Brief cytochalasin-induced disruption of microfilaments during a critical interval in 1-cell C. elegans embryos alters the partitioning of developmental instructions to the 2-cell embryo

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Summary

We are investigating the involvement of the microfilament cytoskeleton in the development of early Caenorhabditis elegans embryos. We previously reported that several cytoplasmic movements in the zygote require that the microfilament cytoskeleton remain intact during a narrow time interval approximately three-quarters of the way through the first cell cycle. In this study, we analyze the developmental consequences of brief, cytochalasin D-induced microfilament disruption during the 1-cell stage. Our results indicate that during the first cell cycle microfilaments are important only during the critical time interval for the 2-cell embryo to undergo the correct pattern of subsequent divisions and to initiate the differentiation of at least 4 tissue types. Disruption of microfilaments during the critical interval results in aberrant division and P-granule segregation patterns, generating some embryos that we classify as 'reverse polarity', 'anterior duplication', and 'posterior duplication' embryos. These altered patterns suggest that microfilament disruption during the critical interval leads to the incorrect distribution of developmental instructions responsible for early pattern formation. The strict correlation between unequal division, unequal germ–granule partitioning, and the generation of daughter cells with different cell cycle periods observed in these embryos suggests that the three processes are coupled. We hypothesize that (1) an 'asymmetry determinant', normally located at the posterior end of the zygote, governs asymmetric cell division, germ–granule segregation, and the segregation of cell cycle timing elements during the first cell cycle, and (2) the integrity or placement of this asymmetry determinant is sensitive to microfilament disruption during the critical time interval.

Key words: C. elegans, microfilaments, partitioning, cytochalasin.

Introduction

One mechanism that has been hypothesized to explain the generation of cell differences in early embryos is that developmental instructions are differentially partitioned to embryonic cells during the early cleavages. Although microsurgical and cleavage-blocking experiments in a number of organisms support this hypothesis, the nature of the developmental instructions and the mechanisms by which they are distributed are still not well understood (see Davidson, 1986 for review). In Caenorhabditis elegans, several lines of evidence indicate the importance of partitioned maternal information (for review see Strome, 1989). (1) In cleavage-blocked embryos, expression of lineage-specific markers occurs only in the cells that would normally give rise to the assayed lineage. This implies that instructions for the expression of these markers are partitioned to the correct lineages during early embryogenesis (Laufer et al. 1980; Cowan and McIntosh, 1985). (2) When the cytoplasmic content of early blastomeres is altered, either by extruding cytoplasm from the embryo or by mixing cytoplasm from different blastomeres, the blastomeres' cell cycling times, cleavage patterns, and developmental fates are altered, implying that cytoplasmic factors are responsible for the behavior of early blastomeres (Schierenberg, 1985; 1988). (3) A recently identified class of mutations, called par (for partitioning-defective) mutations, appear to affect the partitioning of cytoplasmic factors that control cell cycling times, cell division patterns, and differentiation pathways of early blastomeres (Kemphues et al. 1988). In this paper, we present an investigation of the mechanism of cytoplasmic localization and the roles of some of the localized factors in early C. elegans development.
A number of cytoplasmic rearrangements occur during the first cell cycle of *C. elegans* embryos. These include (1) a series of contractions of the anterior portion of the embryo, called pseudocleavage, (Hirsh et al. 1976), (2) streaming of cytoplasm toward the posterior end of the embryo (Nigon, 1960), (3) concentration of foci of filamentous actin in or around the anterior cortex of the embryo (Strome, 1986), (4) segregation of cytoplasmic germ-line or P granules to the posterior cortex of the embryo (Strome and Wood, 1982; Yamaguchi et al. 1983), and (5) movement of the mitotic spindle toward the posterior end of the embryo (Albertson, 1984). The zygote subsequently undergoes an unequal division, generating two blastomeres that differ in size, division pattern, and developmental fate (Sulston et al. 1983).

In previous studies, we determined that microfilaments play a critical role in the cytoplasmic rearrangements described above, but that during this early phase microfilaments are required only during a short (5–10 min) interval about 3/4 of the way through the first cell cycle (Hill and Strome, 1988). In this study, we demonstrate that disruption of microfilaments during the previously defined critical interval has profound and interesting effects on subsequent development; treated embryos often generate cells in a reverse polarity or duplicated pattern and fail to generate differentiated cell types later in development. Our results suggest that microfilaments participate in the segregation of developmental instructions that are responsible for cell division patterns, cell cycle timing, and P-granule segregation patterns. Furthermore, the segregation of instructions that control cell division patterns, cell cycle timing, and P-granule segregation appear to be coupled.

Materials and methods

**Maintenance of *C. elegans***

Wild-type worms were grown at 16°C on NGM plates with *Escherichia coli* strain OP50 as a food source (Brenner, 1974).

**Analysis of the development of embryos ‘pulsed’ with cytochalasin D**

The cytochalasins disrupt the actin cytoskeleton and appear to be relatively devoid of toxic side-effects in cells (Brenner and Korn, 1979; Schliwa, 1986; Toyama and Toyama, 1988). We previously analyzed and discussed the effects of cytochalasins on the microfilament cytoskeleton and on events in the 1-cell *C. elegans* embryo (Strome and Wood, 1983; Hill and Strome, 1988). For our analysis of embryos ‘pulsed’ with cytochalasin D (CD), we divided the first cell cycle into three time intervals defined by developmental events (Hill and Strome, 1988): (1) early, just after the completion of meiosis until the onset of egg pronuclear migration; (2) middle, from the onset of egg pronuclear migration until pronuclear meeting; (3) late, from pronuclear meeting until late anaphase. The middle interval is the ‘critical’ period, when microfilament disruption results in the loss of zygotic asymmetry.

To disrupt microfilaments during these intervals, embryos were dissected on a poly-lysine-coated glass slide from adult hermaphrodite gonads in a drop of egg buffer (EB; 118 mm-NaCl, 40 mm-KCl, 3.4 mm-CaCl₂, 3.4 mm-MgCl₂, 5 mm-Hepes, pH 7.4, 50% fetal calf serum; Edgar and McGhee, 1986) containing 2 μg ml⁻¹ CD (Strome and Wood, 1983). Embryos were overlaid with an 18×18 mm glass coverslip, with high vacuum grease applied along two edges to serve as a spacer. The embryos were permeabilized by applying gentle pressure on the coverslip. After exposure of the embryos to CD for the desired period of time, CD was removed by wicking embryonic culture medium (ECM; 90 mm-NaCl, 50 mm-KCl, 4 mg ml⁻¹ hyaluronic acid, 10% fetal calf serum; Lauffer et al. 1980) under the coverslip from one side to the other, using a paper towel. Success of initial permeabilization was judged by two criteria: (1) the altered appearance of the cytoplasm that is characteristic of CD-treated embryos (Strome and Wood, 1983), and (2) the inhibition of known microfilament-dependent events (Strome and Wood, 1983). After the CD pulse, some embryos were monitored continuously throughout the early cell divisions, for up to 4–6 h at 16°C by Nomarski differential interference contrast microscopy.

The anterior–posterior orientation of embryos was determined by the position of the polar body and by monitoring the migration patterns of the two pronuclei. Whether blastomeres divided equally or unequally was judged using four criteria: (1) the movement of the cell’s nucleus to the cortex and back to the center of the cell, which we have observed to precede unequal divisions but not equal divisions, in both untreated and manipulated embryos (Hill, Ph.D. thesis, Indiana University), (2) the movement of the mitotic spindle toward one end of the cell just prior to an unequal cleavage, (3) the position of the cleavage furrow, and (4) the sizes of the two resulting blastomeres as judged by focusing through the embryo. Cell divisions that could not be placed with confidence into either the ‘equal’ or ‘unequal’ categories were not included in our analyses. The frequency with which CD-pulsed embryos underwent different patterns of division was calculated from the first 50 embryos examined, after which unusually cleaving embryos were preferentially analyzed; >10 embryos from each class were analyzed.

After observing the early divisions, embryos were either fixed and stained for P granules (see below) or were allowed to develop overnight. For long-term incubation, the coverslip was gently removed and the embryos were incubated in ECM at 20°C in a humid chamber. The removal of the coverslip increased embryo viability, presumably by allowing a greater degree of gas exchange.

**Immunofluorescent staining of P granules**

Embryos were fixed for immunofluorescent staining as described by Strome and Wood (1982). Slides with embryos attached were frozen in liquid N₂, the coverslips were removed, and the embryos were fixed in cold methanol, followed by cold acetone, and then air dried. Staining was performed on fixed embryos by the procedure of Strome and Wood (1982). Fixed embryos were incubated at 4°C with 1.5% ovalbumin, 1.5% bovine serum albumin in PBS for 30 min and then with K76, a monoclonal antibody directed against *C. elegans* P granules (Strome and Wood, 1983), for 12 h at 4°C. Slides were washed with multiple changes of PBS, after which the embryos were incubated in fluorescently labelled goat anti-mouse secondary antibody (US Biochemicals) in PBS for 4 h at 4°C and then washed five times in PBS. DAPI was included in the third wash to stain nuclei. The samples were mounted in Gelvatol (Monsanto) mounting fluid.
Assays for differentiation markers
To assay the differentiation of intestinal cells, living embryos were observed using either epi-fluorescence microscopy or polarization microscopy to visualize 'gut granules' (Babu, 1974). Gastrulation was indicated by the internal location of the intestinal and germ-line progenitor (P-granule staining) cells. Muscle differentiation was analyzed by staining embryos with 5.6, a monoclonal anti-myosin antibody kindly provided by D. Miller (Miller et al., 1983), or a rabbit anti-paramyosin antiserum prepared by J. Izant and R. Knowlton (Cowan and McIntosh, 1985). Muscle differentiation could also be assessed by monitoring embryos for twitching. Differentiation of hypoderm was assayed by staining embryos with a monoclonal antibody, MH27, kindly provided by R. Frances and R. Waterston (Priess and Hirsh, 1986); this antibody is directed against an antigen located in the belt desmosomes of hypodermal cells. Hypodermal differentiation could also be assessed by monitoring the smoothing of the surface of the embryo (Priess and Hirsh, 1986).

Photography and microscopy
Fluorescence microscopy was performed on a Zeiss ICM405 inverted microscope equipped with epifluorescence optics and a 40x neofluar objective. Zygotes were examined with 515–560 nm epi-illumination for rhodamine fluorescence, 450–490 nm epi-illumination to visualize fluorescein, and 365 nm epi-illumination to visualize DAPI and autofluorescent gut granules. Living embryos were monitored by Nomarski differential interference contrast microscopy using a 40x planachromat objective. Photographs were taken using Kodak Tri-X film at ASA 1600. Film was developed using Diafine two-bath developer (Acufine Inc., Chicago, IL).

Results
Microfilament disruption outside the critical time interval does not appear to affect subsequent development
We previously reported that brief disruption of microfilament structure in 1-cell C. elegans embryos either before the onset of pronuclear migration or after pronuclear meeting (defined as the early and late time intervals; see Materials and methods) did not affect any of the manifestations of asymmetry normally observed in the first cell cycle (pseudocleavage, posterior meeting of the pronuclei, P-granule segregation, asymmetry of the mitotic spindle, and the generation of two different-sized blastomeres) (Hill and Strome, 1988). To determine whether or not proper microfilament structure is required during the early and late time intervals for correct developmental programming, we pulsed 1-cell embryos with the microfilament inhibitor, cytochalasin D (CD), during these time intervals. The developmental fates of the two blastomeres were then analyzed by monitoring subsequent cell division patterns and by assaying for cell differentiation markers. The blastomeres divided in the same order and with the same patterns as the blastomeres in untreated embryos (Figs 1, 2). The embryos initiated morphogenesis and developed to at least the 'lima bean' stage of embryogenesis, and sometimes continued as far as the late 'comma' stage (Fig. 3) (for a review of C. elegans embryogenesis, see Wood, 1988). Embryos permeabilized in culture medium lacking CD also arrested at the lima bean or comma stage, indicating that the inability to develop further is not a consequence of CD treatment. The CD-treated embryos segregated P granules properly, gastrulated, expressed gut-, hypoderm- and muscle-specific markers correctly (Fig. 3), and twitched at the late 'comma' stage, indicative of formation of

Fig. 1. Nomarski micrographs showing the cleavage orientations during division of a normal 2-cell C. elegans embryo. Anterior is to the left, and posterior is to the right. (A) The anterior cell (AB) divides first. Its spindle is oriented perpendicularly to the anterior–posterior axis. (B) The posterior cell (P1) divides after the anterior cell. It undergoes an unequal division along the anterior–posterior axis. (C) A 4-cell embryo. Bar=10 μm.
Brief microfilament disruption during the critical interval leads to a number of embryonic defects

Alterations in early cell division patterns

We have analyzed the effects of brief microfilament disruption during the critical interval (defined as the middle time interval; see Materials and methods) on blastomere division patterns by monitoring CD-pulsed embryos at 16°C using Nomarski DIC microscopy. Here we report on results obtained through the 8-cell stage of development. After this stage, cell rearrangements within the eggshell make it very difficult to determine cleavage orientation and assess the symmetry versus asymmetry of cell divisions. In 62% (n=50) of the embryos pulsed with CD during the critical interval, the blastomeres of the 2-cell embryo underwent a normal pattern of cell divisions (Fig. 4). That is, the anterior cell (normally AB) of the 2-cell embryo divided symmetrically and perpendicularly to the anterior–posterior axis, while the posterior cell (normally P1) divided asymmetrically along the anterior–posterior axis, and generated a small cell at the posterior end of the embryo (normally P2). In the next round of divisions, the two anterior cells (normally ABa and ABp) again divided perpendicularly to the anterior–posterior axis, while the posterior cells (normally EMS and P2) divided along the anterior–posterior axis. The division of the small posterior cell (normally P2) was unequal and generated a small cell toward the anterior of the embryo (normally P3). This pattern of cell division is identical to that seen in untreated embryos and in embryos exposed to culture medium lacking CD.

The other 38% of the treated embryos underwent one of three unusual patterns of division that occur with approximately equal frequency (see Fig. 10 for a schematic summary of cell division patterns). The first pattern is referred to as 'reverse polarity' (Fig. 5). In these embryos, the anterior cell of the 2-cell embryo underwent an unequal division along the anterior–posterior axis of the embryo (like P1), while the posterior cell divided perpendicularly to the anterior–posterior axis (like AB). The two daughters of the posterior cell again divided perpendicularly to the anterior–posterior axis (like ABa and ABp), while the anterior two cells divided along the anterior–posterior axis (like P2 and EMS). The anterior-most cell (like P2) divided asymmetrically, producing a smaller cell toward the posterior of the embryo (like P3). In summary, these embryos behaved as if the cleavage patterns and polarities of the blastomeres of the 2-cell embryo had been switched.

The second unusual pattern of division is referred to as 'posterior duplication' (Fig. 6). In these embryos, both blastomeres of the 2-cell underwent asymmetric divisions and generated smaller daughter cells toward the ends of the embryo (like Po or P1). These divisions usually occurred directly along the anterior–posterior axis; however, in some cells that showed this pattern of division, the spindle lay at a 45° angle to the anterior–posterior axis. In the next round of divisions, the two internal cells divided perpendicularly to the anterior–posterior axis (like AB), while the cells at each end of the embryo divided asymmetrically along the anterior–posterior axis. These asymmetric divisions generated smaller daughters at the respective ends of the embryo.

Fig. 2. Nomarski micrographs showing the cleavage orientations in an embryo pulsed with CD during the early time interval. Anterior is to the left, and posterior is to the right. (A) The anterior cell divides first. Its spindle is oriented perpendicularly to the anterior–posterior axis. (B) The posterior cell divides after the anterior cell and along the anterior–posterior axis. (C) A 4-cell embryo. Bar=10 μm.
Partitioning in C. elegans embryos (like Po or P1, but not like P2 or P3; Schierenberg, 1987) suggesting a reiteration of the Po or P1 cell division pattern.

The third pattern will be referred to as 'anterior duplication' (Fig. 7). In these embryos, both cells of the 2-cell embryo appeared to cleave symmetrically and perpendicularly to the anterior–posterior axis (similar to AB). Embryos of this class are difficult to analyze.

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Fig. 3. Apparently normal development of embryos pulsed with CD outside the critical time interval. Panels on the left are untreated control embryos. Panels on the right are embryos that were pulsed with CD during the late time interval. Anterior is to the left, and posterior is to the right. (A,B) Nomarski micrographs of embryos at the late 'lima bean' stage of embryogenesis. (C,D) Immunofluorescent staining of P granules in 'lima bean' stage embryos showing two internal, P-granule-positive cells. (E,F) Polarization micrographs of the embryos shown in panels A and B, respectively. Both embryos contain internal birefringent gut granules. (G,H) Confocal laser scanning micrographs of 'comma' stage embryos stained with MH27, a monoclonal antibody that recognizes hypodermal belt desmosomes. (I,J) Confocal laser scanning micrographs of 'comma' stage embryos stained with 5.6, a monoclonal antibody against myosin. Bar=10 μm.
Alterations in cell-cycle timing

The early blastomeres in *C. elegans* embryos have characteristic cell cycling periods (Deppe *et al.* 1978). Because as the 2-cell embryo divides, the dividing cells often rearrange in the eggshell making it difficult to discriminate between symmetric and asymmetric division, as well as to determine the true cleavage orientation. After the rearrangement it is difficult to interpret the subsequent cleavage orientations of the blastomeres. However, the P-granule staining patterns described below provide evidence that this 'anterior duplication' class of embryos exists.

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The sequence of cell divisions probably plays an important role in generating cells in the correct relative spatial positions within the eggshell (Schierenberg, 1985). Embryos pulsed with CD during the critical time interval, but not those pulsed outside the critical interval, showed altered cell cycle periods, which often led to an altered arrangement of cells in the 4-cell
Partitioning in \( C. \) elegans embryos

Fig. 6. Nomarski micrographs showing a 'posterior duplication' embryo treated with CD during the critical time interval. Anterior is to the left, and posterior is to the right. (A) 2-cell stage. (B) The posterior cell divides unequally along the anterior–posterior axis. (C) The anterior cell also divides unequally along the anterior–posterior axis. (D) 4-cell stage. The cells have not yet rearranged in the eggshell and remain in a line. (E) The embryo in panel D continued to divide to a few hundred cells. The focal plane of this embryo is at the embryo's surface. Note that it is rough, indicating an absence of hypodermal differentiation. Bar=10 \( \mu \)m.

Fig. 7. Nomarski micrograph showing an 'anterior duplication' embryo treated with CD during the critical time interval. Anterior is to the left, and posterior is to the right. (A) 2-cell stage. (B) Both cells divided equally and perpendicularly to the anterior–posterior axis. This embryo resembles a normal embryo at the 4-cell stage (see Fig. 1C). The assignment of this embryo to the symmetric class was confirmed by P-granule staining (Fig. 8D). Bar=10 \( \mu \)m.

embryo. In control embryos (exposed to ECM lacking CD), the anterior cell of the 2-cell embryo divided between 2.5 and 5.9 min before the posterior cell (mean=4\( \pm \)1.2 min). In embryos treated with CD during the critical interval, we observed variability in the relative cell cycle timing of the blastomeres of the 2-cell embryo. The anterior cell divided as much as 6.3 min before the posterior cell or as much as 5.2 min after the posterior cell (mean=0.8\( \pm \)3.1 min, where a positive value indicates that the anterior cell divided before the posterior cell). The variability of cell cycle timing in the 2-cell embryos did not correlate with cell size. That is, treating embryos with CD during the critical interval causes the zygote to divide symmetrically or with variable asymmetry (Hill and Strome, 1988). The smaller of the two resulting daughter cells did not always have the longer cell cycle, as is seen in untreated embryos (Deppe et al. 1978). These results are consistent with the hypothesis of Schierenberg (1984) that cell
cycling time is controlled by the quality of the cytoplasm rather than cell size.

However, after the defects in relative cell cycle timing observed in the 2-cell embryo generated after CD treatment, the 4-cell embryo regained normal patterns of relative cell cycling time. That is, after an asymmetric division, the smaller of the two daughter cells had a longer cell cycle and, after a symmetric division, the two daughter cells had approximately equal cell cycling times.

Asymmetric divisions are accompanied by appropriately asymmetric P-granule partitioning

In untreated C. elegans embryos, the asymmetric divisions of the P-cell lineage are accompanied by the partitioning of cytoplasmic germ granules, called P granules, to the smaller of the two daughter cells (Strome and Wood, 1982). Based on the sensitivity of P-granule segregation to microfilament inhibitors, the segregation of P granules is thought to be a microfilament-mediated process (Strome and Wood, 1983). When microfilaments are disrupted during the critical time interval during the 1-cell stage, P granules are distributed to both cells of the 2-cell embryo (Hill and Strome, 1988).

To determine the behavior of P granules during subsequent divisions, we analyzed the distribution of P granules in 4-cell embryos of each of the division patterns described above. In embryos treated with CD, P-granule segregation and asymmetric cell division are coupled. The most dramatic example of this is seen in ‘posterior duplication’ embryos. In 3/3 ‘posterior duplication’ embryos, both blastomeres of the 2-cell divided asymmetrically and segregated P granules to the small cells generated at the ends of the embryo (Fig. 8A). P granules were not seen in the two larger internal blastomeres (Fig. 8A). In 6/6 ‘normal’ embryos, only the cell that divided asymmetrically partitioned P granules, and did so to its smaller daughter (Fig. 8B). The P granules in the cell that divided symmetrically were distributed to both daughter cells (Fig. 8B), and were often faint and perinuclear. In the 2/2 ‘reverse polarity’ embryos in which P-granule staining was seen, P granules were observed only in the small anterior cell and were not visible in any of the other three cells of the embryo (Fig. 8C). (In two other ‘reverse polarity’ embryos, P granules were not visible in any of the cells of the 4-cell embryo.) In 2/2 ‘anterior duplication’ embryos, P granules were found in all 4 cells of the 4-cell embryo (Fig. 8D). Thus, in a total of 14/16 cases in which a cell underwent an asymmetric division, P granules were segregated to the smaller daughter; in the other two cases, no P-granule staining was observed. In 6/10 cases in which a cell underwent a symmetric division, P granules were distributed to both daughters; in the other four cases there was no observable P-granule staining. Therefore, among 20 cells whose daughters stained positively for P granules, there were no exceptions to the rule that P granules are segregated to the smaller daughter during an asymmetric cell division and

<table>
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<th>Pulse time</th>
<th>Embryo class</th>
<th>Expression of markers for:</th>
<th>Development</th>
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<tr>
<td>None</td>
<td>Normal</td>
<td>Hypoderm</td>
<td>Normal</td>
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<tr>
<td>Early</td>
<td>Normal</td>
<td>Gut</td>
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Table 1. Expression of differentiation markers in embryos treated with CD during the early, middle and late intervals in the first cell cycle

*0*/5 embryos showed staining, but 3*/5 had a smooth surface.  
1*/4 embryos showed staining, but 0*/5 had a smooth surface. (One of the embryos whose surface was smooth was lost before staining with the hypodermal antibody was complete.)

are distributed to both daughters during a symmetric division.

Analysis of tissue differentiation in CD-pulsed embryos

To try to determine whether disruption of microfilaments during the critical time interval in the 1-cell stage perturbs the segregation of developmental potential, CD-pulsed embryos were allowed to develop for 12–14 h, and then a number of tissue-specific and morphological markers were used to determine what tissue types had differentiated in these treated embryos. Embryos that showed abnormal early cell divisions (5/5 ‘reverse polarity’ and 5/5 ‘posterior duplication’ embryos) also displayed profound defects in differentiation. The embryos continued to divide to several hundred cells by the end of 14 h, but very few of them displayed differentiation markers (Table 1). None of the embryos underwent morphogenesis into a lima bean shape or showed any signs of elongation into a worm. Because of the difficulty (described above) in assigning embryos to the ‘anterior duplication’ class, these embryos were not included in the analysis of differentiation markers.

Fig. 8. Nomarski and immunofluorescence micrographs of P-granule staining in embryos from each of the cleavage patterns observed after a CD pulse in the critical time interval. Anterior is to the left, and posterior is to the right. (A) A ‘posterior duplication’ embryo. Both cells of this class of embryos undergo an asymmetric division. In this embryo, P granules have been segregated to the two end cells (see Fig. 6). (B) A ‘normal’ embryo. In these embryos, only the posterior cell undergoes an asymmetric division (see Fig. 4). In this embryo, P granules were observed in the small posterior-most cell and in the two large anterior cells. (C) A ‘reverse polarity’ embryo. In these embryos, only the anterior cell undergoes an asymmetric division (see Fig. 5). In this embryo, P granules were observed only in the anterior-most cell. (D) An ‘anterior duplication’ embryo. In these embryos, neither of the cells undergoes an asymmetric division (see Fig. 7). In this embryo, P granules were seen in all four cells of the embryo. Bar=10 μm.
Partitioning in *C. elegans* embryos
In CD-pulsed embryos that underwent apparently normal early cell divisions, a wide variety of differentiation 'phenotypes' were observed (Table 1). Twelve 'normal' embryos were analyzed. Three embryos showed apparently normal differentiation of both gut and hypoderm, contained two internal P-granule-staining cells, and appeared to undergo normal development to at least the lima bean stage; one of these developed to the comma stage and displayed muscle differentiation as assayed by twitching. Two other embryos showed differentiation of gut and hypoderm, but underwent abnormal morphogenesis; one did not appear to have gastrulated correctly, while the other had gastrulated but underwent abnormal elongation (Fig. 9). The latter embryo also twitched, suggesting the formation of muscle tissues. The remainder of the embryos displayed varying degrees of differentiation. Of the remaining seven embryos, all were assayed for gut granules, six were assayed for the position of P-granule staining cells, and five were assayed for hypodermal differentiation.
Three out of seven failed to express gut granules, and four showed gut-granule differentiation in the wrong area of the embryo, presumably due to failure of the intestinal precursor cells to invaginate during gastrulation (Deppe et al. 1978). Three of these apparently gastrulation-defective embryos also contained P-granule staining cells that were not positioned internally. The other three embryos assayed for P granules did not show any visible staining. Four out of five embryos failed to show hypodermal differentiation, as assayed by either the tissue-specific marker or by smoothing of the surface of the embryo; one embryo showed some abnormal surface smoothing but did not adopt the typical lima bean shape. These results indicate that disruption of microfilaments during the critical time interval generally results in the disruption of differentiation in the embryo.

Discussion

The results that we present in this study support the hypothesis that developmental instructions or factors that govern cell division and P-granule segregation patterns and cell cycle timing are partitioned to early blastomeres in C. elegans embryos (Wood et al. 1984; Schierenberg, 1985; Schierenberg and Wood, 1985). Furthermore, our results suggest that the microfilament-based cytoskeleton plays an important role in the partitioning process and that microfilament participation is only required during a short period approximately 3/4 of the way through the first cell cycle of the embryo. When microfilaments are disrupted outside this critical period and are then allowed to recover, the zygote divides into a normal looking 2-cell embryo, and both daughter blastomeres behave like the blastomeres in untreated embryos. These embryos continue to develop apparently normally and express the correct tissue-specific differentiation products at the correct positions in the embryo. In contrast, when the microfilament cytoskeleton is disrupted during the critical time interval, the zygote often undergoes a symmetric first division (Hill and Strome, 1988), and the resulting blastomeres often divide and segregate germ granules in the pattern characteristic of their sister cell in an untreated embryo (see Fig. 10 for a schematic summary).

Schierenberg (1985) previously reported that the potential for a cell to undergo an asymmetric division is localized at the posterior end of the zygote before the critical time interval defined by our experiments. This potential appears to be passed to the posterior daughter, P1, and then to the P-cell daughters generated by each unequal cell division. Our results suggest that after brief disruption of microfilaments during the critical interval, this potential for unequal division or 'asymmetry determinant' can be inherited by either the anterior or posterior cell or in some cases by both cells of the 2-cell embryo. One model that is consistent with our data is that, during the critical time interval, the asymmetry determinant is stabilized at the posterior end of the embryo by microfilaments, and that disruption of microfilaments allows it to shift in position (see Fig. 11). In most cases (62%), disruption of microfilaments during the critical interval does not disrupt the asymmetry determinant severely enough to cause it to
be inherited by the anterior daughter cell. As a result, the blastomeres of the 2-cell embryo undergo the correct pattern of cell divisions. However, in the remaining 38% of the cases, the asymmetry determinant may shift in the cytoplasm or along the membrane of the zygote and become distributed to the anterior or to both blastomeres of the 2-cell embryo. This would result in either the anterior cell or both cells undergoing the series of asymmetric divisions characteristic of the posterior cell in an untreated embryo. The possibility also exists that the asymmetry determinant cannot recover after its disruption, resulting in neither cell undergoing an asymmetric cell division.

Another possible explanation for the variety of cleavage patterns that we see in CD-pulsed embryos is that embryos are actually exposed to slightly different concentrations of CD. Since our permeabilization procedure is a manual disruption of the eggshell and inner vitelline membrane, it is possible that the disruption is more severe in some cases than in others. This could lead to variability in the actual concentration of inhibitor that each embryo is exposed to. However, we think that this possibility is unlikely, since different concentrations of CD in the buffer do not lead to variability in the results, and the concentration of CD that we used for most of the experiments leads to an apparently complete disruption of the microfilament cytoskeleton, as judged by rhodamine-phalloidin staining (Hill and Strome, 1988). We also examined the effect of exposing embryos to CD for different periods of time during the first cell cycle. As long as the entire critical interval is contained within the pulse, the embryos display the same range of phenotypes.

In normal embryos, each germ-line or P cell undergoes an unequal division and partitions P granules to the small P-cell daughter (Strome and Wood, 1982, 1983). Our results provide two pieces of information about the relationship between P-granule segregation and unequal divisions: unequal division and unequal P-granule partitioning appear to be coupled, but the presence of P granules in a blastomere does not insure that the blastomere will undergo an unequal division. That is, after microfilament disruption during the 1-cell stage, both blastomeres contain P granules (Hill and Strome, 1988). In most cases only one of the two blastomeres divided unequally, suggesting that P granules do not cause unequal division. However, in all blastomeres that underwent an unequal division, P granules had been partitioned to the smaller daughter cell. Similarly, in all blastomeres that underwent a symmetric division, P granules were observed in both daughters. The only exceptions to this were two embryos that failed to stain for P granules altogether. This result suggests that unequal divisions and P-granule partitioning are mechanistically coupled. At least two possibilities exist to explain this coupling: (1) The asymmetry determinant that participates in positioning the mitotic spindle is also the target site for P-granule segregation. (2) The segregation of P granules to the posterior cortex of the embryo may be a necessary event in the functioning of the asymmetry determinant.

If this event does not occur, then the spindle does not assume the correct asymmetric position in the cell.

Our data also show that microfilament disruption affects cell cycle timing. It has already been proposed that the division times of C. elegans blastomeres are determined by the quality and not the quantity of each cell's cytoplasm (Schierenberg, 1985). Our results are consistent with this hypothesis, since we do not observe a correlation between cell size and cell cycle timing in the two unusually sized blastomeres generated by a CD-pulsed zygote. However, after the 2-cell stage, when a cell underwent an asymmetric division, the smaller of the two cells had a slower cell cycle period, as observed in untreated embryos. These results suggest that the elements that control cell cycle timing are randomly segregated during the first division after CD treatment, but that during subsequent divisions they are correctly partitioned during asymmetric cell divisions. This latter finding is similar to the one described for P granules, in that the proper segregation of cell cycle control elements appears to be coupled to asymmetric division. One attractive hypothesis to explain the coupling of these processes is that the asymmetry determinant that confers the ability to undergo unequal division.

The existence of the proposed asymmetry determinant may explain the patterns of division and P-granule segregation observed in C. elegans embryos after centrifugation during the first cell cycle (Cowan and McIntosh, unpublished). The 2-cell embryos that were analyzed after centrifugation displayed a range of division and P-granule segregation patterns similar to those that we have described for CD-pulsed embryos. Thus centrifugation may mechanically displace or disrupt the asymmetry determinant. In addition, the existence of an asymmetry determinant could explain the phenotypes of the par mutants (see Introduction). At least some of the par genes may encode elements of the asymmetry determinant or cytoskeletal components, for instance microfilament-associated proteins, involved in positioning it. Interestingly, the embryos produced by par-3 worms display division patterns similar to those seen in CD-treated embryos (Kemphues et al. 1988; K. Kemphues, personal communication), making par-3 a good candidate for a gene that affects the positioning of the asymmetry determinant.

Our results show that disruption of microfilaments during the critical time interval leads not only to effects on early division and partitioning patterns, but also to severe defects in subsequent development of the embryo. The severity of these developmental defects appears to correlate with the severity of the abnormalities observed in the 2-cell embryo. A few embryos that appeared normal at the 2-cell stage underwent apparently normal differentiation events and limited morphogenesis of the embryo. Other ‘normal’ 2-cell embryos and all of the ‘reverse polarity’ and ‘posterior duplication’ embryos displayed defects in differentiation and morphogenesis. There are several possible explanations for our results. (1) Differentiation may be prevented or
extinguished by inhibitory factors that in normal embryos are segregated away from the appropriate blastomeres, but that fail to be partitioned after microfilament disruption during the critical interval. As discussed by Cowan and McIntosh (1985), the presence of inhibitory factors may also explain the results of their analysis of _C. elegans_ blastomeres cleavage-blocked with cytochalasin B. Although such cleavage-blocked blastomeres are known to contain the potential to express multiple differentiation programs, they either failed to express any differentiation markers or only expressed markers for one differentiation program. (2) Microfilament disruption may interfere with the partitioning of somatic lineage-specific factors that must reach a critical threshold concentration for activity. (3) Alterations in cell patterning, caused by the altered patterns of cell division and altered cell cycle times, may interfere with cell-cell interactions and/or the generation of spatial cues that normally influence differentiation. (4) Embryos may be generally 'sick' after microfilament disruption. However, such sickness only occurs in embryos treated with CD during the critical interval; embryos pulsed with CD outside the critical interval develop apparently normally. In any case, our results imply that the potential to undergo gut, muscle and hypodermal differentiation is sensitive to microfilament disruption during the critical time interval, long before these differentiation pathways are initiated.

It has previously been reported that the polarity of insect embryos can be perturbed during early embryogenesis (see Kalthoff, 1979 for review). In many of the resulting double cephalon, double abdomen and reverse polarity embryos, differentiation appears to occur fairly normally, as if the perturbation has simply altered body patterning. The differences between these results and ours in _C. elegans_ may be attributable to differences in the manipulations (CD pulse in _C. elegans_ versus irradiation, centrifugation and cytoplasmic transfer in diptera), or they may reflect fundamental differences in the mechanisms of cell-fate specification in these organisms. In insects, there is good evidence that gradients of information present in the early embryo are responsible for specifying axis position and developmental decisions (Driever and Nusslein-Volhard, 1988a, 1988b; Steward et al. 1988). When the distribution of that information is perturbed, differentiation still occurs, but with incorrect spatial patterning, suggesting that the specification of cell fates has a great deal of plasticity. As discussed earlier, several lines of evidence suggest that in _C. elegans_ developmental decisions may be specified by a variety of factors, all of which must be partitioned correctly at each of the early embryonic divisions. Our results suggest that partitioning during the first cell division is extremely important. Embryos in which this partitioning is perturbed are not able to regulate, and development proceeds aberrantly. Our results also suggest that microfilaments are important during the critical time interval for the organization and/or distribution of developmental instructions, and that certain instructions are partitioned as a unit (the instructions for asymmetric cell division, P-granule segregation, and cell cycling times), while others (differentiation instructions) are likely to be separately controlled. This results in cells of the early embryo displaying characteristic division and P-granule segregation patterns, but failing to display the expected differentiation patterns later in development. These observations suggest that the 'mosaicism' thought to exist in the _C. elegans_ egg is not the result of a single mechanism, but rather results from several different mechanisms, each responsible for a subset of developmental instructions.

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