization at subsequent cleavages occurs by the time of prophase, prior to spindle formation (Figure 2c; Wolf et al., 1983). Third, spindle orientation can be perturbed, either mutationally or with microtubule inhibitors, without altering P-granule segregation, as demonstrated in the following experiments.

The *ts* allele *b244* at the *zyg-9* locus inhibits pronuclear migration and alters the position and orientation of the mitotic spindle in zygotes from mutant hermaphrodites grown at the restrictive temperature. In *zyg-9(b244)* zygotes the egg pronucleus does not migrate to meet the sperm pronucleus. The first spindle forms around the sperm pronucleus at the posterior end of the zygote and does not rotate, remaining perpendicular to the anterior–posterior axis (Figure 5a; Albertson, 1983). As a result, first cleavage in *zyg-9(b244)* zygotes is longitudinal, generating two oblong blastomeres and an anterior cytoplast. The egg pronuclear DNA, which remains anterior during mitosis, is either bisected by the cleavage furrow or incorporated into one of the blastomeres. Despite the altered orientation of the spindle and cleavage furrow in these zygotes, P granules coalesce, become localized at the posterior pole, and so are distributed to both of the resulting blastomeres (Figure 5b). This result suggests that P granules do not segregate either with the microtubules of one half-spindle or with one nucleus, and that the positional information responsible for P-granule segregation is independent of spindle orientation. Furthermore, pronuclear migration is not required for P-granule localization in *zyg-9(b244)* zygotes.

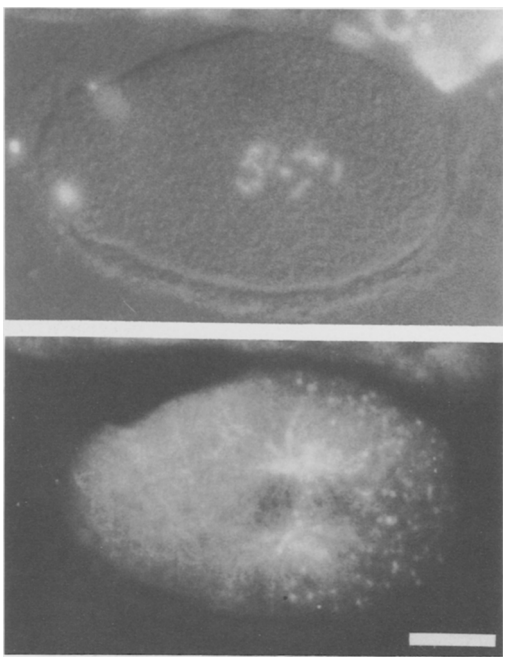


Figure 4. Costaining of P Granules and Microtubules in a Zygote prior to Rotation of the Two Pronuclei

Embryos were fixed and stained simultaneously with K76 antibody and an anti-tubulin antibody, followed by F-GAM. The top shows a Nomarski–DAPI image, and the bottom shows an immunofluorescence image. The asters have not yet rotated to their final positions along the anterior–posterior axis, but P granules are already localized in the posterior cytoplasm. (This embryo is at the stage shown in Figure 2b.) Bar: 10 μm.

These conclusions are supported by experiments using a drug treatment that partially phenocopies the *zyg-9* defect. In wild-type embryos the microtubule inhibitor nocodazole at 5 μg/ml interferes with normal spindle growth but does not prevent mitosis. It also prevents normal migration of the sperm pronucleus to the center of the embryo, presumably by blocking elongation of astral microtubules (Albertson, 1983). Instead, the egg pronucleus migrates to the posterior pole to meet the sperm pronucleus. The spindle remains small, posterior, and oriented perpendicular to the anterior–posterior axis (Figure 6a). The cleavage furrow bisects the spindle to yield an embryo with two oblong blastomeres and an anterior cytoplast (Figure 6b) that closely resembles a *zyg-9(b244)* embryo. As in *zyg-9(b244)* embryos, P granules become localized in the posterior cytoplasm and are distributed to both of the resulting blastomeres (Figure 6e).

Cytoplasmic Microtubules Are Not Required for P-Granule Segregation But Are Required for Pronuclear Migration

The foregoing results indicate that P-granule segregation is independent of mitotic spindle growth and orientation. To test whether P-granule segregation requires the integrity of cytoplasmic microtubules, colcemid, vinblastine, and griseofulvin were used to inhibit microtubule polymerization

in newly fertilized zygotes. Effects of these inhibitors are seen within 1 min in embryos made permeable as described in Experimental Procedures. When such embryos are exposed to concentrations of these inhibitors that prevent mitosis (50 μg/ml colcemid, 10 μg/ml vinblastine, or 20 μg/ml griseofulvin), several of the early events following fertilization are affected. (For comparison with the early events in an untreated wild-type zygote, see Figure 1.) As shown in Figure 7 and summarized in Table 1, migration of the egg pronucleus toward the sperm pronucleus as well as centripetal migration of the sperm pronucleus are inhibited in drug-treated zygotes. Both pronuclei remain at the positions they occupied when drug was added, until after the time of nuclear envelope breakdown (Figure 7b). In fact, the two pronuclei usually remain at their respective poles through at least one cycle of nuclear envelope breakdown and reformation. (In some embryos treated when pronuclear migration was already under way, pronuclear migration stopped until after nuclear envelope breakdown, and then the egg pronuclear DNA slowly moved posteriorly, perhaps as a result of the observed streaming of cytoplasm from anterior to posterior.) Despite the inhibition of pronuclear migration in drug-treated zygotes, pseudocleavage occurs normally (Figure 7a), and P-granule segregation is unaffected; granules become localized and stay at the posterior periphery of the zygote (Figure 7d, Table 1).

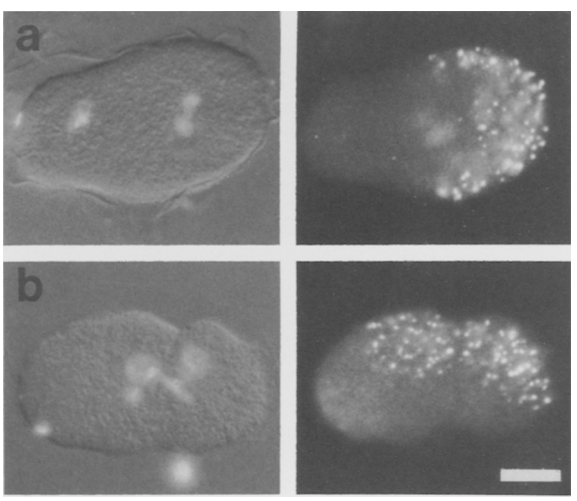


Figure 5. Immunofluorescence Staining of P Granules in *zyg-9(b244)* Embryos

Embryos were obtained from mutant hermaphrodites that had been transferred to 25°C for 5 hr. The left panels show Nomarski-DAPI images, and the right panels show immunofluorescence images. (a) Embryo during first cleavage. The nuclei derived from the sperm pronucleus (right) are at anaphase and show the orientation of the mitotic spindle perpendicular to the anterior–posterior axis. The egg pronucleus is still anterior. P granules are localized in the posterior cytoplasm. (b) Two-cell embryo after a longitudinal first cleavage showing the distribution of P granules to both resulting blastomeres. The blastomeres have rotated within the eggshell so that one blastomere lies posterior to the other, and the anterior cytoplast has fused with the anterior blastomere. (An analogous sequence of events can be seen in Figure 6.) Bar: 10 μm.

The processes of pronuclear migration and mitosis could be more sensitive to microtubule inhibitors than is P-granule segregation, such that the levels of inhibitors used were sufficient to inhibit only the former processes. To test this possibility, embryos were treated with higher concentrations of inhibitor (100 μg/ml colcemid, 50 μg/ml vinblastine, and 100 μg/ml griseofulvin.) These high concentrations result in the same inhibition of pronuclear migration as low concentrations but still do not affect pseudocleavage or P-granule localization (data not shown).

Evidence for disruption of microtubules in drug-treated zygotes was obtained by staining with anti-tubulin antibody, which revealed no microtubule fibers (Figures 8a, 8b). Microtubule arrays were present in other embryos, on the same slide, that had not been rendered permeable to drugs (Figure 8c). Thus we conclude that intact microtubules are not required for P-granule segregation, whereas they are required for the centripetal migration of the two pronuclei. As seen in *zyg-9(b244)* zygotes, pronuclear migration is not required for P-granule segregation.

Microfilament Function Is Required for Several Manifestations of Asymmetry, Including Segregation of P Granules

Another cytoskeletal component involved in motility, cleavage, and maintenance of cell shape is actin microfilaments. Cytochalasins D and B are inhibitors of microfilament-mediated processes. Zygotes made permeable to concentrations of cytochalasin D or B sufficient to prevent cleavage (1 μg/ml cytochalasin D or 8 μg/ml cytochalasin B)

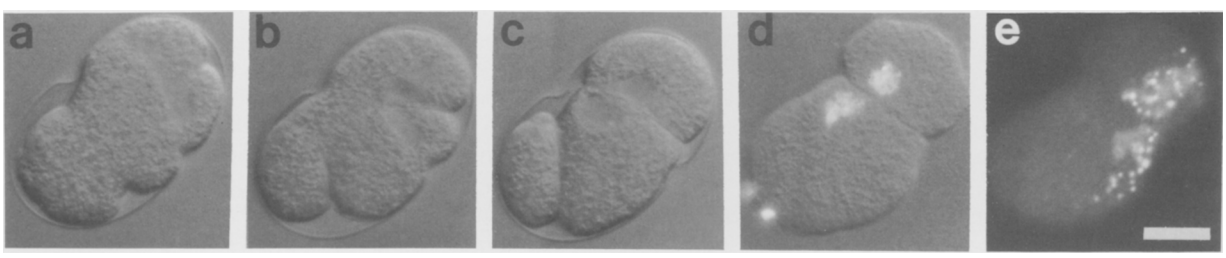


Figure 6. Effects of Nocodazole on Spindle Orientation and P-Granule Distribution

(a, b, c) Nomarski images of a living embryo in the presence of 5 μg/ml nocodazole. (a) The spindle is located posteriorly and oriented approximately perpendicular to the anterior–posterior axis. (b) The zygote is undergoing a longitudinal first cleavage and generating an anterior cytoplast. (c) The embryo is shown after cleavage and rotation of the blastomeres in the eggshell. (d) Nomarski-DAPI image of the same embryo, which was fixed after 20 min of observation. (e) Immunofluorescence image showing P granules in both blastomeres of the same embryo. Bar: 10 μm.

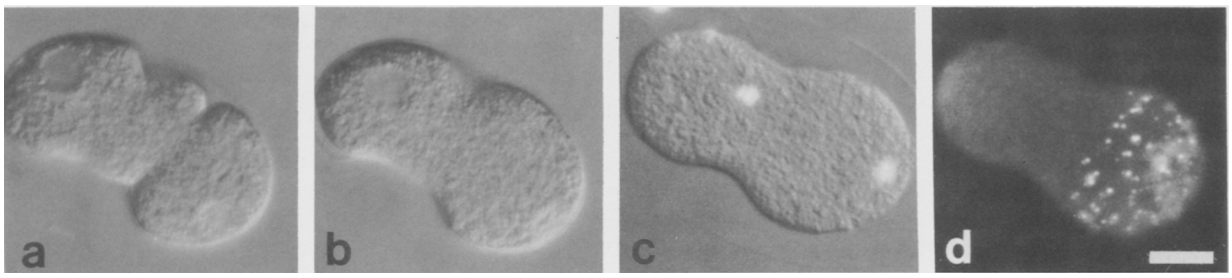


Figure 7. Effects of Colcemid on the Early Events following Fertilization

(a, b) Nomarski images of a living embryo rendered permeable to 50 μg/ml colcemid. (a) Shows contractions of the anterior membrane and pseudocleavage. (b) Shows inhibition of pronuclear migration until nuclear envelope breakdown. (c) Nomarski–DAPI image of the same embryo, which was fixed after 12 min of observation. (d) Immunofluorescence staining of P granules in the same embryo, showing their posterior localization. Bar: 10 μm.

Table 1. Effects of Inhibitors on Events between Fertilization and First Cleavage in a Wild-Type Zygote

	Microtubule Inhibitors			Microfilament Inhibitors	
Event	Colcemid (50 μg/ml)	Vinblastine (10 μg/ml)	Griseofulvin (20 μg/ml)	Cytochalasin D (1 μg/ml)	Cytochalasin B (8 μg/ml)
Pseudocleavage	+ 13/13	+ 9/9	+ 8/8	− 7/7	− 5/5
Migration of the egg pronucleus	− } 26/26[a]	− } 24/24[a]	− } 25/28[a,b]	+[c]	+[c]
Migration of the sperm pronucleus	− }	− }	− }	+[c]	+[c]
Rotation of the two pronuclei				+	+
Formation of the spindle				+ } 10/10	+ } 8/8
Swinging of the posterior aster				−	−
Asymmetric location of the spindle				−	−
Different aster morphologies				− }	− }
Localization of P granules at the posterior periphery	+ 12/12	+ 13/14[d]	+ 11/14[e]	− 20/22[f]	− 14/14

Embryos were rendered permeable to inhibitor either before or after pseudocleavage and then monitored for the occurrence of subsequent events. Therefore, the later events were scored in a larger number of embryos. Not all embryos followed by Nomarski microscopy were analyzed for P granules; some of the embryos were stained with anti-tubulin antibody to analyze microtubules, and some embryos were lost during fixation and staining. + indicates that the event occurs apparently normally, and − indicates an inhibitory effect of drug treatment. Numbers indicate the fraction of embryos analyzed that displayed the indicated behavior.

[a] In embryos in which pronuclear migration was prevented by a microtubule inhibitor, spindles did not form; therefore, other spindle-related events could not be monitored.

[b] In 3 of the 28 embryos, the egg pronucleus moved very slowly toward the posterior pole prior to and during breakdown of the nuclear envelope.

[c] The pronuclei migrated and met in the center of the zygote.

[d] In 1 of the 14 embryos, granules extended from the posterior pole down the center of the embryo.

[e] In 3 of the 14 embryos, P granules were present through the central core of cytoplasm as well as at the posterior pole. Higher concentrations (100 μg/ml) of griseofulvin seem toxic and often cause embryo disintegration. Such toxicity could explain the anomalous distribution of P granules in embryos treated with 20 μg/ml griseofulvin. This pattern of staining was not seen in untreated or colcemid-treated zygotes and was seen in 1 of 14 vinblastine-treated zygotes.

[f] In 20 of 22 embryos, P granules were present in the center of the zygote; in the other 2, granules were localized in the posterior cytoplasm.

appear unable to develop normal embryonic asymmetry by several criteria, as shown in Figure 9 and summarized in Table 1.

In embryos treated very early, polar body extrusion is inhibited. The contractions of the anterior membrane and pseudocleavage that normally accompany migration of the pronuclei are not seen (Figures 9a, 9b; Table 1). Although cytochalasin does not inhibit migration of the two pronuclei, it does affect the position at which they meet. Normally, the egg pronucleus meets the sperm pronucleus in the posterior hemisphere of the zygote, usually within approximately 20%–40% of the egg length from the posterior pole (Figure 1c). In cytochalasin-treated embryos, the egg pronucleus migrates only as far as the center of the embryo, where it remains until the sperm pronucleus arrives there also (Figure 9b).

Cytochalasin also affects the asymmetry of the first mitotic spindle. In normal zygotes the spindle is asymmetric in at least three respects (see Figure 1): first, the posterior aster swings from side to side early in mitosis; second, as mitosis progresses the spindle assumes a more and more asymmetric position along the anterior–posterior axis, the posterior aster being much closer to the posterior pole of the zygote than the anterior aster to the anterior pole; third, late in mitosis the two asters have strikingly different morphologies, the posterior (P1) aster being flattened and disc-shaped, compared to the large round anterior (AB) aster (Figure 1g). These asymmetries of the mitotic spindle

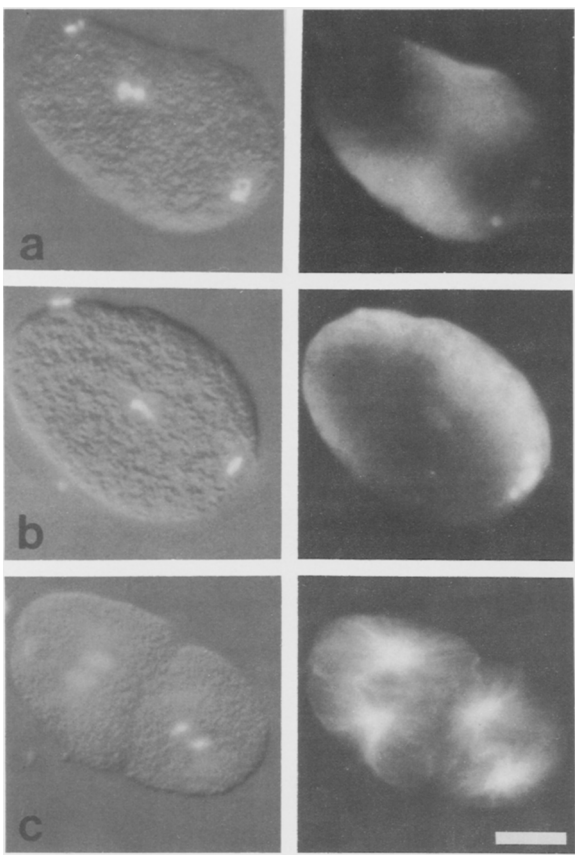


Figure 8. Staining of Colcemid-Treated Embryos with Anti-tubulin Antibody

The embryos were exposed to 50 μg/ml colcemid for 12 min, followed by fixation and staining with anti-tubulin antibody. The left panels show Nomarski–DAPI images, and the right panels show immunofluorescence images. (a, b) Two zygotes in which colcemid has arrested pronuclear migration. The anti-tubulin antibody does not stain fibers but rather stains diffuse masses of cytoplasmic material, presumably depolymerized tubulin. (c) Two-cell embryo that was not rendered permeable to inhibitor, showing staining of microtubule arrays. Bar: 10 μm.

are not seen in cytochalasin-treated embryos: the posterior aster swings much less or not at all; the spindle remains centrally and symmetrically located (Figures 9d–9f); and the anterior and posterior asters are similar in morphology (Figures 9e, 9f). Staining of cytochalasin-treated zygotes with anti-tubulin antibody corroborates the similarity of the anterior and posterior asters and reveals apparently normal microtubule arrays and spindles, although spindles become multipolar in the absence of cytokinesis (data not shown).

In addition to affecting spindle asymmetry, cytochalasin treatment affects the ability of C. elegans zygotes to partition P granules asymmetrically. In untreated embryos, P granules are distributed apparently randomly in the cytoplasm during interphase and are present at the periphery when they are becoming asymmetrically localized. In cytochalasin-treated embryos, P granules do not assume a peripheral location and aggregate in the center of the embryo (Figure 9i, Table 1). When such treated embryos are allowed to undergo one or more rounds of mitosis, P granules remain in the center of the cleavage-blocked zygote and are no longer segregated. When later-stage embryos are treated with cytochalasin, P granules show the same behavior—namely, aggregation in the center of the cleavage-blocked P blastomere.

Discussion

The filamentous systems of the cytoskeleton may serve any of at least three functions in the directed movement of cellular constituents: provision of a structural framework, generation of motive force, and control of directionality. An example in which microtubules serve a structural function is provided by the erythrophore system; microtubules seem to provide the tracks along which pigment granules are translocated (Beckerle and Porter, 1983). Microtubules in flagella and cilia are involved in generating motive force (Warner, 1979), and astral microtubules determine the plane of cleavage and thus provide directionality during cytokinesis (Mazia, 1961). Actin filaments are believed to serve a structural function in maintenance of cell shape (Burgess and Schroeder, 1977; Taylor and Condeelis, 1979) as well as participating with myosin to generate force, for instance during muscle contraction and during cytokinesis of nonmuscle cells (Goldman et al., 1976; Pollard and Weihing, 1974; Inoue and Stevens, 1975). Actin filament contraction may also provide the motive force for ooplasmic segregation in ascidian embryos (Zalokar, 1974; Jeffery and Meier, 1983). An elegant example of directionality being provided by microfilaments is the highly organized streaming of cytoplasm along bundles of unidirectional actin filaments in characean algae (Kersey et al., 1976). Our results indicate that both microtubules and microfilaments participate in directed movement of several cellular components in C. elegans zygotes, and that the movements directed by these two filament systems, while concurrent, are independent.

Microtubules

Because P granules become localized normally in the posterior cytoplasm of *zyg-9(b244)* zygotes and nocodazole-treated wild-type zygotes in which the first mitotic spindle is perpendicular to the normal wild-type orientation, we conclude that P granules are not segregated via association with spindle microtubules. The results of treating C. elegans embryos with other microtubule inhibitors demonstrate that intact cytoplasmic microtubules are also not required for granule localization. Colcemid, vinblastine, and griseofulvin are chemically unrelated microtubule poisons that prevent microtubule assembly by binding to tubulin dimers, probably at different sites (Wilson and Bryan, 1974; Wehland et al., 1977). Griseofulvin may bind microtubule-associated proteins as well (Sloboda et al., 1976; Wehland et al., 1977). All three inhibitors quickly permeate C. elegans embryos and result in complete inhibition of mitosis. Furthermore, staining of treating embryos with anti-tubulin antibody demonstrated the absence of detectable microtubule fibers, indicating efficient microtubule disruption.

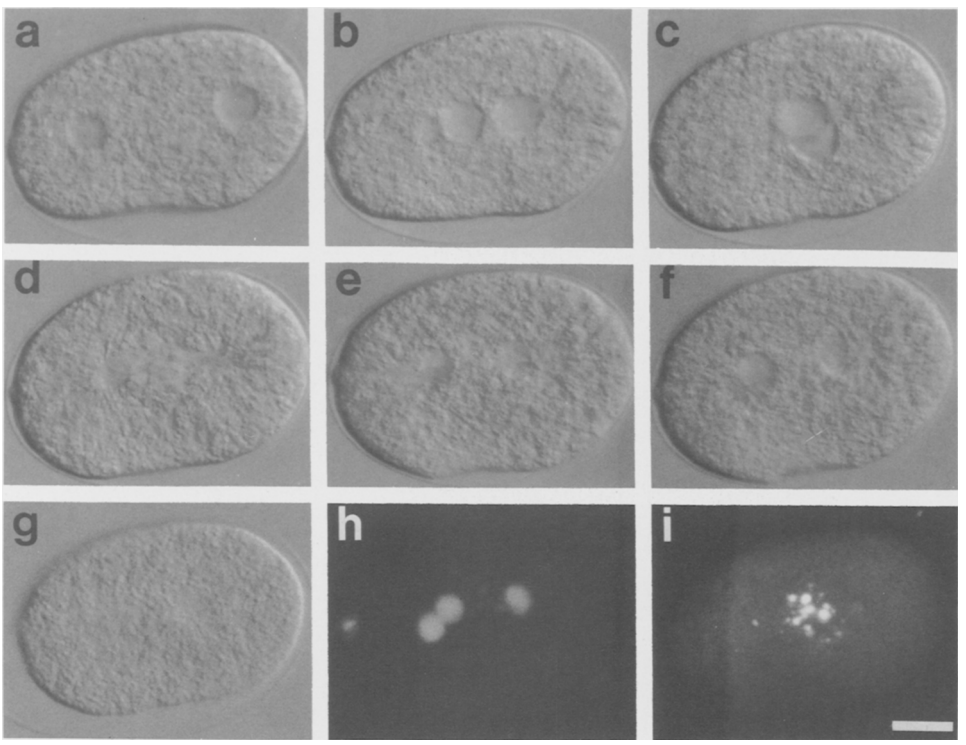


Figure 9. Effects of Cytochalasin D on Early Events following Fertilization

(a–f) Nomarski images of a living zygote rendered permeable to 1 μg/ml cytochalasin D. (a, b) Pronuclear migration in the absence of pseudocleavage or other contractions of the membrane. The pronuclei meet in the center of the zygote. (c) Rotation of the two pronuclei. (d) Formation of the first mitotic spindle. Note that the asters are approximately equidistant from the poles. (e) Anaphase. (f) Telophase. The reforming nuclei in (e) and (f) are symmetrically positioned with respect to the ends of the zygote, and the asters distal to each nucleus appear similar. (g, h) Nomarski and DAPI images of the same zygote, which was observed for 26 min, then fixed. This zygote was treated early enough that cytochalasin D prevented extrusion of the second polar body, and the extra DNA is seen next to one of the telophase nuclei. (i) Immunofluorescence staining showing P granules in the center of the zygote. Bar: 10 μm.

Nevertheless, P granules appear to segregate normally in the presence of these drugs. Although these results show that P-granule segregation is microtubule-independent, an earlier involvement of microtubules cannot be ruled out. All the zygotes analyzed were exposed to drug during or after completion of meiosis II. Therefore, our experiments do not address the possibility that microtubules play an initial role in establishing embryonic polarity or setting up an actin-mediated motility system.

Although colcemid, vinblastine, and griseofulvin do not prevent segregation of P granules, they do inhibit migration of the egg pronucleus. The benzimidazole derivative benomyl shows similar inhibition (Dr. Donna Albertson, personal communication). Evidence that these effects are directly due to disruption of microtubules and not to more general effects on cell integrity or viability is that the normally concurrent events of pseudocleavage and P-granule segregation are not inhibited. Pronuclear migration is also mediated by microtubules in sea urchin zygotes; microtubules can be seen extending from the sperm microtubule-organizing centers to the egg pronucleus, and pronuclear migration is inhibited by griseofulvin, vinblastine, colchicine, and colcemid, but not by cytochalasin B (Bestor and Schatten, 1981; Schatten and Schatten, 1981; Zimmerman and Zimmerman, 1967). In C. elegans, the sperm asters are not yet well formed when the egg pronucleus starts to migrate, and microtubules have not been observed connecting the two pronuclei. The effect of microtubule inhibitors on migration of the egg pronucleus is the first indication of a role of microtubules in this process.

Microfilaments

Inhibition experiments with cytochalasins B and D indicate that microfilaments in the C. elegans zygote are involved in processes that generate asymmetry, including P-granule segregation. Both cytochalasins prevent actin filament elongation by binding to sites at the fast-polymerizing end of actin filaments (Brenner and Korn, 1979; Flanagan and Lin, 1980; MacLean-Fletcher and Pollard, 1980). We used cytochalasin D for most experiments because it has a higher affinity for actin than does cytochalasin B (Flanagan and Lin, 1980) and does not interfere with hexose transport, as does cytochalasin B (Tanenbaum, 1978). However, both cytochalasins gave the same results. In embryos treated with these drugs, pseudocleavage is inhibited; the membrane does not undergo visible contractions; the pronuclei meet in the center of the zygote rather than the posterior; and the resulting spindle shows none of the normal asymmetries in behavior, position, and morphology.

Likewise, the P granules fail to become asymmetrically localized, but instead aggregate in the central region of cytoplasm. These results indicate that actin filaments are involved either directly in positioning P granules or indirectly in maintaining the integrity of other cytoskeletal functions required for manifestation of zygotic polarity. Again the normal occurrence of other early events such as pronuclear migration and spindle formation indicates that the observed effects of cytochalasins are not simply results of general cell debility.

Interestingly, the *ts* allele *g48* at the *emb-27* locus causes effects similar to those seen with cytochalasins. Mutant zygotes are defective in extruding polar bodies and in cleavage (Denich et al., submitted). The embryos accumulate nuclei, although mitoses often appear aberrant. P granules coalesce in these mutant embryos, but they do not assume a peripheral or asymmetric location; instead they aggregate in the center of the embryo, as in cytochalasin-treated embryos.

In C. elegans, as in other embryos (Kirschner et al., 1980; Gerhart et al., 1981), fertilization appears to trigger a series of directed intracellular movements that are mediated by microtubules and microfilaments. We have shown that some of these events, which normally occur concurrently, can be uncoupled by drugs or mutation and therefore appear to be independent. Microtubule inhibitors and a mutation at the *zyg-9* locus prevent pronuclear migration without affecting pseudocleavage or P-granule localization. Microfilament inhibitors prevent pseudocleavage and proper P-granule localization without preventing pronuclear migration. Manifestation of asymmetry in general appears to depend on microfilament-mediated events. Two of these events, pseudocleavage and P-granule segregation, occur concurrently, suggesting that they could be mechanistically related.

Experimental Procedures

Nematode Strains and Maintenance

Wild-type (N2) Caenorhabditis elegans strain Bristol was cultured at 16°C on agar plates with Escherichia coli as food source (Brenner, 1974). Strains carrying the temperature-sensitive mutations *zyg-9(b244), emb-27(g48), fem-2(b245),* and *fer-(hc1* or *hc24)* were maintained at the same (permissive) temperature (Wood et al., 1980; Denich et al., submitted; Edgar, op. cit; Ward and Carrel, 1979). Mutant embryos were obtained from gravid *zyg-9(b244)* or *emb-27(g48)* hermaphrodites that had been transferred to the restrictive temperature, 25°C, for 5 hr. Unfertilized oocytes were obtained from *fem-2(b245)* or *fer-1(hc1* or *hc24)* hermaphrodites that had been raised at the restrictive temperature, 25°C.

Antigen Preparation and Immunization

The antigen was a homogenate of early C. elegans embryos obtained from synchronously growing young adult worms. To obtain synchronous populations, embryos were isolated by dissolving gravid adults with 1% hypochlorite, 0.5 M KOH for 3–5 min, and allowed to hatch onto agar plates without food for 12 hr. The newly hatched larvae were transferred to plates with E. coli to initiate larval growth. When a majority of the worms contained several early embryos, the animals were dissolved as described above and the embryos were isolated and washed by centrifugation. Early embryos (2×10^5 to 5×10^5) were disrupted in 0.5 ml phosphate-buffered saline (PBS, pH 7.4; 150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$) by sonication (two 1 min bursts at setting 7 of a Branson sonifier). A female BALB/c mouse was injected intraperitoneally with 0.5 ml embryo homogenate emulsified with 0.5 ml complete Freund's adjuvant, and the animal was boosted intravenously 27 and 34 days later with 0.2 ml embryo homogenate in PBS.

Hybridoma Fusion

Three days after the last intravenous hyperimmunization, the mouse was sacrificed, its spleen removed, and 1.5×10^8 splenic lymphocytes were fused with 1.25×10^7 SP2/0 myeloma cells by using 30% (w/v) polyethylene glycol 1000, as described by Izant and McIntosh (1980). The fused cells were separated into ninety-six 1 ml wells, and when cell growth was apparent (2–3 weeks after fusion), samples of spent tissue culture medium from each well were assayed by immunofluorescence microscopy. Hybrid cells producing antibodies of interest were cloned twice, either in soft agar containing tissue culture medium (Izant and McIntosh, 1980) or by limiting dilution using a procedure modified from Mishell and Shigi (1980). Spent tissue culture medium from the antibody-producing hybrid cell line K76 was used for the experiments described in the text.

Fixation and Immunofluorescence Staining of Embryos

For screening the hybridoma supernatant medium, large quantities of C. elegans embryos were prepared by NaOCl/KOH digestion of gravid adults. A small drop containing embryos including all developmental stages in H_2O was placed in each well of an 8-well Teflon-coated slide (Cel-Lin Assoc.) treated with polylysine. Each slide was covered with another standard slide, frozen on dry ice, fixed in methanol at 4°C, and air dried as previously described (Strome and Wood, 1982). Each well was incubated with 30 μl of 1.5% ovalbumin/1.5% bovine serum albumin in PBS for 1 hr at room temperature, followed by 30 μl of hybridoma culture fluid for 6–12 hr at 4°C. The slides were washed in PBS at 16°C for 1 hr, and each well was incubated for 6–12 hr at 4°C with 30 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG serum (F-GAM; U. S. Biochemicals) diluted 1:100 in PBS. This secondary antibody detected both mouse IgG and IgM, the latter presumably via the κ light chain. The slides were washed in PBS at 16°C for 90 min, treated with diamidinophenylindole (DAPI) hydrochloride (0.5 μg/ml; Boehringer Mannheim) in PBS, rinsed with H_2O, and mounted in Gelutol (Monsanto) mounting fluid.

Oocytes and early-stage embryos used to analyze early events were obtained by cutting hermaphrodites open in a small drop of M9 salts solution (Brenner, 1974) on a polylysine-treated slide. A coverslip was placed over the sample, and the slide was immersed in liquid nitrogen for 2 min. The coverslip was removed, and the embryos were fixed in methanol at 4°C for 20 min, followed by acetone at 4°C for 10 min, and then air dried. Immunofluorescence staining of embryos was carried out as described above, using the K76 monoclonal antibody to stain P granules and a monoclonal antibody directed against Drosophila α-tubulin (kindly provided by Dr. Margaret Fuller) to stain microtubules. The supernatant culture medium containing anti-tubulin antibody was diluted 1:50 before use.

Treatment of Embryos with Inhibitors

Stock solutions of inhibitors were prepared as follows: cytochalasin D (Sigma) at 2 mg/ml in 95% ethanol, cytochalasin B (Sigma) at 10 mg/ml in 95% ethanol, nocodazole (Aldrich) at 5 mg/ml in dimethylsulfoxide, colcemid (Sigma) at 1 mg/ml in H_2O, vinblastine sulfate (Lilly) at 10 mg/ml in methanol, and griseofulvin (Sigma) at 100 mg/ml in dimethyl formamide. Stock solutions were stored at 4°C and added to embryonic culture medium (50% fetal calf serum; 80 mM NaCl; 20 mM KCl; 10 mM $MgCl_2$; 5 mM HEPES, pH 7.2) immediately before use.

Pronuclear-stage zygotes were cut out of hermaphrodites, transferred to polylysine-treated slides, resuspended in embryonic culture medium containing inhibitor, covered with a coverslip, and rendered permeable to external solutions by repeatedly deforming the eggshell with gentle pressure on the coverslip (Laufer et al., 1980). Nocodazole permeates pronuclear-stage embryos without pressure. All the other inhibitors require cracking of the eggshell and vitelline membrane to be effective. Embryos rendered permeable in the presence of embryonic culture medium alone or medium containing the same concentration of solvent as added with the various inhibitors underwent normal early development. Embryos were observed with a Wild dissecting microscope during preparation and pressure treatment, and then examined immediately with Normarski optics to assess penetration of inhibitors. Penetration was judged both by the integrity of the vitelline membrane and eggshell and by the consistency of the cyto-

plasm, which took on an altered appearance in the presence of inhibitors, and was confirmed subsequently by observation and staining as described in the text.

Microscopy

A Zeiss Photomicroscope III equipped with Nomarski differential interference contrast and epifluorescence optics was used for observation and photography, in a room maintained at about 25°C. Kodak Tri-X film was exposed at ASA 1600 and developed in Diafine Two-Bath Developer (Acufine, Inc.). Living embryos rendered permeable to inhibitors were observed and photographed with Nomarski optics and then were frozen, fixed, and stained with antibody as described above. Fixed embryos were photographed first with 440–490 nm epi-illumination to visualize fluorescein immunofluorescence and then simultaneously with visible transmitted light and 365 nm epi-illumination to visualize the embryo and DAPI-stained chromosomes. Embryos are oriented anterior-left, posterior-right in all photographs.

Acknowledgments

We are grateful to Jonathan Izant for helpful advice on monoclonal antibodies and to L. S. B. Goldstein, Richard McIntosh, Kenneth Kemphues, and Mary Beckerle for valuable discussions. This research was supported by a postdoctoral fellowship to S. S. from the National Institutes of Health (F32-HD05845) and by grants to W. B. W. and S. S. from the National Institutes of Health (HD14958) and the American Cancer Society (CD-96).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 25, 1983

References

Albertson, D. G. (1983). Formation of first cleavage spindle in nematode embryos. Dev. Biol., in press.

Beams, H. W., and Kessel, R. G. (1974). The problem of germ cell determinants. Int. Rev. Cyt. *39*, 413–479.

Beckerle, M. C., and Porter, K. R. (1983). Analysis of the role of microtubules and actin in erythrophore intracellular motility. J. Cell Biol. *96*, 354–362.

Bestor, T. H., and Schatten, G. (1981). Anti-tubulin immunofluorescence microscopy of microtubules present during pronuclear movements of sea urchin fertilization. Dev. Biol. *88*, 80–91.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Brenner, S. L., and Korn, E. D. (1979). Substoichiometric concentrations of cytochalasin D inhibit actin polymerization. J. Biol. Chem. *254*, 9982–9985.

Burgess, D. R., and Schroeder, T. E. (1977). Polarized bundles of actin filaments within microvilli of fertilized sea urchin eggs. J. Cell Biol. *74*, 1032–1037.

Eddy, E. M. (1975). Germ plasm and the differentiation of the germ cell line. Int. Rev. Cytol. *43*, 229–280.

Flanagan, M. D., and Lin, S. (1980). Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. J. Biol. Chem. *255*, 835–838.

Gerhart, J., Ubbels, G., Black, S., Hara, K., and Kirschner, M. (1981). A reinvestigation of the role of the grey crescent in axis formation in *Xenopus laevis*. Nature *292*, 511–516.

Goldman, R., Pollard, T., and Rosenbaum, J., eds. (1976). Cell Motility. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Illmensee, K., and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. Proc. Nat. Acad. Sci. USA *71*, 1016–1020.

Illmensee, K., and Mahowald, A. P. (1976). The autonomous function of germ plasm in a somatic region of the *Drosophila* egg. Exp. Cell Res. *97*, 127–140.

Inoue, S., and Stephens, R., eds. (1975). Molecules and Cell Movement. New York: Raven Press.

Izant, J. G., and McIntosh, J. R. (1980). Microtubule-associated proteins: a monoclonal antibody to MAP2 binds to differentiated neurons. Proc. Nat. Acad. Sci. USA *77*, 4741–4745.

Jeffery, W. R., and Meier, S. (1983). A yellow crescent cytoskeletal domain in ascidian eggs and its role in early development. Dev. Biol. *96*, 125–143.

Kersey, Y. M., Hepler, P. K., Palevitz, B. A., and Wessells, N. K. (1976). Polarity of actin filaments in characean algae. Proc. Nat. Acad. Sci. USA *73*, 165–167.

Kirschner, M., Gerhart, J. C., Hara, K., and Ubbels, G. A. (1980). Initiation of the cell cycle and establishment of bilateral symmetry in *Xenopus* eggs. Symp. Soc. Dev. Biol. *38*, 187–215.

Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature *256*, 495–497.

Laufer, J. S., Bazzicalupo, P., and Wood, W. B. (1980). Segregation of developmental potential in early embryos of Caenorhabditis elegans. Cell *19*, 569–577.

MacLean-Fletcher, S., and Pollard, T. D. (1980). Mechanism of action of cytochalasin B on actin. Cell *20*, 329–341.

Mahowald, A. P. (1977). The germ plasm of *Drosophila*: an experimental system for the analysis of determination. Am. Zool. *17*, 551–563.

Mazia, D. (1961). Mitosis and the physiology of cell division. In The Cell. (New York: Academic Press), pp. 80–412.

Mishell, B. B., and Shigi, S. M. (1980). Selected Methods in Cellular Immunology. (San Francisco: Freeman and Co.).

Pollard, T. D., and Weihing, R. R. (1974). Actin and myosin and cell movement. CRC Crit. Rev. Biochem. *2*, 1–65.

Schatten, G., and Schatten, H. (1981). Effects of motility inhibitors during sea urchin fertilization. Exp. Cell Res. *135*, 311–330.

Sloboda, R. D., Malawista, S. E., Van Blaricom, G., Creasy, W. A., and Rosenbaum, J. L. (1976). Griseofulvin: association with tubulin and inhibition of *in vitro* microtubule assembly. J. Cell Biol. *70*, 290a.

Strome, S., and Wood, W. B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. Proc. Nat. Acad. Sci. USA *79*, 1558–1562.

Sulston, J., Schierenberg, E., White, J., and Thomson, N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Dev. Biol., in press.

Tanenbaum, S. W., ed. (1978). Cytochalasins: Biochemical and Cell Biological Aspects. (Amsterdam: Elsevier/North Holland Biomedical Press).

Taylor, D., and Condeelis, J. (1979). Cytoplasmic structure and contractility in amoeboid cells. Int. Rev. Cytol. *56*, 57–144.

von Ehrenstein, G., and Schierenberg, E. (1980). Cell lineages and development of *Caenorhabditis elegans* and other nematodes. In Nematodes as Biological Models, *1*, B. M. Zuckerman, ed. (New York: Academic Press), pp. 1–71.

Ward, S., and Carrel, J. A. (1979). Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. Dev. Biol. *73*, 304–321.

Warner, F. D. (1979). Cilia and flagella: microtubule sliding and regulated motion. In Microtubules, K. Roberts and J. S. Hyams, eds. (New York: Academic Press), pp. 359–380.

Wehland, J., Herzog, W., and Weber, K. (1977). Interaction of griseofulvin with microtubules, microtubule protein and tubulin. J. Mol. Biol. *111*, 329–342.

Whittaker, J. R. (1982). Muscle lineage cytoplasm can change the developmental expression in epidermal lineage cells of ascidian embryos. Dev. Biol. *93*, 463–470.

Wilson, E. B. (1925). The Cell in Development and Heredity. (New York: McMillan).

Wilson, L., and Bryan, J. (1974). Biochemical and pharmacological properties of microtubules. Adv. Cell Mol. Biol. *3*, 22–72.

Wolf, N., Priess, J., and Hirsh, D. (1983). Segregation of germline granules in early embryos of *Caenorhabditis elegans:* an electron microscopic analysis. J. Embryol. Exp. Morphol. *73*, 297–306.

Wood, W. B., Hecht, R., Carr, S., Vanderslice, R., Wolf, N., and Hirsh, D. (1980). Parental effects and phenotypic characterization of mutations that

affect early development in *Caenorhabditis elegans*. Dev. Biol. *74*, 446–469.

Zalokar, M. (1974). Effect of colchicine and cytochalasin B on ooplasmic segregation of ascidian eggs. Wilhelm Roux's Arch. Dev. Biol. *175*, 243–248.

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

Note Added in Proof

While this article was in preparation, two other monoclonal antibodies that also appear to stain P granules were reported by Yamaguchi et al., Dev. Growth Differn. *25*, 121–131, 1983.