Control of Cleavage Spindle Orientation in *Caenorhabditis elegans*: The Role of the Genes *par-2* and *par-3*

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ABSTRACT

Polarized asymmetric divisions play important roles in the development of plants and animals. The first two embryonic cleavages of *Caenorhabditis elegans* provide an opportunity to study the mechanisms controlling polarized asymmetric divisions. The first cleavage is unequal, producing daughters with different sizes and fates. The daughter blastomeres divide with different orientations at the second cleavage; the anterior blastomere divides equally across the long axis of the egg, whereas the posterior blastomere divides unequally along the long axis. We report here the results of our analysis of the genes *par-2* and *par-3* with respect to their contribution to the polarity of these divisions. Strong loss-of-function mutations in both genes lead to an equal first cleavage and an altered second cleavage. Interestingly, the mutations exhibit striking gene-specific differences at the second cleavage. The *par-2* mutations lead to transverse spindle orientations in both blastomeres, whereas *par-3* mutations lead to longitudinal spindle orientations in both blastomeres. The spindle orientation defects correlate with defects in centrosome movements during both the first and the second cell cycle. Temperature shift experiments with *par-2(it5ts)* indicate that the *par-2(+)* activity is not required after the two-cell stage. Analysis of double mutants shows that *par-3* is epistatic to *par-2*. We propose a model wherein *par-2(+)* and *par-3(+)* act in concert during the first cell cycle to affect asymmetric modification of the cytoskeleton. This polar modification leads to different behaviors of centrosomes in the anterior and posterior and leads ultimately to blastomere-specific spindle orientations at the second cleavage.

POLARIZED asymmetric cell divisions play key roles in development in plants (LYNDON 1990) and animals (WILSON 1925; DAVIDSON 1986), but there are few experimental systems well suited for studies of the mechanisms controlling such divisions. Studies of polarized divisions in yeast have resulted in the identification of a number of genes that interact to establish the division polarity by assembling a group of proteins at a defined site on the cortex (DRUBIN 1991; MADDEN *et al.* 1992). The cytoplasmic microtubules appear to interact with this site in an actin-dependent manner to orient the mitotic apparatus (PALMER *et al.* 1992). Studies of the asymmetric polar divisions of algae (ALLEN and KROPF 1992) and sea urchins (DAN 1984; SCHROEDER 1987; HOLY and SCHATTEN 1991) indicate that similar interactions between astral microtubules and the cortex play a role in oriented divisions in metazoans as well, but the relationship between the processes in yeast and in metazoan development is unclear.

The oriented asymmetric early cleavages of the nematode *Caenorhabditis elegans* have provided an additional opportunity to study the mechanisms controlling polarized divisions in metazoan development (NIGON *et al.* 1960; HIRSH *et al.* 1976; SULSTON *et al.* 1983; STROME 1993). Early stage embryos can be manipulated experimentally (WOOD 1988a; SCHIERENBERG and STROME 1992), and mutants have been identified that alter the early cleavage patterns (WOOD *et al.* 1980; KEMPHUES *et al.* 1988b; MAINS *et al.* 1990).

The first two cleavages have been particularly well studied (NIGON *et al.* 1960; HIRSH *et al.* 1976; ALBERTSON 1984; HYMAN and WHITE 1987; HYMAN 1989). A schematic summary of the major events of these two cleavages is shown in Figure 1.

The orientation of the first cleavage spindle along the long axis of the egg is apparently mediated by interactions between the astral microtubules and the cortex (HYMAN and WHITE 1987). The cleavage is unequal, producing a large cell (AB) in the anterior and a smaller cell (P_1) in the posterior. This unequal division is either a consequence of a migration of the spindle toward the posterior pole at metaphase (KEMPHUES *et al.* 1988b), a migration of the posterior aster toward the posterior pole during anaphase (ALBERTSON 1984) or both. At the second cleavage division, the two blastomeres behave differently. In both blastomeres, the duplicated centrosomes migrate along opposite sides of

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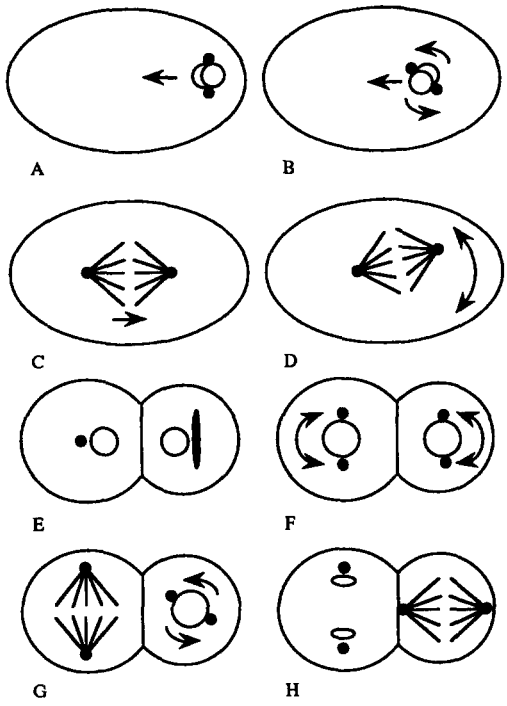

FIGURE 1.—Schematic summary of spindle and centrosome behavior during the first two cleavages of *C. elegans*. ● centrosomes; ○, represent nuclei; lines, microtubles of the metaphase spindle; astral microtubules are not shown. (A) After meeting in the posterior, the two pronuclei and associated sperm centrosomes migrate to the center of the zygote (P_0). (B) As they migrate, the centrosome-nuclear complex rotates to align along the long axis. (C) At metaphase, the spindle migrates to the posterior pole. (D) During anaphase, the posterior centrosome swings side to side while the anterior centrosome remains relatively stationary. (E) At telophase, the posterior centrosome changes shape. (F) In early prophase, the centrosomes of both daughter cells duplicate and each daughter centrosome migrates along the nuclear envelope to a position 90° from its starting point. (G) In the anterior cell (AB), the spindle forms along the axis defined by the position of the centrosomes at the end of their migration. In the posterior cell (P_1), after the centrosomes migrate, but before the spindle forms, the centrosome-nuclear complex rotates through 90°. (H) The AB nucleus completes its transverse division, and the P_1 spindle forms longitudinally along the axis defined by the final position of the centrosomes.

the nuclear envelope to final positions 90° from their starting point (HYMAN and WHITE 1987). In the AB blastomere, the spindle forms along this newly defined axis and thus is oriented tranverse to the long axis of the egg. In the P_1 blastomere, an additional 90° rotation of the centrosome-nuclear complex occurs before the formation of the spindle (HYMAN and WHITE 1987; HYMAN 1989); as a result, the P_1 spindle is aligned along the long axis of the egg. The rotation is mediated by an interaction between one of the centrosomes and a site on the anterior cortex (HYMAN 1989). The potential to undergo this rotation is restricted to the posterior cytoplasm in late one-cell embryos (SCHIERENBERG 1985, 1988) and may come to reside there as a consequence of a microfilament-dependent event (or events) during the first cell cycle (HILL and STROME 1990). Pulses of cytochalasin within a critical period during the first cell cycle lead to variable effects on centrosome rotation at the second cleavage, including blockage of the rotation in P_1, ectopic rotation in AB or a combination of the two, leading to an apparent reversal of embryonic polarity.

In addition to the dramatic differences exhibited by the AB and P_1 centrosomes and spindles, there are also differences in behavior of the anterior and posterior centrosomes during the division of the zygote, P_0 (NIGON *et al.* 1960; HYMAN and WHITE 1987; MORTON *et al.* 1991). At anaphase of the zygotic division, the posterior centrosome undergoes a series of rapid lateral migrations, whereas the anterior centrosome remains relatively immobile (NIGON *et al.* 1960). During telophase, just after the completion of cytokinesis, the posterior centrosome changes shape, elongating transverse to the long axis of the egg and becoming disc shaped as seen by Nomarski microscopy in living animals (HYMAN and WHITE 1987). The significance of the posterior-specific centrosome behaviors is unknown.

The products of at least four maternally acting genes, named *par-1* through *par-4* for *par*titioning defective, are required for normal cleavage patterns and proper cytoplasmic localization (KEMPHUES *et al.* 1988b). Mutations in two of these genes, *par-2* and *par-3*, are particularly interesting with respect to their cleavage defects. Mutations in both genes were found to produce equal first cleavages because of a failure in posterior migration of the first cleavage spindle. However, mutations in the two genes showed different and quite dramatic effects on the orientation of the second cleavage spindles. In embryos from *par-2* mutant mothers, the spindles of both blastomeres oriented transversely (see Figure 2B). In contrast, embryos from *par-3* mutant mothers exhibited all possible combinations of transverse and longitudinal orientations. The longitudinal orientation (see Figure 2C), however, was most common (KEMPHUES *et al.* 1988b).

Because the original analysis was based on a single *par-3* mutation and only two *par-2* mutations, it was possible that the differences in cleavage spindle orientation were due to unusual properties of the alleles and that strong loss-of-function mutations in both genes might show similar phenotypes. In addition, it was not clear whether the defect in *par-3* was best interpreted as randomized spindle orientation or longitudinal spindle orientation nor was it understood how the two genes contribute to spindle pattern.

In this paper we report the isolation and analysis of additional *par-2* and *par-3* mutations. We present evidence that strong loss-of-function mutations in *par-2*

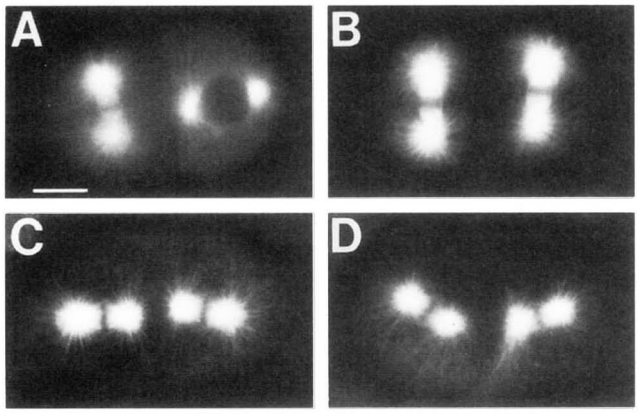

FIGURE 2.—Spindle orientation in wild-type, *par-2* and *par-3* mutant embryos as revealed by immunofluoresence visualization of tubulin. (A) Wild-type embryo. (B) *par-2 (it5)* embryo grown at 25°. (C) *par-3(it62)* embryo. (D) *par-2(it5) par-3(it62)* double mutant embryo. The slight deviation from strictly longitudinal orientation show by this embryo is reflective of the variation show by both *par-3* and *par-2 par-3* embryos. Bar, 10 μm.

block the P_1-specific spindle rotation, resulting in transverse orientation of second cleavage spindles in both blastomeres, whereas strong loss-of-function mutations in *par-3* cause ectopic rotation in the AB cell, resulting in longitudinal orientations of second cleavage spindles, and that the likely time of action of *par-2* is during the first cell cycle. We also show that *par-3* is epistatic to *par-2*. We interpret the double mutant phenotype to mean that neither *par-2(+)* nor *par-3(+)* is required for P_1-specific spindle rotation to occur. Instead, the two genes act in concert to prevent the rotation in AB while allowing it to occur in P_1.

MATERIALS AND METHODS

Nematode strains and growth: Wild-type (N2) and mutant strains of *C. elegans* var. Bristol were maintained as described by BRENNER (1974) and are described in WOOD (1988b). The strain *qC1 dpy-19(e1259) glp-1(q339)* was provided by J. AUSTIN and J. KIMBLE. The remainder of the strains were obtained from the Caenorhabditis Genetics Center.

All strains were grown at 20° except for temperature-sensitive alleles, which were maintained at 15–16°, the permissive temperature, and shifted to the restrictive temperature, 25°, for analysis of the mutant phenotype. Nomenclature conforms to standard rules (HORVITZ *et al.* 1979). The terms "*par-2* embryos" and "*par-3* embryos" refer to embryos produced by homozyous *par-2* and *par-3* mothers.

Screen for mutants failing to complement *par-2(e2030)*: For the screen, *daf-7 III*; *dpy-11 V* hermaphrodites were treated with 23 mM EMS (BRENNER 1974) and then mated to *par-2(e2030)* males at 16°. *daf-7* is closely linked to *par-2* and *dpy-11* marks self-progeny. Individual non-Dpy progeny were picked and selfed at 16° and those that gave rise to many dead embryos and infertile F2's were scored as putative *par-2* mutants. Screening of 2652 chromosomes resulted in recovery of one new *par-2* allele, *it46*.

Screen for maternal-effect lethal mutations closely linked to *daf-7*: In this screen *daf-7*; *dpy-11* hermaphrodites were mutagenized with 23–57 mM EMS and mated to N2 males. Individual non-Daf, non-Dpy hermaphrodites were picked and selfed at 25°. Five Daf-7 F2 segregants from each F1 were transferred to 16° for recovery from dauer and then were scored for maternal-effect lethality (Mel). For each mutation identified, 8–10 non-Daf siblings from the corresponding F1 (*daf-7 m/+ +*) plate were picked individually to recover the mutation. *par-2* allelism was determined by complementation tests with *it5*ts. From 8747 chromosomes screened, three linked maternal-effect lethal mutations were identified: one mutation that complemented *par-2* and two *par-2* alleles, *it49* and *it53*.

Screens for maternal-effect lethal mutations closely linked to *lon-1* or *sma-3*: Young adult hermaphrodites of genotype *lon-1/sma-3* or *lon-1/sma-3 III*; *egl-23 him-3 IV* were treated with 25 mM EMS. Heterozygous F1 progeny, 11,586, were singly plated to every third well of 24-well culture plates and grown at 25°. From each well, two F2 Sma worms and two F2 Lon worms were picked into the adjacent two wells scored and for maternal-effect lethality. New mutations were recovered by picking F3 *lon-1/sma-3* hermaphrodites from the original F1 well. Each of the new maternal-effect lethal mutants was tested again for linkage to the chromosome *III* marker by picking larger numbers of segregants homozygous for the markers. One new *par-3* mutation, *it54*, was isolated.

Screen for mutations failing to complement *par-3(it54)*: Young gravid *lon-1* hermaphrodites were irradiated with 6,000 rads of γ-radiation (Gamma Cell 1000 ^{137}Cesium source with a dose rate of 947 rads/min) and crossed to *lon-1/qC1* males. Progeny males of genotype *lon-1*/qC1* were crossed to hermaphrodites of genotype *fer-1*(ts) *I*; *lon-1 par-3(it54) unc-36/qC1 III* at 25°. (At 25° *fer-1* hermaphrodites have no functional sperm.) From each successful cross, two F2 Lon non-Unc worms were picked to a second plate and scored for maternal-effect lethality. If neither worm gave viable progeny, the *lon-1*/qC1* hermaphrodites were recovered from the original F1 plate and *lon-1** progeny were retested. One new *par-3* allele, *it136*, was recovered from 2,298 mutagenized third chromosomes screened.

Additional *par-2* and *par-3* mutations: The *par-2* allele *jb2* was isolated by D. LEVITAN and D. STINCHCOMB (LEVITAN *et al.* 1994) in a screen for mutator-induced maternal-effect lethal mutations. We recovered the *par-3* allele *it62* in a general screen for EMS-induced Mel mutations. Seven additional *par-2* alleles and two additional *par-3* alleles were isolated in screens for maternal-effect lethal mutations that affected differentiation of intestine (MORTON *et al.* 1991); four of these, *par-2(it58, it87)* and *par-3(it71, it91)*, are included in this analysis.

Screen for deficiencies in the *par-2* region: For the screen, *dpy-1* hermaphrodites were exposed to 6,000 rads gamma irradiation and then mated to *daf-7* males at 25°. Rare Daf non-Dpy F1 worms were identified by visual inspection and transferred to 16° for recovery from the dauer stage. F1 Daf animals, for which approximately one fourth of the embryos failed to hatch and which segregated no Dpy F2 progeny, potentially carried large deficiencies, on the assumption that homozygotes for such deficiencies would be zygotic lethal. Six Daf mutants were isolated from ~2,000 F1 progeny. One of these segregated no Dpy progeny and failed to complement *par-2(it5)* and *unc-45 (e286*ts). This mutation was designated *itDf1*. Unfortunately, the *itDf1* mutation was lost before we could carry out all of the desirable crosses.

Mapping: For genetic mapping, two- and three-factor crosses were performed as described by BRENNER (1974). *par-2* maps between *daf-7* and *dpy-1* ~1 map unit from *daf-7*; *par-3* maps between *daf-4* and *sma-4* about 0.05 map unit from

daf-4. (Map data can be obtained from the Caenorhabditis Genetics Center.)

Construction of *par-2 par-3* double mutants: Strains of genotype *daf-7 par-2(it5*ts) *lon-1 par-3/ daf-7 par-2(it5*ts) + + were constructed by recombination and maintained at 15°. Daf Lon segregants were shifted to 25° as L4 larvae and dissected as adults to obtain double mutant embryos.

Expressivities of maternal-effect lethal and agametic phenotypes: Expressivities of the maternal-effect lethal (Mel) and agametic phenotypes were calculated using procedures described previously (KEMPHUES *et al.* 1988b). For *par-2* mutations with <1% of embryos surviving the maternal effect lethality, data on agametic animals were compiled from survivors produced by >50 mutant parents. For *par-2/ itDf1*, the calculation for percentage of dead embryos was modified because of the contribution of zygotic lethals from the homozygous deficiency: mel (%) = [total dead embryos − (1/4) entire brood]/(3/4) entire brood × 100.

Tests for amber suppression and male rescue: *par-2* mutations *e2030*, *it5*ts, *it46*, *it49*, *it53* and *it58* and *par-3* mutations *e2074*, *it54*, *it62*, *it71* and *it91* were tested with *sup-7* (*st5*) *X*, an amber-suppressing mutant tRNA gene (WATERSTON 1981; WILLS *et al.* 1983). For *par-2* alleles, *daf-7 par-2/ dpy-18*; *sup-7* hermaphrodites were constructed and picked onto individual plates for selfing. Daf segregants were scored for Par phenotypes. The amber mutation *dpy-18* (*e364*) (WATERSTON 1981) was used to monitor the presence of the *sup-7* mutation, and the *daf-7* (*e1372*ts) mutation is not suppressed by *sup-7* (*st5*) (GOLDEN and RIDDLE 1984).

For suppression tests of *par-3* alleles, *lon-1 par-3/ + +*; *sup-7/ +* hermaphrodites were constructed and allowed to self. Lon progeny from these plates (*sup-7*, *sup-7/ + +*) were picked individually and scored for maternal-effect lethality. Two *par-3* mutations showed suppression by this test. Twenty-four of 29 *lon-1 par-3*(*e2074*) animals and 17 of 68 *lon-1 par-3*(*it71*) animals were suppressed for the Mel phenotype. The ratios indicate that although one copy of *sup-7* is sufficient to suppress the weak allele *e2074*, two copies are required to suppress the strong allele *it71*.

par-2 alleles *it46*, *it49*, *it53*, *it58* and *jb2* and *par-3* alleles *it54*, *it62*, *it71* and *it91* were tested for male rescue using methods described by KEMPHUES et al. (1988a).

Observing early development in live embryos: Early embryos were dissected out of gravid hermaphrodites in H_2O and either transferred to a 5% agar pad on a microscope slide (SULSTON and HORVITZ 1977) or transferred to a polylysine-coated slide (KIRBY *et al.* 1990). The former method applies pressure to the embryos, whereas the latter method does not. The specimens were examined with Nomarski optics under a Zeiss microscope equipped with a video camera. Development of embryos from before the pronuclear migration stage to stages after the four-cell was videorecorded. Data were collected directly or by reviewing time-lapse videorecordings. Statistical comparisons were done using unpaired *t*-tests.

Tracing movement of AB and P_1 centrosomes: The centrosome movements were traced on an acetate sheet from the video screen. The starting time for tracing was when the two centrosomes in the one-cell embryo completed the rotation to align with the anteroposterior axis. The center of the centrosomes was the point for tracing. Measurements of telophase centrosomes in newly formed two-cell embryos were taken from tracings made just when the first cytokinesis appeared complete.

Spindle orientation: The orientations of cleavage spindles at the two-cell stage were determined from videorecordings of developing embryos mounted by each of the two techniques described above. Two positions were scored as a function of the angle of the spindle axis relative to the long axis of the embryo: longitudinal = ≤45°, transverse = >45°. In wild-type AB and *par-2* AB and P_1 blastomeres, spindles do not deviate from 90° by more than 30°.

Indirect immunofluorescence: Staining with K76 or OI-CID4 antibodies (gifts from S. STROME) specific to P granules followed the procedure of STROME and WOOD (1983). Spindles were visualized using a monoclonal antibody against Drosophila α-tubulin (gift from M. FULLER) according to procedures previously described (ALBERTSON 1984; KEMPHUES *et al.* 1986). Immunofluorescence assays for terminal differentiation markers followed KEMPHUES et al. (1988b).

Determination of the temperature-sensitive period: For temperature shift analysis, embryos were dissected out of *it5*ts gravid hermaphrodites and shifted to 16° at 2-hr intervals before or after the two-cell stage (at interphase) or to 25° at 1-hr intervals. The difference in time at the two temperatures was based on the observation that the development of *C. elegans* at 25° is almost twice as fast as at 16° (HIRSH and VANDERSLICE 1976). The two-cell interphase, which lasts ~5 min, was chosen as a reference point for timing of the shifts, because the embryos at this stage are easy to identify under a dissecting microscope. The shifted embryos were allowed to develop and then scored for viability. For shifts up before the two-cell stage, worms from 16° were shifted to 25°. Then at successive 1-hr intervals after the shift, two-cell embryos were cut from the shifted worms at room temperature (21–23°) and returned within 5 min to 25°. For shifts up at the two-cell and later stages, two-cell embryos were dissected out and shifted to 25° or were retained at 16° for multiples of 2 hr before being shifted to 25°. Shifts down were carried out by reciprocal procedures.

RESULTS

New alleles of *par-2* and *par-3*: The *par-2* and *par-3* genes were intitially identified by three maternal-effect lethal mutations (mel) with defects in cytoplasmic localization (KEMPHUES *et al.* 1988b). Throughout the paper we refer to embryos from homozygous *par* mothers as "*par* embryos" or "mutant embryos." The mutations also exhibit dramatic effects on the orientation of the second cleavage spindles, with *par-2* embryos having both spindles transverse (Figure 2B) and the *par-3* embryos often having both spindles longitudinal (Figure 2C). To better understand the role of these genes in cleavage patterning, we analyzed additional alleles isolated in our laboratory and other laboratories (see MATERIALS AND METHODS). In this report we describe the results of our analysis of six new *par-2* mutations and five new *par-3* mutations. Some aspects of some of the mutations have been reported elsewhere (KIRBY *et al.* 1990).

All *par-2* and all but one of the *par-3* mutations are maternal-effect lethal. That is, homozygous mutants from heterozygous mothers are viable, but embryos from homozygous mutant mothers die. The mutations can be arranged in a series from strongest to weakest based on expressivity of the maternal lethality at 25° (Table 1). Many mutations in both genes also express a maternal-effect sterile phenotype (Mes); surviving

TABLE 1

Expressivities of Mel and Mes phenotypes of *par-2* and *par-3* mutations

Mutation[c]	Mel phenotype: dead embryos (%)[a]			Mes phenotype: agametic worms among viables (%)[b]	
	16°	20°	25°	16°	20°
par-2					
it87	3	9	27	87	78
e2030[d]	5	8	45	71	73
it5[d]	26	52	98	76	94
jb2	63	84	99	99	99
it49	74	95	99	100	100
it46	90	91	99	99	100
it58	90	97	99	100	100
it53	89	98	99	100	100
par-3					
it54	94	97	99	100	99
e2074[d]	96	98	99	91	94
it62	100	100	100	—	—
it71	100	100	100	—	—
it91	100	100	100	—	—
it136[e]	—	—	—	—	—

[a] Average of percentages of dead embryos among the entire broods produced by ≥10 hermaphrodites (~250 embryos per hermaphrodite).

[b] Average of percentages of agametic worms among the progeny of ≥10 homozygous parents. In cases with expression of the Mel phenotype at levels of 99%, ≥40 of the rare survivors were scored for the agametic phenotype.

[c] *par-2* mutations *it46, it49, it53* and *it58* were also homozygous for *daf-7(e1372)*; *par-3* mutations were all also homozygous for *lon-1(e185)*. *lon-1par-3(+)* and *daf-7 par-2(+)* do not produce significant amounts of lethality or sterility.

[d] Data from KEMPHUES *et al.* (1988b).

[e] *it136* is closely linked to a nonmaternal lethal mutation and could not be analyzed as a homozygote.

progeny from the homozygous mutant mothers are agametic. The maternal effects are strict; neither lethality nor sterility is rescued in heterozygous (m/+) progeny of homozygous mutant mothers (male rescue test, see MATERIALS AND METHODS). Mutations in both genes show some temperature sensitivity. The *par-2* allele *it5*, in particular, exhibits strong temperature sensitivity that has been useful in temperature shift experiments described below.

Embryonic phenotypes of strong *par-2* and *par-3* mutations: The newly isolated strong *par-2* and *par-3* alleles exhibit embryonic phenotypes that are similar to those described previously (KEMPHUES *et al.* 1988b). Mutations in both genes result in symmetric first cleavages, synchronous subsequent cleavages, altered spindle orientiations and abnormal distributions of P granules, but the stronger mutations exhibit greater expressivity of some phenotypes. For example, P granule distribution is more severely disrupted in *par-3(it71)* than in *par-3(e2074)* (KIRBY 1992), and as discussed below, the proportion of embryos with longitudinal second cleavage spindles is larger among *it71* embryos.

Immunofluorescence assays for markers of specific differentiated cell types in terminal stage embryos have revealed that most differentiated cell types can be present in *par-2* and *par-3* embryos (KEMPHUES *et al.* 1988b; CHENG 1991; KIRBY 1992). Certain cell types, however, are present in excess in most embryos (*e.g.*, body wall and pharyngeal muscle). One cell type, intestine, is absent from most embryos from mothers mutant for the strong alleles. The expressivity of this phenotype correlates with the expressivity of the Mel and Mes phenotypes (*cf.* Tables 1 and 2) and can be used as a sensitive indicator of the severity of the mutant phenotype (see below).

***par-2* and *par-3* phenotypes result from loss-of-function mutations:** All mutations are completely recessive and can be placed in an allelic series based on expressivities of the Mel phenotype (see Table 1). Further analysis, described below, leads us to conclude that the strong mutations approach the null condition.

We have obtained *par-2* mutations at frequencies consistent with loss-of-function at typical *C. elegans* loci (BRENNER 1974) (see MATERIALS AND METHODS). Furthermore, the two strong alleles, *it49* and *it53*, behaved similar to a deletion of *par-2* (*itDf1*, see MATERIALS AND METHODS) when heterozygous with the weak allele *e2030* (Table 3). In these tests, the Mel phenotypes of *it49/e2030*, *it53/e2030* and *itDf1/e2030* were indistinguishable. Surprisingly, the intestinal differentiation phenotype was *less* expressed in *it53* hemizygotes than in homozygotes (Table 2), and the Mes phenotype of the *itDf1/e2030* worms was slightly less severe than that of *it49/e2030* or *it53/e2030* (Table 3). None of the tested alleles of *par-2* are suppressible by the amber-suppressing tRNA, *sup-7(st5)*. Recent molecular evidence has shown that *par-2(lw32)*, isolated by J. SHAW, is an opal mutation that should result in a protein truncated to one third of its normal length (LEVITAN *et al.* 1994). Because *lw32* is indistinguishable from *it53* in expressivity (K. J. KEMPHUES, unpublished data), we believe that the phenotype exhibited by *it53* is likely to be the null phenotype.

Two alleles of *par-3*, *e2074* and *it71*, are suppressible by the amber-suppressing tRNA mutation *sup-7(st5)* and thus are likely amber mutations (see MATERIALS AND METHODS). Although it is clear that the weak allele *e2074* is not a null mutation, the combination of a strong phenotype and amber mutation are consistent with *it71* being a null allele.

Two observations, however, could be interpreted to mean that the null phenotype of *par-3* is nonmaternal lethality. First, from a screen to recover maternal-effect

TABLE 2

Intestinal differentiation[a] in *par-2* and *par-3* mutants

Genotype	Percent with gut granules	*n*	Genotype	Percent with gut granules	*n*
par-2(it5ts)	20	126	*par-3(e2074)*	58	257
par-2(it49)	20	109	*par-3(it54)*	69	207
par-2(jb2)	19	181	*par-3(it62)*	48	220
par-2(it46)	15	114	*par-3(it71)*	28	213
par-2(it53)	8	396	*par-3(it91)*	31	214
par-2(it58)	3	352	*par-3(it54)/par-3(it71)*	34	214
par-2(it53)/itDf1	25	122	*par-3(it136)let/par-3(it71)*	21	233
			par-3(it136)let/par-3(it54)	36	215

[a] Differentiation of intestine is scored by the presence of gut granules in terminal stage embryos (CHITWOOD and CHITWOOD 1974; LAUFER *et al.* 1980).

lethal mutations in *par-3*, we recovered *par-3* alleles at a frequency five times lower than expected for loss-of-function mutations (1/11,000 *vs.* 1/2,000). Second, a gamma-induced mutation, *it136*, that fails to complement *par-3* mutations is linked by <0.1 map unit to a zygotic (nonmaternal) lethal mutation. The maternal and zygotic lethality could be due to a single mutation in *par-3*. On the other hand, the zygotic lethality could be the result of a second mutation in a closely linked vital locus or a deletion that affects *par-3* and a linked essential gene. If so, the deletion must be small, because the *it136* chromosome complements mutations in *daf-4* and *sma-4*, the markers immediately flanking *par-3*. We have been unable to recover deletions that span the *daf-4 sma-4* interval and therefore remove the *par-3* locus.

One possible way to distinguish between the two alternative explanations for the zygotic lethality of *it136* was to examine *trans*-heterozygotes of *it136* and other *par-3* mutant alleles. If the *it136* chromosome carried a single severe loss-of-function *par-3* allele rather than a double mutation, it was possible that *trans*-heterozygotes of *it136* and other alleles would show a more severe phenotype than homozygotes for any of the other *par-3* alleles. They do not. Expressivity of the intestinal phenotype in embryos from *it136/it54* mothers is identical to that of embryos from *it71/it54* mothers (Table 2). Similarly, the expressivity of the intestinal defect in *it136/it71* is very close to that of *it71/it71*. We conclude that the phenotype exhibited by *it71* probably represents the null phenotype for the maternal role of *par-3*, but our data do not rule out a possible zygotic role.

TABLE 3

Comparison of Mel and Mes expressivities between strong *par-2* mutants and a *par-2* deletion

Genotype	Mel phenotype: dead embryos (%)[a]	Mes phenotype: agametic worms among viables (%)[b]
e2030/itDf1	67.3 ± 11.5	90.9 ± 7.3
e2030/daf-7 it49	65.1 ± 17.6	99.9 ± 0.2
e2030/daf-7 it53	65.4 ± 14.1	99.1 ± 1.7

Values are means ± SD.

[a] Average of percentages of dead embryos among the entire broods produced by ≥10 worms. Worms were grown at 25°.

[b] Average of percentages of steriles among the entire broods produced by the same worms as footnote *a*.

The *par-2(it5ts)* temperature-sensitive period ends by the two-cell stage: Temperature shift experiments can provide information about the time of synthesis or the time of action of a gene product (SUZUKI 1970; WOOD *et al.* 1980). The *it5* mutation is strongly temperature sensitive (Table 1), allowing us to carry out reciprocal temperature shifts as described in MATERIALS AND METHODS. The results are shown in Figure 3. The proportion of inviable embryos gradually increases with later shifts down during oogenesis. In contrast, the shift-up experiments show an abrupt transition in proportion of inviable embryos near the time of fertilization, with shifts at or later than mid-two-cell stage having no effect. We interpret the gradual slope of the shift-down curve to mean that *par-2* protein is synthesized during oogenesis. Based on the shift-down data alone, the protein could be acting over a long period during oogenesis or could be accumulating during oogenesis for use in early embryos. The abrupt transition in the shift-up curve, however, is not consistent with action over a long period in oogenesis. Thus, we conclude that the *par-2* gene product accumulates during oogenesis but acts during a period beginning in late oogenesis and ending by two-cell interphase.

***par-2* and *par-3* mutations alter spindle orientation in a locus-specific fashion:** As described in the Introduction, in wild-type embryos, the anterior-posterior polarity is reflected in the orientations of the spindles at the second cleavage. The anterior blastomere (AB) orients its spindle transverse to the long axis of the embryo; the posterior blastomere (P_1) orients its spindle longitudinally (Figure 2A). As previously reported, the mutations

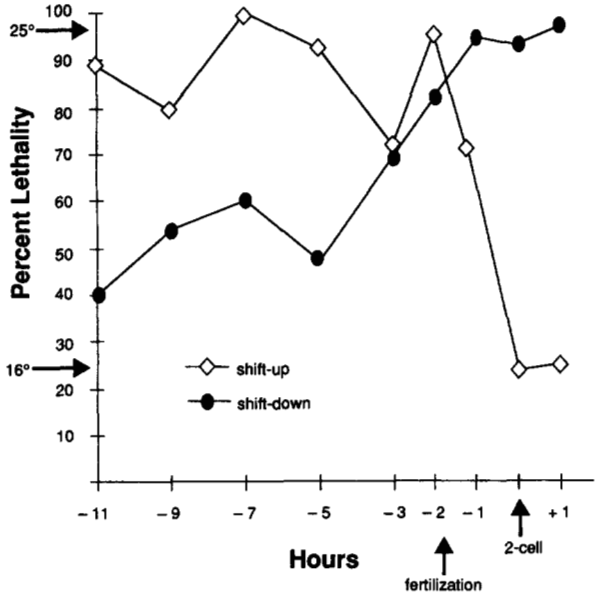

FIGURE 3.—Data from temperature shift experiments with *par-2(it5ts)* embryos. ◇, the percentage of embryonic lethality among a minimum of 60 individual embryos shifted from permissive (16°) to restrictive (25°) temperatures at the indicated times (shift up). ●, the percent lethality among a minimum of 60 individual embryos shifted from restrictive to permissive temperature at the indicated times (shift down). Arrows on the ordinate at 98 and 26% indicate the degree of lethality for embryos grown continously at 25 and 16°, respectively. The time is normalized to hours at 16° (see MATERIALS AND METHODS).

par-2(it5) and *par-3(e2074)* affect this pattern differently (KEMPHUES *et al.* 1988b). To better understand the role of these genes, we have examined cleavage spindle behavior in the strong *par-2* and *par-3* mutations.

In embryos mutant for strong *par-2* alleles, the AB spindle is like wild-type but the P_1 spindle orients transversely (Figure 2B and Table 4). In strong *par-3* alleles, the majority of mutant embryos orient both spindles along the long axis (Figure 2C and Table 4). Thus, loss-of-function mutations in the two genes lead to opposite effects on spindle orientations. The *par-3* mutants exhibit more variability than *par-2* mutants. The *par-2* mutations affect only the P_1 spindle; no *par-2* mutant embryos ever exhibit altered AB spindle orientation. In contrast, although most *par-3* embryos show defects only in the orientation of the AB spindle, some *par-3* embryos have alterations in the orientation of both spindles; some "wild-type" cleavage patterns recorded in Table 4 are the result of reversals of polarity, with the anterior cell dividing longitudinally and the posterior cell dividing transversely.

In the course of these experiments we noted two additional gene-specific defects in spindle behavior. First, in nearly half of the *par-2* embryos examined (11/25 *it5*, 14/29 *it46*), the first mitotic anaphase began with the spindle axis about 45° off of the longitudinal axis (not shown). In wild-type embryos, the first mitotic anaphase takes place only after the centrosome-nuclear complex has completed its rotation and the centrosomes are aligned along the anterior/posterior axis (HYMAN and WHITE 1987). Second, in *par-3*, the spindle orientation at second cleavage is more sensitive to pressure than in *par-2* and wild-type cells. Embryos mounted on agar pads are flattened by the weight of the coverslip (SULSTON *et al.* 1983). For some of our observations we used a mounting method that does not flatten the embryos (KIRBY *et al.* 1990). We found that two-cell *par-3* embryos on agar pads were less likely to exhibit a longitudinal spindle orientation in both blastomeres (Table 4). Flattened *par-3* embryos show a higher proportion of transverse and "wild-type" spindle orientations as compared with undisturbed embryos. Flattening does not affect wild-type or *par-2* spindle orientation.

***par-2* and *par-3* mutations affect centrosome shape and movements:** As described in the Introduction, the centrosomes of *C. elegans* embryos undergo polarized behaviors during the first two cleavages. To examine the behavior of centrosomes in *par-2* and *par-3* mutants, we videotaped several embryos from mothers of each genotype during early development and monitored centrosome shapes and movements.

In *par-2* embryos, both first-division centrosomes behave like the wild-type anterior centrosome. Both remain relatively stationary during anaphase. As shown in Table 5, the extent of lateral swing of both *par-2* centrosomes is less than the anterior centrosome in wild-type. Both also remain spherical during telophase and the following early interphase like the wild-type anterior centrosome (Table 5 and Figure 4B). In addition, the 90° rotation of the centrosome-nuclear complex seen in wild-type P_1 cells does not occur in *par-2* embryos. It is presumably this failure in centrosome rotation that is the basis for the transverse spindle orientation in *par-2* P_1 cells.

In *par-3* embryos both first-division centrosomes behave more like the wild-type posterior centrosome. Both exhibit lateral swings during anaphase of the first mitosis (Table 5). In addition, both centrosomes change shape at telophase, elongating transversely to form a disc (Figure 4C). Measurements shown in Table 5 indicate that the amplitude of the swing and the magnitude of the elongation in both posterior and anterior centrosomes of *par-3* are not quite as large as the wild-type posterior centrosome ($P > 0.1$ in unpaired *t*-tests) but are not significantly different from each other ($P < 0.05$). To determine whether the P_1-like spindle orientations in two-cell stage *par-3* embryos were arising by the same mechanism as in wild-type, we watched several early embryos through the second division and monitored the centrosome position. In *par-3* embryos exhibiting longitudinal spindle orientations, the *par-3*

TABLE 4

Spindle orientation at second cleavage in *par-2* and *par-3* embryos

par genotype	Mounting method[a]	No. of embryos	% both spindles longitudinal	% both spindles transverse	% wild-type[b]
par-2 (it5) (25°)	Flattened	39	0	100	0
par-2 (it46)	Flattened	34	0	100	0
par-3 (it71)	Fixed	48	77	0	23
par-3 (it71)	Unflattened	33	88	0	12
par-3 (it71)	Flattened	53	56	11	32
par-3 (it62)	Fixed	53	79	4	17
par-2 (it5) par-3 (it62) (25°)	Unflattened	19	63	—[c]	—[c]
par-2 (it5) par-3 (it71) (25°)	Unflattened	34	88	3	9

[a] Embryos were prepared in three ways (see text): "flattened" embryos were mounted on agar pads; "unflattened" embryos were mounted to prevent coverslip pressure on the embryos and "fixed" embryos were extruded from gravid adults by slight pressure and then fixed and treated with DAPI and anti-tubulin antibodies.

[b] These embryos had one transverse and one longitudinal spindle but include both reverse and normal polarity. We could not always score the position of the polar bodies to determine anterior-posterior polarity and so did not distinguish between them when collecting the data. However, both normal and reverse polar patterns are consistently observed.

[c] Only longitudinal orientations were scored in this data set.

centrosome-nuclear complexes in both anterior and posterior cells undergo the 90° rotation similar to the wild-type P_1 cell, suggesting that the basis for longitudinal spindle orientations in *par-3* blastomeres is the same as in wild-type P_1 cells. The rotation is not identical to that seen in the wild-type P_1, however. It is more variable than wild-type. In wild-type, the rotation occurs in late prophase (HYMAN and WHITE 1987); in *par-3* embryos some rotations occurred as late as anaphase. In addition, as noted above, the rotation is more sensitive to pressure on the embryo.

***par-2 par-3* double mutants:** In an effort to understand the role of *par-2* and *par-3* in spindle behavior in early embryos, we constructed *par-2 par-3* double mutant strains. We placed two strong *par-3* alleles (*it62* and *it71*) in double mutant combination with the temperature-sensitive allele *it5*. At the nonpermissive temperature, the behavior of second division spindles in *it5* embryos is indistinguishable from that of the strong nonconditional *par-2* alleles (Tables 1 and 4). Hermaphrodites doubly homozygous for *par-2* and *par-3* mutations were grown at the nonpermissive temperature; embryos were then dissected out and examined. As shown in Figure 2 and Table 4, *par-3* is epistatic to *par-2* with respect to spindle behavior at the two-cell stage. Indeed, for every feature of early development thus far examined for which the two phenotypes are distinguishable, the *par-3* phenotype is epistatic. This includes several events of the first cell-cycle (KIRBY *et al.* 1990) as well as the centrosome behavior we have described here (Figure 4).

DISCUSSION

At the second cleavage division of *C. elegans*, the two blastomeres behave differently. In the AB blastomere

TABLE 5

Lateral swing of centrosomes and centrosome shape changes

	Extent of centrosome swing[a]				Centrosome shape[b]		
Genotype	*n*[c]	Lateral swing (anterior)	Lateral swing (posterior)	P:A ratio[d]	*n*[c]	Anterior axial ratio	P:A axial ratio
N2 (wild-type)	5	12 ± 4	22 ± 3	2 ± 0.5	11	1.2 ± 0.2	4.4 ± 1
daf-7 par-2(i46)	4	10 ± 4	9.5 ± 5	1.2 ± 0.4	4	1.2 ± 0.1	1.2 ± 0.2
lon-1 par-3(it71)	5	18 ± 5	16 ± 4	0.9 ± 0.1	29	2.1 ± 0.6	2.2 ± 0.8

Values are means±SD.

[a] Lateral centrosome swing was determined as the distance between the points of closest apposition of the center of the centrosome to the opposite sides of the embryo and is given as a percentage of embryo with (see MATERIALS AND METHODS).

[b] Centrosome shape was measured at telophase as "axial ratio," the ratio of the transverse axis to the longitudinal axis.

[c] Number of embryos scored.

[d] Average of the ratios of the extent of posterior to anterior swing for individual embryos.

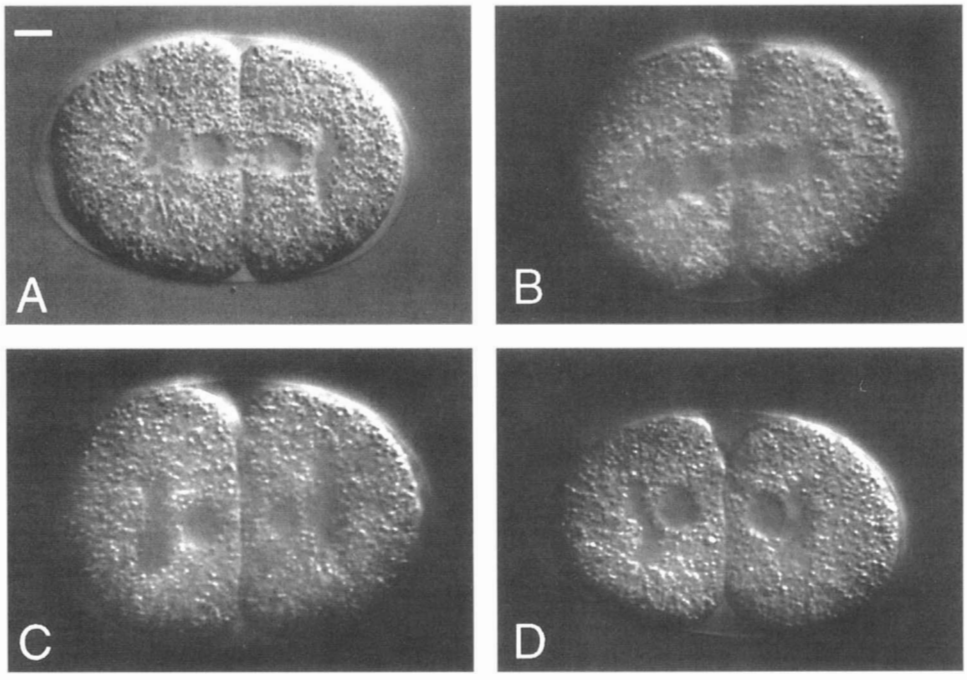

FIGURE 4.—Centrosome shape in wild-type and *par* mutant embryos. (A) Wild-type; (B) *par-2(it5)*; (C) *par-3(it62)*; (D) *par-2(it5) par-3(it62)* double mutant. Bar, 10 μm.

the spindle orients tranverse to the long axis; in the P_1 blastomere the spindle orients along the long axis. Mutations in two maternally expressed genes, *par-2* and *par-3*, had been shown to have dramatic effects upon the orientation of the two-cell stage cleavage spindles in addition to defects in cytoplasmic localization (KEMPHUES *et al.* 1988b). We discuss here the results of further analysis of these two genes with particular emphasis on their role in control of spindle orientation.

We studied eight mutations in *par-2* and six mutations in *par-3*, including strong loss-of-function alleles at each locus. We found that although strong mutations at each locus consistently affect the same early events, the phenotypes of *par-2* mutations are clearly distinguishable from *par-3* mutations. Strong mutations in both genes exhibit phenotypes previously described (KEMPHUES *et al.* 1988b): first cleavage blastomeres are of equal size, P granules localize improperly and cell fates and numbers are altered. The differences between mutations at the two loci include previously reported differences in events of the first cell cycle (KIRBY *et al.* 1990), a less severe effect on P-granule localization in *par-2* mutants (N. CHENG, unpublished results) and differences in centrosome behavior during the first cleavage.

The most striking difference between *par-2* and *par-3* mutants is their opposite effect on orientation of the second division spindles. Mutations in *par-2* cause a failure of the 90° rotation of the centrosome-nuclear complex in the P_1 cell leading to transverse orientation of the spindle. In contrast, mutations in *par-3* cause ectopic 90° rotation of the centrosome-nuclear complex in the AB cell leading to longitudinal orientation of the spindle.

We propose that *par-2(+)* and *par-3(+)* are exerting their effects on second division as a secondary consequence of defects during the first cell cycle. We base this hypothesis on the results of our temperature shift experiments showing that the likely time of action of *par-2* is during the first cell cycle as well as on our previously reported observations of defects during the first cell cycle in both *par-2* and *par-3* mutants (KEMPHUES *et al.* 1988b; KIRBY *et al.* 1990). This hypothesis is also consistent with the observation that pulses of cytochalasin during the first cell cycle variably produce phenocopies of either the *par-2* or *par-3* spindle orientation defects (HILL and STROME 1990).

Our observations of alterations in the behavior of the first division centrosomes in *par-2* and *par-3* mutants can be interpreted in light of this hypothesis. In wild-type embryos, the two centrosomes of the first division spindle behave differently. The posterior centrosome swings laterally during anaphase and changes shape during telophase, whereas the anterior centrosome remains stationary and constant in shape. In *par-2* mutants both centrosomes behave like the anterior centrosome, whereas in *par-3* mutants both behave more like the posterior centrosome. Thus, the centrosome behavior at the end of the first division correlates with the orientation of the second division spindle. We suggest that *par-2(+)* and *par-3(+)* are required for local alterations of the cytoskeleton during the first cell cycle that are necessary for proper spindle orientation at the next division. Polarized centrosome behavior during the first division may be reflective of those local changes.

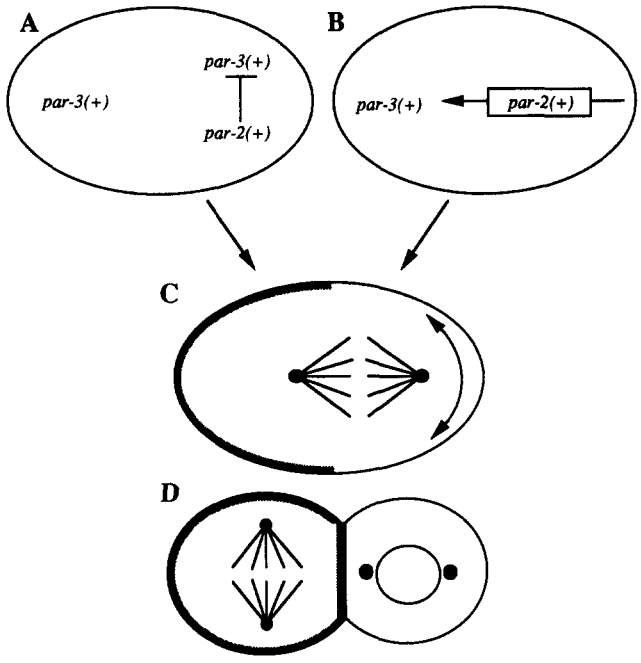

FIGURE 5.—Model for the role of the *par-2* and *par-3* genes in spindle orientation. (A and B) Alternative models explaining possible relationships between *par-2*(+) and *par-3*(+). In A, *par-2*(+) inhibits the activity of *par-3*(+) in the posterior of the one-cell embryo. In B, *par-2*(+) localizes *par-3*(+) activity to the anterior. (C) In either case, *par-3*(+) activity could lead to a modification of the cytoskeleton (here shown arbitrarily as an alteration in the anterior cortex). As a consequence of this modification, the first division spindle is asymmetrically placed and the posterior centrosome swings laterally (double-headed arrow). (D) As a further consequence of the asymmetric cytoskeletal modification, two-cell stage spindles are differently oriented.

The finding that *par-3* is epistatic to *par-2* was unexpected but informative about the roles of these two genes in controlling oriented cleavages. In the absence of both genes, the spindles of *both* cells rotate; thus neither gene is required for the rotation. This leads us to propose the model, summarized in Figure 5, that *par-3*(+) acts to prevent spindle rotation and *par-2*(+) is required to restrict the *par-3*(+) activity to the AB cell. *par-2*(+) could do this either by localizing the *par-3*(+) product to the anterior or by acting to inhibit or modify the *par-3*(+) activity in the posterior. We cannot distinguish between these two hypotheses, but one observation supports the latter. The rotation of the P_1 centrosome-nuclear complex is more easily perturbed in *par-3* mutant embryos than in wild-type embryos. This implies that *par-3*(+) gene activity normally contributes to the stability of the spindle position in the P_1 cell. If this is correct, *par-3*(+) is probably being acted upon by *par-2*(+) in the posterior of the embryo.

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