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Identification of Genes Required for Cytoplasmic Localization in Early C. elegans Embryos

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Summary

We have isolated and analyzed eight strict maternal effect mutations identifying four genes, *par-1*, *par-2*, *par-3*, and *par-4*, required for cytoplasmic localization in early embryos of the nematode C. elegans. Mutations in these genes lead to defects in cleavage patterns, timing of cleavages, and localization of germ line–specific P granules. Four mutations in *par-1* and *par-4* are fully expressed maternal effect lethal mutations; all embryos from mothers homozygous for these mutations arrest as amorphous masses of differentiated cells but are specifically lacking intestinal cells. Four mutations in *par-2*, *par-3*, and *par-4* are incompletely expressed maternal effect lethal mutations and are also grandchildless; some embryos from homozygous mothers survive and grow to become infertile adults due to absence of functional germ cells. We propose that all of these defects result from the failure of a maternally encoded system for intracellular localization in early embryos.

Introduction

The early embryonic blastomeres of Caenorhabditis elegans show striking differences in behavior during development. The first cleavage divides the fertilized egg into a large blastomere called AB and a small blastomere called P_1 (see Sulston et al., 1983). These sister blastomeres have different cell cycle periods and patterns of cleavage, producing different numbers and types of differentiated cells. For example, in normal development AB always divides before P_1 and only P_1 produces intestinal cells.

Although the early blastomeres of C. elegans differ in size, experimental studies have indicated that qualitative differences, rather than differences in the absolute amount of cytoplasm, are responsible for their distinctive behaviors. AB continues to divide more rapidly even after the size of AB is reduced to, or below, that of P_1 through the removal of cytoplasm (Wood et al., 1984; Schierenberg, 1984; Schierenberg and Wood, 1985). Similarly, the unique ability of P_1 to generate intestinal cells is the result of qualitative differences in the cytoplasm it inherits (Laufer et al., 1980; Cowan and McIntosh, 1985; Schierenberg, 1985; Edgar and McGhee, 1986).

A possible mechanism for generating differences between sister blastomeres is for developmentally important substances, often called determinants, to be unequally distributed in the egg or early embryo (see Davidson, 1986, for review). The oocytes and early embryos of many types of animals contain a nonuniform distribution of cytoplasmic components, although the developmental significance of these components is not known. For example, the vegetal half of a frog oocyte is highly enriched in a maternally derived mRNA called Vg1 (Rebagliati et al., 1985), and in the embryo of C. elegans, germ line–specific granules called P granules are associated with the posterior (vegetal) pole of the embryo just before the first cleavage (Strome and Wood, 1982; Wolf et al., 1983). Like the Vg1 transcript in frogs (Melton, 1987), the P granules of C. elegans initially are distributed uniformly throughout the oocyte and only later become associated with the posterior pole. These observations suggest that the oocytes of frogs and the early eggs of nematodes contain systems for cytoplasmic localization, possibly by either unequal segregation or position-dependent stabilization of macromolecules.

The well-developed genetics of C. elegans (Brenner, 1974; Herman and Horvitz, 1980) provides an opportunity to undertake a mutational analysis of cytoplasmic localization in early development. Because genes required for cytoplasmic localization in the early cleavages are expected to be expressed during oogenesis, mutations in such genes are likely to be maternal effect lethal mutations. Due to the difficulties of maintaining embryonic lethal mutations in stock, previously reported screens for maternal effect lethal mutations in C. elegans have relied upon the identification of temperature-sensitive embryonic lethal mutations (Hirsh and Vanderslice, 1976; Hirsh et al., 1977; Miwa et al., 1980; Wood et al., 1980; Cassada et al., 1981) or were limited to regions of the genome for which balancers were available (Meneely and Herman, 1979; Sigurdson et al., 1984). Most of the temperature-sensitive maternal effect lethal mutations exhibit additional phenotypes (most commonly larval lethality or defective gonadogenesis), suggesting that many of the identified genes encode general metabolic functions (Hirsh et al., 1977; Wood et al., 1980). Furthermore, the number of genes being sampled by temperature-sensitive embryonic lethal mutations has been estimated to be greater than a thousand (Wilkins, 1986). If this is correct, the probability of identifying temperature-sensitive mutants in the small subset of these genes with significant roles in early development is low. Because it is likely that nonconditional mutations in genes with general metabolic functions would be homozygous lethal regardless of the mother's genotype, screens for nonconditional maternal effect lethal mutations could reduce the genes being sampled to a more practical number. Therefore, we have used a new screening method to isolate large numbers of nonconditional maternal effect lethal mutants and have examined these for defects in early development.

We report here the identification of four new, maternally

acting genes that are required for proper cytoplasmic localization in early embryos. Mutations in these genes lead to abnormal localization of P granules and abnormal cleavage patterns and can lead to failure to specify or maintain intestine and germ line cells.

Results

Identification and Genetic Characterization of Mutations Defective in Cytoplasmic Localization

Using the screening method described in Experimental Procedures and Figure 7, we were able to recover maternal effect lethal mutations at a frequency of about 1 per 20 haploid genomes screened. To identify maternal effect lethal mutations causing defective early cleavage patterns, 1-cell embryos cut from newly identified mutants were examined under the compound microscope and scored for defects in relative sizes of blastomeres, orientation of spindles, and timing of early cleavages. Many mutants have such defects at early stages. Most of these fall into three general categories: mutants with nuclear abnormalities (multiple nuclei or poor nuclear morphology), mutants with abnormal cytokinesis, and mutants with very slow cleavages. Embryos with these kind of catastrophic defects usually arrest at early stages of embryogenesis. Mutants in these classes were not maintained.

A fourth category of mutant, producing embryos that exhibit altered early cleavage patterns with no catastrophic defects in mitosis or cytokinesis and that arrest at late stages of cellular proliferation, can be identified. Eight such mutations result in at least three of the following early cleavage defects: equal first cleavage, altered orientation of second cleavages, synchronous early cleavages, and abnormalities in localization of P granules.

These eight mutations identify four genes, which we have designated as partioning defective: *par-1*, *par-2*, *par-3*, and *par-4* (*par-1* was previously named *zyg-14* [Hirsh et al., 1985]). The mutations were mapped by linkage analysis and meiotic recombination; linked mutations were tested for complementation. The results gave the following linkage information and gene assignments: *par-1* (*b274,e2012,it32*)*V*; *par-2*(*e2030,it5*ts)*III*; *par-3*(*e2074*)*III*; *par-4*(*it33,it47*ts)*V* (map positions are given in Experimental Procedures). All mutations were found to be completely recessive, strict maternal effect lethal mutations. That is, all homozygous progeny from heterozygous mothers are phenotypically wild type, but embryos produced by homozygous mutant mothers exhibit the mutant phenotype regardless of the genotype of the embryo (Hirsh et al., 1977; Wood et al., 1980; see Experimental Procedures).

Strict maternal effect lethal mutations are typically interpreted as indicating that the genes they identify are specifically expressed in oogenesis for use in embryogenesis, but it is known that rare mutations in genes with vital functions outside of oogenesis can also exhibit maternal effects (Perrimon et al., 1986). Such mutations tend to occur as single alleles in large collections of female sterile mutations (Perrimon et al., 1986; K. J. K., unpublished data). The *par* mutants do not appear to be such atypical mutants in more generally required genes. The three alleles of *par-1* described here were obtained at a frequency of 1.7×10^{-4}, a rate near to that expected for loss-of-function mutants (Brenner, 1974). We have subsequently obtained additional recessive alleles at all of the *par* loci (K. J. K., N. C., and D. G. M., unpublished data). However, we do not yet know whether any of these are null mutations and hence cannot rule out with certainty the possibility that the genes have additional functions outside of embryogenesis.

Table 1. Expressivity of Lethal and Grandchildless Phenotypes

Mutation	16°C	20°C	25°C
par-1 (*b274*)	100[a]	100	100
(*e2012*)	100	100	100
(*it32*)	100	100	100
par-2 (*e2030*)	5 (71)[b]	8 (73)	45 (79)
(*it5*)	26 (76)	52 (94)	98 (96)
par-3 (*e2074*)	96 (91)	98 (94)	99 (99)
par-4 (*it33*)	100	100	100
(*it47*)	96 (35)	99 (50)	100

[a] Values represent the percentage of lethality of 1500–4000 embryos per allele for each temperature.
[b] Numbers in parentheses indicate the percentage of agametic worms among surviving adult progeny from homozygous parents.

All mutations are completely penetrant, but four are incompletely expressed (Table 1). Surprisingly, a large proportion of the surviving progeny from homozygotes for all incompletely expressed *par* mutations exhibit abnormal gonadal morphology and fail to produce gametes. The significance of this grandchildless phenotype is discussed below.

Because these are maternal effect mutants, homozygous *par* embryos only display mutant phenotypes when they are produced by *par* homozygous mothers. Throughout the text "*par* embryo" and "*par* mutant" will refer to embryos produced by homozygous *par* mothers. Also, unless stated otherwise, *par-1* will refer to allele *b274*, *par-2* to allele *it5*ts at 25°C, and *par-4* to allele *it33*.

par Embryos Have Abnormal Positioning of the Early Mitotic Spindles

The first 2 blastomeres of a wild-type C. elegans embryo have different sizes; the anterior blastomere (AB) is always larger than the posterior blastomere (P_1). The first 2 blastomeres in *par-4* mutants have this wild-type pattern of size differences (Figure 1; Table 2). In contrast, the first 2 blastomeres in most *par-1*, *par-2*, or *par-3* embryos are nearly identical in size (Figure 1; Table 2). The relative sizes of sister cells are determined by the position of the

Table 2. Relative Sizes of AB Blastomeres

Genotype	AB/Total	Number of Embryos
N2 (wild type)	57 ± 2	57
par-1 (*b274*)	53 ± 2	39
par-2 (*it5*)	51 ± 2	35
par-3 (*e2074*)	52 ± 1	21
par-4 (*it33*)	57 ± 3	37

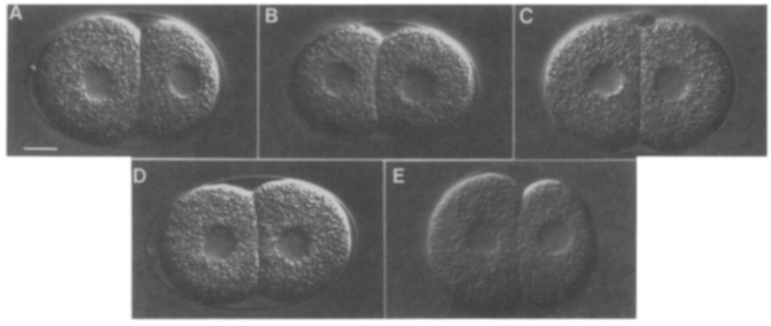

Figure 1. Equal Sized Blastomeres in 2-Cell *par* Embryos

Nomarski micrographs of 2-cell embryos from mothers of wild-type (A) and of genotypes *par-1* (B), *par-2* (C), *par-3* (D), and *par-4* (E) embryos. The slight differences in axial ratios of the *par-1*, *par-2*, and *par-3* embryos relative to wild type represent normal variability. However, *par-4* homozygotes have a consistent tendency toward production of eggs with a more spherical shape. The shape of the eggs does not seem to be responsible for the defects, since wild-type eggs with similar shapes are viable and *par-4* eggs with normal shape are nonetheless defective (K. J. K., J. R. P., and D. G. M, unpublished observations). Bar, 10 μm.

mitotic spindle in many animal cells (Rappaport, 1971). In newly fertilized, wild-type C. elegans embryos, the female and male pronuclei join and move together toward the center of the egg. The mitotic spindle forms at this position, then migrates posteriorly prior to the first cleavage. The cleavage furrow bisects the spindle apparatus, thus producing daughter blastomeres with different sizes (Albertson, 1984). In *par-1*, *par-2*, and *par-3* mutant embryos, the first spindle forms at the correct time and approximate position, but the posterior migration of the spindle complex does not occur (Figure 2).

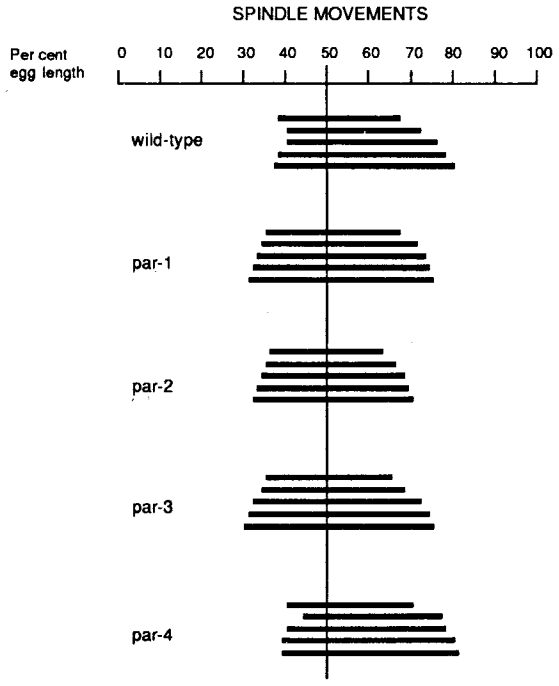

Figure 2. Spindle Migration in *par* Embryos

Each bar represents distance between the two asters of the mitotic spindle and is the average of at least 8 embryos for each genotype. The uppermost bar in each group represents the position of the spindle immediately after the rotation of the asters (see text). The lower bars represent the position of the spindle at successive times during the remainder of mitosis up to the first appearance of the cleavage furrow.

At the second cleavage in wild-type C. elegans embryos, the AB blastomere divides transversely while the P_1 blastomere divides longitudinally (Figure 3A). This orthogonal relationship of cleavage axes is due to a specific rotation of the P_1 spindle complex before division (Hyman and White, 1987). All *par* mutations lead to abnormal second cleavage patterns, including *par-4*, which has a wild-type pattern of first cleavage. In nearly 100% of *par-2* embryos, 25% of *par-1* embryos, and 20% of *par-4* embryos, the P_1 blastomere divides transversely rather than longitudinally (Figure 3C). The orientation of the second cleavage in *par-3* is highly variable. The most common orientation is that shown in Figure 3D, in which both the anterior and posterior blastomeres divide longitudinally. However, other *par-3* embryos exhibit a wild-type cleavage pattern, some have a "reversed wild-type" pattern in which the anterior blastomere divides longitudinally and the posterior divides transversely; in others both blastomeres divide transversely.

Subsequent cleavages in the *par* embryos are difficult to interpret in terms of the wild-type pattern. Because several cellular events, including spindle orientation, cell cycle rate, and spatial constraints, contribute to the wild-type pattern (Laufer et al., 1980; Schierenberg, 1985), it is likely that the abnormal first and second cleavages observed in the *par* embryos contribute significantly to later pattern abnormalities.

par Mutant Embryos Have Altered Timing of Early Cleavages

Each of the early blastomeres in a wild-type C. elegans embryo has a characteristic cell cycle rate such that blastomeres tend to divide sequentially rather than synchronously (Deppe et al., 1978; Sulston et al., 1983). For example, AB always divides before P_1, and one of the daughters of P_1 (called EMS) always divides before the other daughter (P_2). In contrast to the wild-type division sequence, all of the early blastomeres in the *par* embryos tend to divide synchronously (Figure 3). *par-1* and *par-4* embryos cleave synchronously up until the fourth or fifth cleavage, when synchrony gradually breaks down in an apparently random fashion. Loss of synchrony can occur earlier in synchronous *par-2* and *par-3* embryos, and as

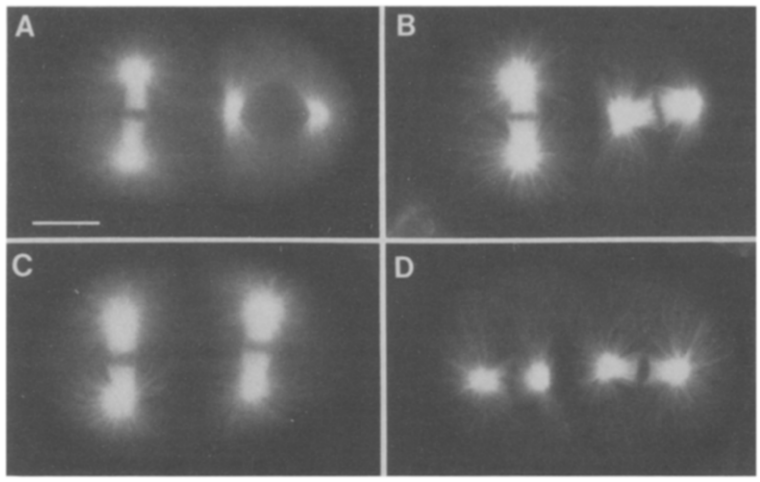

Figure 3. Cell Division Synchrony and Spindle Orientation in 2-Cell *par* Embryos

Immunofluorescence images of 2-cell embryos stained with anti-tubulin antibody. (A) Wild-type embryo showing transverse, metaphase spindle in anterior cell (left) and longitudinal, prophase spindle in posterior cell. (B) Synchronous *par-1* embryo. (C) Synchronous *par-2* embryo with transverse spindles. (D) Synchronous *par-3* embryo with longitudinal spindles. Bar, 10 μm.

expected from the variable expression in these mutants, there is variability in the extent of synchrony and some mutant embryos exhibit a normal division sequence.

par Mutants Fail to Localize P Granules Properly

Wild-type C. elegans oocytes contain a uniform distribution of cytoplasmic granules, called P granules (Strome and Wood, 1982, 1983). After fertilization, the P granules become localized to the posterior pole of the egg and thus are partitioned by the first cleavage into the posterior cell, P_1. At the next cleavage, all P granules are partitioned into P_2, the posterior daughter of P_1. During the next 2 cleavages, the P granules are partitioned successively into the P_3 blastomere (a daughter of P_2) and the P_4 blastomere (a daughter of P_3). The P_4 blastomere divides only once during embryogenesis, and during this division, the P granules are distributed equally to both daughter cells. These 2 daughters proliferate during post embryonic development and are the sole progenitors of the germ line.

Each of the *par* mutations leads to defects in the localization of P granules. *par-1* embryos fail to segregate P granules entirely, such that P granules are distributed equally to all early blastomeres (Figure 4B). *par-4* embryos resemble *par-1* embryos, except that the posteriormost blastomere tends to contain more P granules than other blastomeres (Figure 4F). In *par-4*(*it47*ts), a temperature-sensitive allele of *par-4*, the failure to properly localize P granules correlates with temperature: P granules were not localized in 29%, 87%, and 98% of *par-4*(*it47*ts) embryos cultured at 16°C, 20°C, and 25°C, respectively.

par-2 embryos have either no or incomplete localization of P granules. A wild-type 4-cell embryo contains P granules only in the P_2 blastomere (Figure 4A). In 100 4-cell stage *par-2* embryos examined, 58 had P granules in all 4 blastomeres and 6 had P granules in 3 of the blasto-

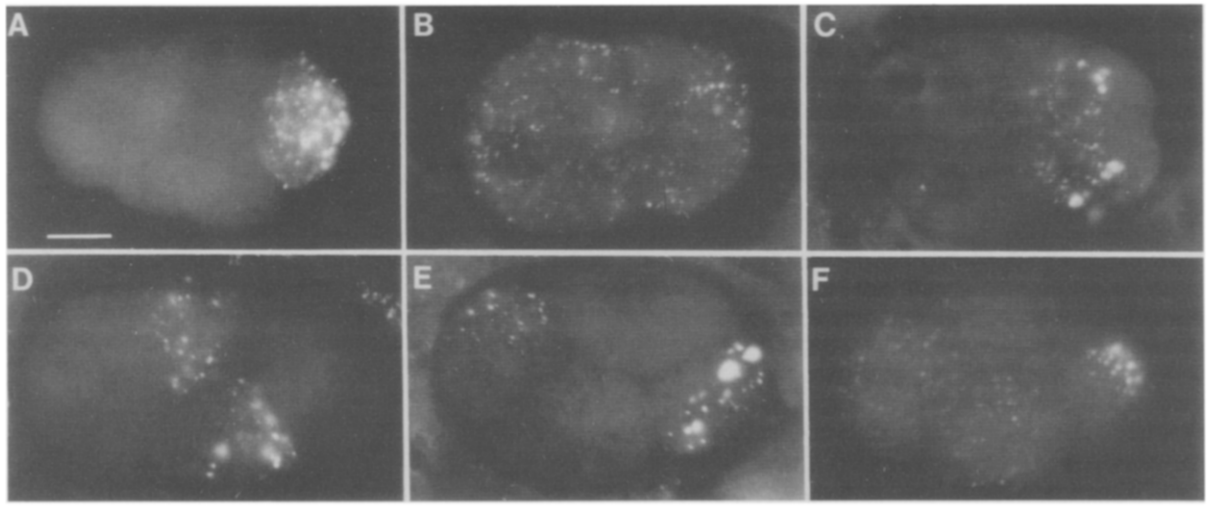

Figure 4. P Granule Distributions in 4-Cell *par* Embryos

Immunofluorescence images of 4-cell embryos stained with anti–P granule antibody. (A) Wild-type. (B) *par-1* embryo. (C) *par-2* embryo. (D and E) *par-3* embryos. (F) *par-4* embryo. Bar, 10 μm.

meres. Thirty-five of the embryos had P granules only in P_2 and its sister (EMS) (Figure 4C), implying that the P granule localization was normal during the first cleavage in these animals. Finally, 1 mutant embryo had P granules only in P_2, the wild-type pattern. The weak *par-2* allele, *e2030*, results in embryos in which P granule localization is relatively normal during the first few cleavages but fails in the later divisions.

par-3 embryos exhibit the same range of defects in P granule localization seen in *par-2* embryos, with the interesting exception that some *par-3* embryos appear to localize P granules anteriorly as well as posteriorly. Many 4-cell stage *par-3* embryos contain P granules in either the 2 middle or the 2 polar blastomeres (Figures 4D and 4E). These patterns appear to arise from incomplete partitioning of P granules at the first cleavage, followed by either anterior or posterior localization of P granules at the second cleavage.

In wild-type embryos the vast majority of the P granules are exclusively segregated into the germ line precursors (P_1, P_2, P_3, and P_4), as described above. However, early cleavage stage embryos occasionally contain a very few P granules in somatic cell precursors that are sisters of the germ line precursors, indicating that the partitioning of P granules was incomplete at the previous division. The "stranded" P granules appear to disintegrate quickly, because at later cleavage stages P granules are present only in the germ line cells (Strome and Wood, 1982). The apparent instability of P granules in somatic cell precursors is consistent with the idea that the early C. elegans blastomeres contain qualitatively different types of cytoplasm.

Interestingly, *par* mutants show differences in their ability to maintain ectopic P granules. In *par-1* and *par-4* embryos, which do not segregate P granules during the early cleavages, the P granules quickly disappear from all cells during embryogenesis. In *par-2* and *par-3* embryos, which show abnormal segregation of P granules during the early cleavages, P granules may persist in novel blastomeres throughout embryogenesis.

par Mutant Embryos Produce Differentiated Cell Types

Although the early cleavage patterns in the *par* mutants are abnormal, the embryos continue to cleave, arresting as amorphous masses made up of large numbers of apparently fully differentiated cells (Figure 5). Indeed, *par-1* embryos have abnormally large cell numbers. The mean nuclear number in terminal stage *par-1* embryos is 809 ± 58 (n = 22), compared with the wild-type value of 533 ± 21 (n = 27). After an incubation period equivalent to the time required for wild-type embryogenesis, all *par* embryos contain numerous cells that resemble several classes of wild-type cells. Refractile nuclei characteristic of programmed cell deaths (Sulston and Horvitz, 1977) can be seen with Nomarski optics (Figure 5a), and cells expressing differentiation markers characteristic of pharyngeal muscles and body wall muscles can be seen with immunofluorescence microscopy (Table 3; Figure 5e). Electron microscopy, carried out thus far only for *par-1(e2012)*, shows that the pharyngeal cells are often orga-

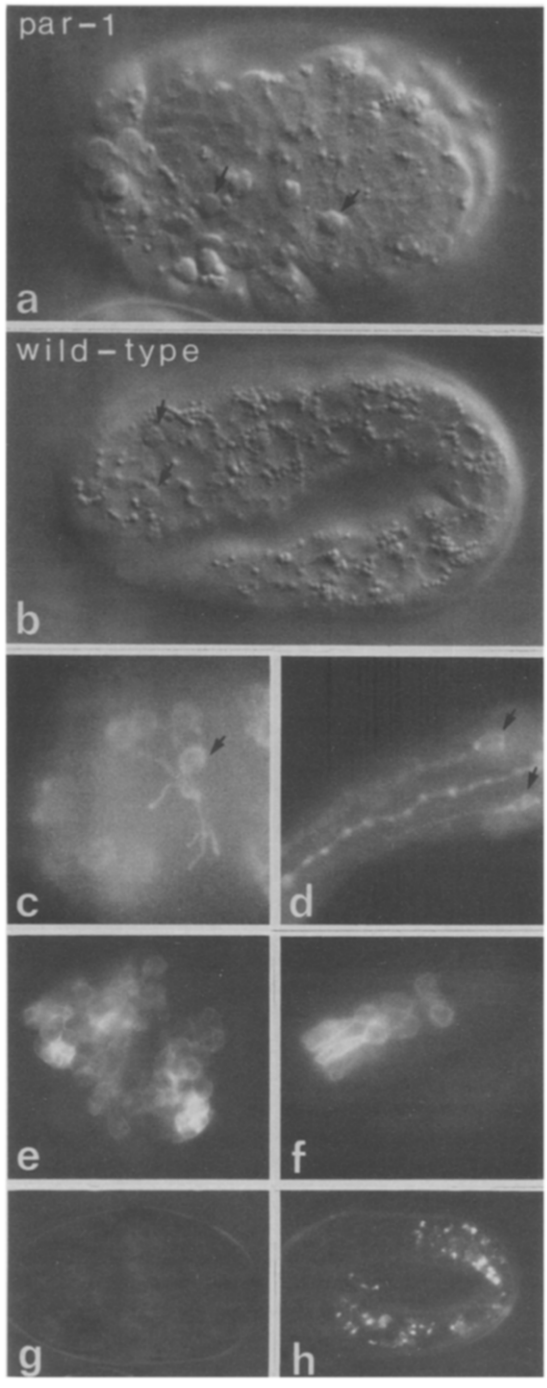

Figure 5. Cell Differentiation in *par-1(e2012)* Mutant Embryos

(a and b) Nomarski micrographs illustrating cell deaths (arrows) in *par-1* (a) and wild-type (b) embryos. (c and d) Immunofluorescence images of *par-1* (c) and wild-type (d) embryos stained with ICB4, a monoclonal antibody that recognizes certain neurons in the head of wild-type C. elegans larvae. Processes characteristic of neurons are indicated by arrows. (e and f) Immunofluorescence images of *par-1* (e) and wild-type (f) embryos stained with an antibody, 3NB12, that recognizes a subset of pharyngeal muscles in wild-type embryos. The *par-1* embryo (e) contains many more than the normal number of cells recognized by 3NB12 in wild-type embryos. (g and h) Polarization micrographs illustrating birefrengent granules that are present in wild-type embryos (h) but are not present in *par-1* embryos. Magnification: 1340× (a, b, c, and d); 720× (e and f); 680× (g and h).

Table 3. Differentiated Cells in Terminal Stage Embryos of Representative *par* Mutants

Mutation	Temperature (°C)	Intestinal[a] Granules (%)	Pharyngeal[a] Myosin (%)	Body Wall[a] Myosin (%)
par-1 (*e2012*)	16	0 n = 506[b]	94 n = 48	94 n = 238
par-2 (*it5*ts)	25	20 n = 126	92 n = 340	100 n = 254
par-3(*e2074*)	16	79 n = 282	95 n = 79	100 n = 186
par-4(*it33*)	20	0 n = 315[c]	43 n = 47	13 n = 85

[a] Values indicate percentage of embryos scored as positive for the indicated marker.
[b] At 25°C some (<1%) *par-1*(*e2012,b274*) embryos produce intestinal granules.
[c] Values for the ts allele *par-4*(*it47*ts) correlate with temperature, from 30% at 16°C to 0.2% at 25°C.

nized into multiple, distinct clusters, each of which approximates the single, wild-type pharynx in structure. Similar clusters of pharyngeal cells can be seen under the light microscope in other *par* embryos. Similarly, neuronal cells (scored only in *par-1*(*e2012*) embryos) differentiate characteristic processes (Figure 5c).

Detailed cell lineage analysis of *par* embryos has not yet been undertaken. However, the terminal phenotypes of these embryos suggest that at least some lineages are profoundly altered. Although all *par* embryos produce differentiated cells, the numbers of cells of any one type produced in an embryo can vary from none to numbers in excess of wild-type (Figure 5e; Table 3). Intestinal differentiation is most severely affected. Intestinal cells can be identified in wild-type embryos by morphology, by the presence of birefringent granules (Laufer et al., 1980), or by a histochemical stain for an intestinal cell–specific esterase (Edgar and McGhee, 1986). Almost no terminal stage *par-1* or *par-4* embryos and only 20% of *par-2* embryos produce any intestinal cells (Figure 5; Table 3). Interestingly, the failure to produce intestinal cells correlates with the strength of the mutation. All of the completely expressed mutations lead to absence of intestinal differentiation (Table 1; Table 3).

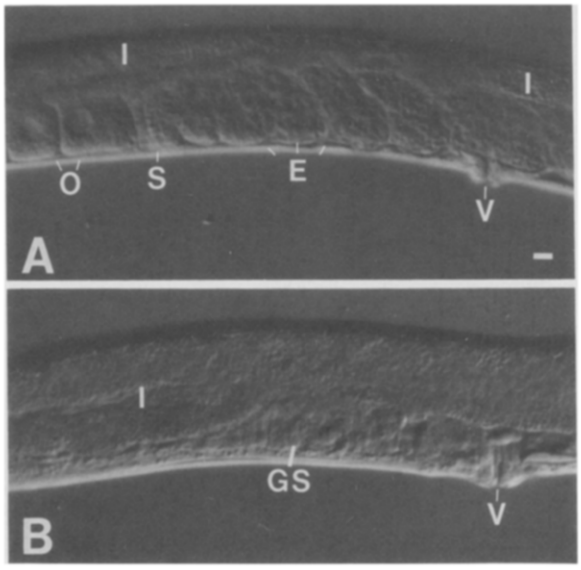

Figure 6. Agametic Phenotype of *par-3* Mutant

Nomarski micrographs of portions of adult hermaphrodites. (A) hermaphrodite with wild-type gonad containing oocytes, sperm, and developing embryos. (B) *par-3* hermaphrodite showing somatic gonad devoid of germ cells. The intestine occupies space normally occupied by the gonad. E, embryos; GS, somatically derived gonad sheath; I, intestine; O, oocytes; S, spermatheca containing sperm; V, vulva. Bar, 10 μm.

Mutations in Three of the *par* Genes Result in a Grandchildless Phenotype

Four of the *par* mutations produce some progeny that complete embryogenesis successfully and hatch (Table 1). Some of the escapers are severely abnormal and die during larval development or develop into adults with abnormal body shape. However, many other larvae develop into morphologically normal adults but lack mature gametes (Table 1; Figure 6). Agametic worms have what appear to be normal somatic gonads containing a functional anchor cell, since a vulva is produced. These worms do not stain for P granules. However, the P granule antibody K76 also stains the spermatheca of wild-type worms, and this staining is seen in the agametic animals. The agametic phenotype is a strict maternal effect; it is not expressed in homozygotes from heterozygous mothers and is not rescued by mating to wild-type males.

Larvae that grow to agametic adults appear to be lacking the P_4-derived germ cell precursors. For example, of 50 newly hatched *par-3* larvae examined, 34 were morphologically abnormal and died as larvae, but 16 grew to adults. Only 2 of these had detectable germ cell precursors at hatching, and only these 2 produced functional gametes as adults. In most newly hatched larvae we could identify at least one of the two precursors to the somatic gonad (Kimble and Hirsh, 1979), and agametic adults had a seemingly normal somatic gonad. Many newly hatched *par-2*(*e2030*) and *par-2*(*it5*ts) larvae also lack germ cell precursors.

Discussion

Mutations at the four *par* loci lead to abnormalities in three features of early embryogenesis: cleavage pattern, timing of cleavages, and partitioning of P granules. However, the *par* mutants do not appear to be defective in general processes of mitosis, cytokinesis, or cellular metabolism: *par* embryos cleave at rates comparable to wild-type and produce large numbers of euploid cells. At terminal stage, *par* embryos exhibit cellular differentiations typical of

most of the wild-type embryonic cell types. However, embryos from mothers homozygous for strong *par* mutations fail to produce intestinal cells, and adult escapers from mothers homozygous for weak mutations are lacking germ cells.

It is possible that the defects observed in the *par* embryos are an easily triggered cascade of events resulting from different types of perturbations occurring in the 1-cell embryo. However, several lines of evidence argue against this interpretation. First, the vast majority of maternal effect lethal mutants that we have examined, as well as those reported previously (Wood et al., 1980; Denich et al., 1984; Kemphues et al., 1986), do not have phenotypes similar to the *par* mutants. For example, almost all of the maternal effect C. elegans mutants we have observed produce intestine-specific granules; even embryos with severe aneuploidy undergo at least some intestinal cell–specific differentiation (Kemphues et al., 1986; K. J. K. and J. R. P., unpublished data). Moreover, differentiation of intestine-specific markers is relatively insensitive to severe experimental perturbations such as cleavage blocking (Laufer et al., 1980; Cowan and McIntosh, 1985; Edgar and McGhee, 1986), blastomere isolation (Laufer et al., 1980; Priess and Thomson, 1987), or fusion of adjacent early blastomeres (Schierenberg, 1985). Similarly, most maternal-effect lethal mutants segregate P granules properly (K. J. K. and D. G. M., unpublished data). Finally, mutations at each *par* locus affect the same processes in locus-specific ways. For example, all three *par-1* mutations completely block the localization of P granules at all divisions, whereas all *par-2* mutations (including three partially characterized alleles not discussed here) often do not detectably affect the first division localization of granules. Therefore we conclude that the shared pattern of pleiotropy caused by mutations in the *par* genes is not due to an easily triggered series of secondary effects. Rather, we conclude that the *par* genes function in a common process required for proper timing and patterning of cleavages, intestinal differentiation, and P granule localization.

Specifically, we propose that the *par* genes encode products required for a maternally derived system for intracellular localization in the early embryo. The most compelling evidence for this interpretation is the direct observation of abnormal distributions of P granules and abnormal positioning of cleavage spindles in *par* embryos. The synchronous cleavages in *par* embryos are consistent with this view. Studies of wild-type C. elegans embryos indicate that cytoplasmic differences are responsible for the differences in cleavage rates of wild-type blastomeres (Schierenberg, 1984, 1985; Schierenberg and Wood, 1985). Thus, cleavage synchrony could result from improper localization of cytoplasmic regulators of cell cycle rate. The failure of many *par* embryos to produce intestinal cells is also consistent with a defect in cytoplasmic localization. Intestinal cell differentiation in wild-type embryos has been shown to be dependent upon qualitative segregation of cytoplasm (Laufer et al., 1980; Wood et al., 1984; Schierenberg, 1985; Cowan and McIntosh, 1985; Edgar and McGhee, 1986). Wild-type 1-cell embryos that are prevented from dividing by cytochalasin, a treatment that also disrupts P granule localization and spindle placement (Strome and Wood, 1983), do not produce intestine-specific markers, yet embryos that are cleavage-arrested after they have completed the first division can produce these markers.

The germ line seems also to be especially sensitive to mutations in the *par* genes. All incompletely expressed mutations result in a grandchildless phenotype. Many of the *par* embryos that hatch lack germ cells and grow to be agametic, but otherwise normal, adults. Because some hatching embryos have abnormal somatic lineages as indicated by nongonadal morphological defects, it is possible that absence of germ cells is a secondary effect of undetected somatic lineage abnormalities. However, the correlation between abnormal distribution of germ line–specific granules and this grandchildless phenotype of the weak *par* mutations suggets that P granules or some other segregated cytoplasmic components are required for germ line specification or maintenance.

Actin microfilaments have been shown to be required for both the wild-type pattern of P granule localization (Strome and Wood, 1983) and the proper positioning of the mitotic spindle in wild-type C. elegans embryos (Hyman and White, 1987), both of which are defective in *par* mutants. The *par* mutations do not map to the chromosomal locations of any of the four actin genes in C. elegans (Files et al., 1983; Albertson, 1985), indicating that the *par* genes do not encode actin proteins. However, they may encode products required for the specialized functions or distributions of actin microfilaments in the early embryo. Molecular analysis of the *par* genes and their products should provide an insight into the mechanism by which the early asymmetries in C. elegans embryos are established.

Experimental Procedures

Genetic Analysis

Maintenance of C. elegans strains, linkage analysis, and recombination mapping were carried out using standard techniques (Brenner, 1974). Mutations utilized were obtained from the Caenorhabditis Genetics Center and included *him-3(e1147) IV* (Hodgkin et al., 1979), *lin-2(e1309) X* (Horvitz and Sulston, 1980), *egl-23(n601) IV* (Trent et al., 1983), *daf-7(e1372) III* (Riddle et al., 1981), *dpy-21(e428) V* (Hodgkin, 1983), *rol-4(sc8) V* (Cox et al., 1980), *lon-1(e185) III*, and *sma-3(e491) III* (Brenner, 1974).

Screen for Maternal Effect Lethal Mutations

Young adult hermaphrodites of genotype *him-3;lin-2* or *egl-23 him-3* were treated with 25–40 mM ethyl methanesulfonate according to the procedure of Brenner (1974). F1 progeny were cloned to individual culture dishes at 25°C and allowed to produce F3 (Figure 6). When the dishes contained young F3 larvae, they were scored for surviving F2 adults. Because *egl-23* and *lin-2* homozygotes fertilize but do not lay their eggs, they are consumed by their progeny. Therefore, the only expected F2 survivors are those that failed to produce F3 progeny. Included in this class are fertilization-defective, gonadogenesis-defective, and maternal effect lethal mutations. Newly induced zygotic lethal mutations are not detected because homozygous F2 animals do not survive to adulthood. Maternal effect lethals were identified by the presence of fertilized (refractile) unhatched eggs in the body cavities of surviving F2. (Gonadogenesis-defective mutants do not produce eggs and usually have visibly abnormal gonad morphology; unfertilized eggs are not refractile.) Mutant strains were maintained initially by cloning individual F3 progeny and selecting clones that produced long-

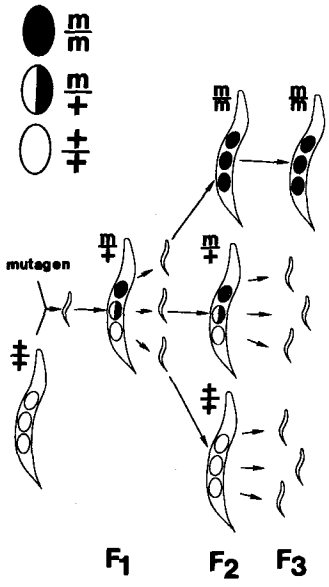

Figure 7. Isolation of Maternal Effect Lethal Mutations in C. elegans

Egg laying defective animals were mutagenized, and their progeny were cloned onto single culture dishes. F1 animals that have acquired a maternal effect lethal mutation (m/+) produce viable eggs (+/+, m/+, and m/m) that hatch within and consume the parent. These F2 animals grow to be adults, but now the m/m animals produce only inviable eggs and are therefore not consumed. These are visible as F2 survivors (see text for details).

lived F4 progeny containing embryos, then repeating this selection in subsequent generations. To identify temperature-sensitive mutations, 10–20 siblings of homozygous mutants were cloned at 16°C. Inability to maintain the selection at 16°C indicated that the mutation was temperature-sensitive. Subsequently, homozygous clones were established and maintained at 16°C. The *par* mutations were identified as follows. The mutation *b274* was identified by directly scoring early embryos for cleavage defects among approximately 150 maternal effect lethal mutants obtained from 1700 F1 progeny of mutagenized hermaphrodites. In a second screen, approximately 4000 F1 progeny of mutagenized parents were prescreened for the production of maternal effect lethal mutants that arrested with large numbers of cells but no morphogenesis (i.e., the terminal phenotype of *b274* embryos). Based on observations of early cleavages of embryos from this selected set, three mutations (*e2012*, *e2030*, and *e2074*) were identified. Because *par* mutations lead to the production of embryos that arrest at late stages of cellular proliferation and differentiation but that lack cells expressing intestine-specific differentiation markers (discussed in Results) we also screened approximately 3000 F1 progeny of ethyl methanesulfonate–treated hermaphrodites and identified three mutations that result in embryos lacking intestinal granules (*it32* and *it33*). The mutation *it47* was identified by the same strategy among approximately 1000 F1 progeny of parents treated with gamma rays. An eighth ethyl methanesulfonate–induced mutation (*it5*ts) was identified fortuitously among the discards of an unrelated mutagenesis. Because we have obtained three alleles of *par-1* via ethyl methanesulfonate mutagenesis, we can calculate a mutation frequency for *par-1* of 1.7×10^{-4} (3 mutations per 17,400 chromosomes). Thus, taking into account the concentration of ethyl methanesulfonate used in our screens, mutations in *par-1* occur at a frequency close to 5×10^{-4}, the frequency of X chromosome lethal mutations in C. elegans (Brenner, 1974).

Designated alleles of a given *par* gene both fail to complement each other and map to the same genetic interval by 3-factor recombination analysis with closely linked markers. The *par-1* locus maps 1.5 map units to the right of *rol-4 V*; *par-2* is located 1.5 map units to the right of *daf-7 III*; *par-3* lies 0.1 map unis to the right of *lon-1 III*; *par-4* is 0.6 map units to the right of *dpy-21 V*. Map data have been submitted to the Caenorhabditis Genetics Center.

After mapping, nonconditional mutations were maintained as heterozygotes using closely linked morphological markers (eg., *lon-1 par-3 +/++ sma-3*). All mutations were outcrossed at least 3 times. Mutations were identified as strict maternal effect mutations by one of two tests. In the most rigorous, individual hermaphrodites were allowed to deplete their own sperm. This was monitored by transferring hermaphrodites to new plates daily until they laid only unfertilized oocytes. These hermaphrodites were then mated to wild-type males, thus ensuring that any fertilized eggs would represent outcross progeny. Occasionally these old hermaphrodites were unwilling or unable to mate. Therefore, for a few strains several independent matings of single young homozygous hermaphrodites to five wild-type males were carried out. Failure to increase the percentage of viable progeny or the percentage of fertile survivors in such crosses indicated that the mutations were strict.

Expressivity of Lethal and Agametic Phenotypes

Individual L4 hermaphrodites of each genotype were placed on petri dishes incubated at 16°C, 20°C, or 25°C and transferred daily until the hermaphrodites laid only unfertilized oocytes. Eggs were counted, and hatching was monitored. Agametic phenotypes of the surviving adult progeny were scored under the dissecting microscope based on gonad morphology. Gonads lacking germ cells differ from wild-type in that they are smaller, are transparent in their proximal as well as their distal portions (maturing oocytes in proximal wild-type gonads appear dark under the dissecting microscope), and tend to be confined to a small section of the body near the vulva.

Blastomere Size Measurements

Embryos at the 2-cell stage were mounted on agar pads (Sulston and Horvitz, 1977) and viewed with a Zeiss photomicroscope III equipped for videomicroscopy. The image of the focal plane with the largest cross-sectional area was used to trace the outline of the blastomeres onto acetate sheets, and the areas of each blastomere tracing were determined using a planimeter. Relative size of the AB cell for each genotype was based on the average ratio of the AB area to the sum of AB and P1 areas for several embryo.

Measurements of Spindle Movements

Development of several embryos from the early pronuclear stage through the 4-cell stage was recorded on time-lapse videotape for each genotype. Spindle position was marked by the distance from the center of the aster to the anterior or posterior membrane of the embryo. Each measurement was normalized to total embryo length and averaged over several embryos. The first measurement was taken when the growing spindle had completed its 90° rotation to become aligned with the anterior–posterior axis (Albertson, 1984). In wild-type embryos the posterior aster swings laterally 5 or 6 times during late metaphase and anaphase. Subsequent measurements were taken every other time the posterior aster crossed the medial position in its swing. For *par-2* embryos, in which the aster does not swing (N. C., unpublished data), measurements were taken at times corresponding to the times of the swings in wild type.

Scoring Gonad Primordia in First Stage Larvae

Plates containing 10–30 gravid adults were incubated overnight at 20°C. Newly hatched first stage larvae were identified by size and mounted individually on agar pads for microscopy. After scoring for the presence of gonad primordia, the larvae were recovered from the pads to seeded plates and allowed to mature. The adult phenotype was scored. Wild-type worms were not adversely affected by this procedure.

Analysis of Terminal Phenotypes

Embryos at the 1-cell to 4-cell stage were cut from adult hermaphrodites, transferred to a drop of water on a siliconized microscope slide, and incubated for the time required for wild-type 1-cell embryos to reach hatching (19, 16, and 12 hr at 16°C, 20°C, and 25°C, respectively). The embryos were scored for the presence of intestine-specific rhabditin granules under polarized light (Chitwood and Chitwood, 1950) and

for the presence of cell death remnants (Sulston and Horvitz, 1977) using Nomarski optics. In addition, some embryos were prepared for staining with mouse monoclonal antibodies specific for C. elegans body wall myosin heavy chain A (antibody 5.6 of Miller et al., 1983), pharyngeal myosin heavy chain (antibody 9.2.1 of Epstein et al., 1982), pharyngeal precursor cell membrane (antibody 3NB12 of Priess and Thomson, 1987), and certain head neurons (antibody ICB4 of Okamoto and Thomson, 1985). Alternatively, some embryos were prepared for electron microscopy (Priess and Hirsh, 1986).

Antibody Staining

Previously described procedures were used to prepare embryos for immunofluorescence microscopy for anti-tubulin (Albertson, 1984; Kemphues et al., 1986), 3NB12 and ICB4 (Priess and Thomson, 1987), and anti–P granule (Strome and Wood, 1983). For visualization of myosin, embryos were mounted on polylysine-coated microscope slides, frozen in liquid nitrogen, fixed in −20°C methanol for 5 min, postfixed in −20°C acetone for 5 min, and air dried. The myosin antibodies were diluted 1/1000 in 50% goat serum in PBS.

Nuclear Counts

Terminal stage embryos were incubated briefly in a 1:1:2 solution of 4%–6% NaOCl:1 M KCl:H_2O to soften the eggshell, rinsed, treated with chitinase (US Biochemicals) for 5 min, then squashed gently under a siliconized coverslip onto a gelatin- and polylysine-coated microscope slide (0.1% gelatin and 0.1% polylysine) in an approximately isotonic salt solution (40 mM NaCl, 60 mM KCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 10 mM HEPES [pH 7]). The slides were then frozen on dry ice, the cover slips were pried off with a razor blade, and the slides were immersed in 3:1 ethanol:acetic acid for 5 min. The slides were rehydrated through an ethanol series and stained with .05 μg/ml diamidinophenylindole for 2 min, washed in water, and mounted in Gelvatol (Monsanto). Nuclear counts were made from back-projected 35 mm negatives of individual squashed embryos.

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