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DNA Replication Defects Delay Cell Division and Disrupt Cell Polarity in Early *Caenorhabditis elegans* Embryos

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In early *Caenorhabditis elegans* embryos, asymmetric cell divisions produce descendants with asynchronous cell cycle times. To investigate the relationship between cell cycle regulation and pattern formation, we have identified a collection of embryonic-lethal mutants in which cell divisions are delayed and cell fate patterns are abnormal. In *div* (for division delayed) mutant embryos, embryonic cell divisions are delayed but remain asynchronous. Some *div* mutants produce well-differentiated cell types, but they frequently lack the endodermal and mesodermal cell fates normally specified by a transcriptional activator called SKN-1. We show that mislocalization of PIE-1, a negative regulator of SKN-1, prevents the specification of endoderm and mesoderm in *div-1* mutant embryos. In addition to defects in the normally asymmetric distribution of PIE-1, *div* mutants also exhibit other losses of asymmetry during early embryonic cleavages. The daughters of normally asymmetric divisions are nearly equal in size, and cytoplasmic P-granules are not properly localized to germline precursors in *div* mutant embryos. Thus the proper timing of cell division appears to be important for multiple aspects of asymmetric cell division. One *div* gene, *div-1*, encodes the B subunit of the DNA polymerase α-primase complex. Reducing the function of other DNA replication genes also results in a delayed division phenotype and embryonic lethality. Thus the other *div* genes we have identified are likely to encode additional components of the DNA replication machinery in *C. elegans*. **© 2000 Academic Press**

Key Words: **cell cycle; cell polarity; *div* genes; DNA replication; DNA polymerase B subunit; PIE-1; SKN-1.**

INTRODUCTION

After fertilization, the newly formed *Caenorhabditis elegans* zygote undergoes a sequence of five asymmetric and asynchronous cell divisions. These early cleavages produce six "founder" cells called AB, MS, E, C, D, and P_4 (Fig. 1; Deppe *et al.,* 1978; Sulston *et al.,* 1983). While unequal and asynchronous cleavages produce each founder, most of the descendants of any one founder cell subsequently undergo roughly equal and synchronous divisions, a pattern largely responsible for their original description and naming. Experimental manipulations, including the isolation or rearrangement of early embryonic cells, indicate that both induced and intrinsic cell polarities result in founder cells acquiring their distinct developmental potentials upon birth (Schierenberg and Wood, 1985; Priess and Thompson, 1987; Schierenberg, 1987; Goldstein, 1992). While genetic and molecular studies have identified localized developmental regulators that combinatorially specify founder cell fates (reviewed in Schnabel and Priess, 1997; Bowerman, 1998), little is known about the temporal regulation of early embryonic cell divisions, or whether cell division timing is important for properly specifying founder cell fate.

The asynchrony of cell division in early *C. elegans* embryos contrasts with the synchronous cell cycle timing observed in two other rapidly developing animals, the

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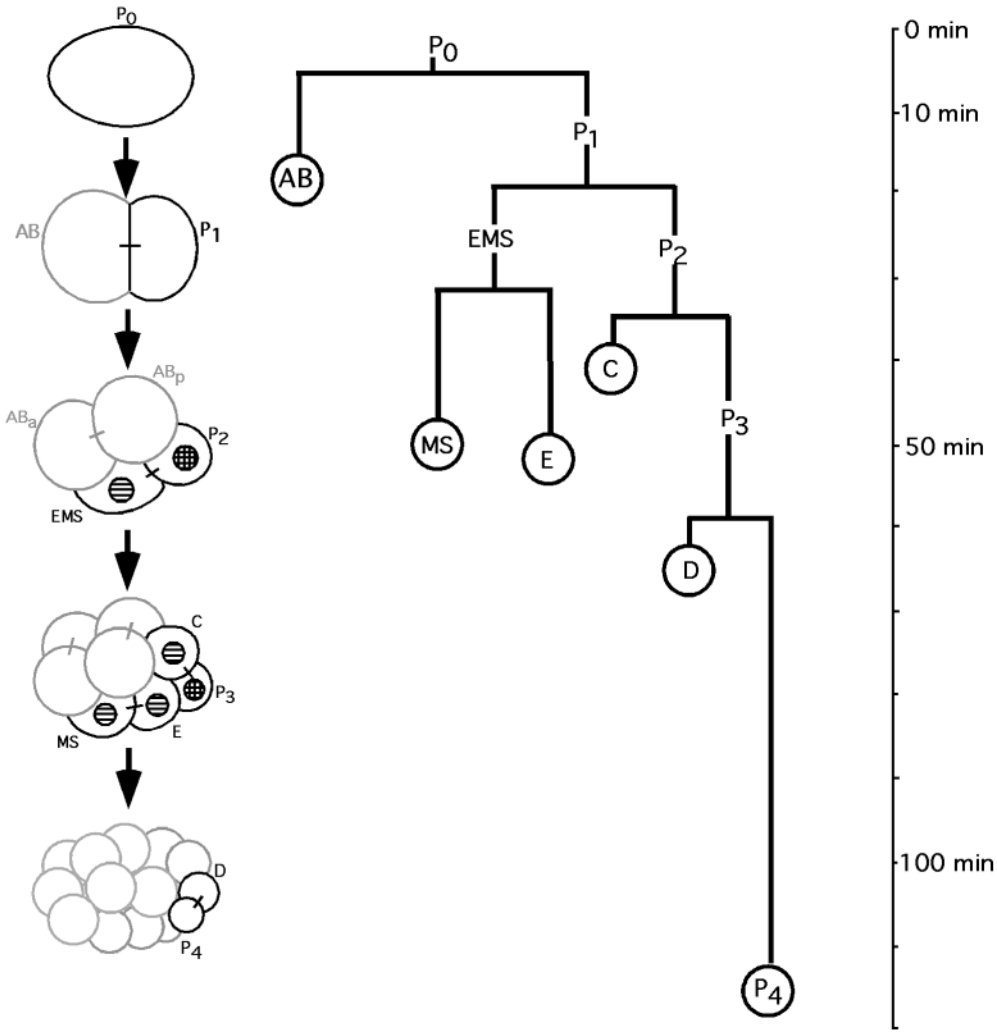

FIG. 1. Cell cycle timing in early *C. elegans* embryos. The early embryo undergoes five asymmetric cell divisions that result in six "founder" cells (labeled in the schematic embryos on the left; circles in the lineage diagram to right; see Schierenberg and Wood, 1985). Sister blastomeres in the schematic drawings on the left are indicated by short connecting lines, and founder cells are shown in bold. Horizontal lines in the nuclei of EMS, P_2, MS, E, C, and P_3 indicate localization of SKN-1 protein, while vertical lines in the nuclei of P_2 and P_3 indicate PIE-1 protein localization. In the lineage diagram on the right, vertical bars indicate time, horizontal bars indicate cell division, anterior daughters are positioned to the left, and posterior daughters to the right. Founder cells are circled at the time of their first division.

amphibian *Xenopus laevis* and the insect *Drosophila melanogaster* (Newport and Kirchner, 1982; Foe *et al.*, 1993). In embryos from both *Xenopus* and *Drosophila*, rapid early cell cycles consist only of DNA replication (S phase) alternating with mitosis (M phase), without intervening gap phases (G1 and G2), for regulating the timing of cell division. Likewise, an analysis of DNA replication and cell division during embryogenesis in *C. elegans* has shown that differences in the rate of DNA replication and hence the duration of S phase—not the introduction of G1 or G2 phases—are responsible for the asynchrony of early embryonic divisions (Edgar and McGhee, 1988; Azzaria and McGhee, 1992). Thus *C. elegans* resembles other rapidly developing animal embryos in lacking G1 and G2 phases during early development, even though the divisions that produce founder cells are asynchronous.

Experimental manipulations of wild-type *C. elegans* embryos have provided some insight into cell cycle control. For example, when isolated at birth and grown in culture medium, most embryonic cells continue to exhibit asynchronous cleavages (Schierenberg, 1987). Furthermore, cells divide prematurely if a more slowly dividing embryonic cell is fused with the cytoplast left after enucleation of a neighboring, more rapidly dividing cell (Schierenberg and

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Wood, 1985). Thus cytoplasmic factor(s) present within founder cells can modulate the timing of cell division.

Genetic insight into the regulation of cell division timing has come from the identification of six maternally expressed *par* genes. Mutational inactivation of any one *par* gene results in a complete, or nearly complete, loss of asynchrony in early embryonic cell divisions (for a review, see Rose and Kemphues, 1998). Embryonic cells in *par* mutant embryos not only divide synchronously but also exhibit other losses of asymmetry, ultimately producing highly abnormal patterns of cell fate due to the improper partitioning of developmental regulators. Some *par* genes are conserved in other organisms and appear to be of general importance for regulating cell polarity (reviewed in Kemphues, 2000). How the *par* gene products might regulate the timing of cell division in early *C. elegans* embryos remains unknown.

To investigate the role of cell division timing during embryogenesis in *C. elegans,* we have identified and characterized a collection of embryonic-lethal mutants in which early cell divisions are delayed but remain asynchronous. We have cloned one of the genes we identified in our screen, *div-1,* and show that it encodes the B subunit of a predicted DNA polymerase α-primase complex. Defects in DNA replication in *div-1* mutants delay cell division, and the delays are accompanied by defects in the early asymmetric cleavages that produce embryonic founder cells. We conclude that the correct execution and timing of cell division are essential for proper cell fate patterning in early *C. elegans* embryos.

TABLE 1

div[a] Genes and Alleles

Alleles	Gene	Linkage group	Terminal phenotype[b]
*or17, or27, or148*ts, *or345*ts	*div-1*	III	Skn-like
*or182*ts, *or234*ts	*div-2*	III	Skn-like
*or209*ts	*div(or209*ts*)*	III	Skn-like
*or235*ts	*div(or235*ts*)*	III	Variable differentiation
*or291*ts	*div(or291*ts*)*	X	Skn-like
*or310*ts	*div(or310*ts*)*	I	Skn-like
*or335*ts, *or366*ts	*div(or335*ts, *or366*ts*)*	I	Variable Morphogenesis
*or361*ts	*div(or361*ts*)*	IV	Variable differentiation
*or364*ts	*div(or364*ts*)*	III	Skn-like
*or389*ts	*div(or389*ts*)*	II	Variable differentiation

Note. Other *div* alleles with a Skn-like terminal phenotype are *or294*ts, *or396*ts, *or426*ts, *or438*ts, and *or442*ts. *div* alleles with variable differentiation include *or395*ts, *or400*ts, *or401*ts, *or414*ts, *or422*ts, *or439*ts, *or440*ts, and *or441*ts. Those with variable morphogenesis include *or315*ts, *or394*ts, *or397*ts, *or398*ts, *or399*ts, *or410*ts, *or415*ts, *or418*ts, *or421*ts, *or427*ts, *or431*ts, *or432*ts, *or435*ts, and *or437*ts.

[a] The gene names *div-1* and *div-2* have been submitted to the *C. elegans* genetic map database. The remaining *div* alleles may prove to be allelic to previously named genes, and thus have not been named.

[b] The terminal phenotype of *div* embryos was assessed using Nomarski optics (see Materials and Methods).

MATERIALS AND METHODS

Strains and Alleles

N2 Bristol was used as the wild-type strain; the standard nematode culture methods and genetics were as described (Brenner, 1974). Mutations and balancer chromosomes used are listed by chromosome as follows: *dpy-5(e61),* I; *rol-6(e187),* II; *daf-7(e1372*ts*), dpy-1(e1), dpy-17(e164), emb-13(g6), emb-34(g62), unc-32(e189), unc-50(e306), unc-69(e587), zyg-7(b187), qC1* (inversion balancer: *dpy-19 glp-1*), III; *him-8(e1489), unc-5(e53),* IV; *dpy-11(e224), him-5(e1490)* V; *lin-2(e1309), lon-2(e678),* X. The strain *unc-32(e189) div-1(or17) dpy-18(e364) pie-1(zu127)/qC1* III was constructed by looking for UncDpy progeny produced by crossing *dpy-18 pie-1/unc-32 div-1* males to *dpy-18 unc-32* hermaphrodites and then balancing the recombinant chromosome with *qC1*. The *axEx73[pJH3.92; pRF4]* transgenic strain containing the *pie-1*::GFP construct was provided by G. Seydoux. To examine PIE-1::GFP distribution in *div-1(or27)* mutant embryos, the strain *div-1(or27) unc-32/qC1; axEx[pJH3.92;pRF4]* was constructed and used to look at embryos from RolUnc mothers.

Genetic Analyses

The *div* alleles were isolated in screens for both nonconditional and temperature-sensitive (*ts*) maternal-effect embryonic-lethal mutations, using previously described methods (Kemphues *et al.,* 1988; O'Connell *et al.,* 1998), except that *ts* mutants were isolated solely on the basis of conditional embryonic lethality. Temperature-sensitive mutants were maintained at the permissive temperature (15°C). L4 larvae or young adults were shifted to the restrictive temperature (25°C) and grown for at least 4 h before collection of early embryos for phenotypic analysis. *div-1(or148*ts*), div-2(or182*ts*),* and *div(or209*ts*)* hermaphrodites grown at 15°C produce 99.2% (255/257), 97.3% (327/336), and 78.5% (194/247) hatching embryos, respectively. At 25°C, all three mutant alleles result in the production of 100% dead embryos: 0/214, 0/181, and 0/178 embryos hatched, respectively. The 40 conditional mutants listed in Table 1 were identified by examining early mitotic cell divisions in mutant embryos from a collection of about 700 heat-sensitive, embryonic-lethal mutants. Other mutants identified in this screen have been, or will be described, elsewhere (Severson *et al.,* 2000).

Temperature-sensitive *div* mutants were isolated in a *lin-2* background (Kemphues *et al.,* 1988; O'Connell *et al.,* 1998). To outcross the homozygous *ts* mutants, young adult egg-laying mutant mothers were mated with either *him-8* or *him-5* males; the *lin-2* allele *e1309* is leaky and about 10% of the adults lay embryos. F1 outcross hermaphrodites were singled onto plates and allowed to produce self-progeny at 15°C. Young adult F2 hermaphrodites from these broods were singled onto plates and grown overnight at 15°C to produce a stock of viable progeny. These F2 parents were then transferred to fresh plates for 2–4 h at 25°C. The worms were

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then transferred again to fresh plates and grown at 25°C overnight. Roughly one quarter of the F2s proved to be *ts/ts* in all cases, producing dead embryos after the upshift. We obtained *ts/ts, him/him,* and *non-him* strains, and we excluded the *lin-2* X chromosome from outcrossed strains. The *div* alleles *or17, or27, or148*ts, *or182*ts, *or209*ts, *or234*ts, *or235*ts, *or291*ts, *or294*ts, *or310*ts, *or315*ts, *or335*ts, *or345*ts, *or361*ts, *or364*ts, *or366*ts, and *or389*ts were outcrossed; the alleles *or394*ts, *or395*ts, *or 396*ts, *or397*ts, *or398*ts, *or399*ts, *or400*ts, *or401*ts, *or410*ts, *or414*ts, *or415*ts, *or418*ts, *or421*ts, *or422*ts, *or426*ts, *or427*ts, *or431*ts, *or432*ts, *or435*ts, *or437*ts, *or438*ts, *or439*ts, *or440*ts, *or441*ts, and *or442*ts remain in the original mutagenized *lin-2* background. The *div-1* alleles *or17, or27,* and *or148*ts were outcrossed between 2 and 7 times each. The nonconditional alleles were also isolated in an egg-laying defective *lin-2(e1309)* background, as described previously (Kemphues *et al.,* 1988). The nonconditional alleles *or17* and *or27* were outcrossed by mating *him-8* males with *orX/+; lin-2* hermaphrodite egg-layers. Male outcross progeny were mated into a *dpy-17 unc-32/qC1* III strain to obtain *orX/qC1; him-8* and *orX/qC1* strains. We detected *orX/orX* animals by looking for 1/4 of the progeny of parental *orX/+* hermaphrodites producing all dead embryos at room temperature (either by looking for *lin-2/lin-2* animals filled with dead embryos or by singling out candidates onto individual plates). The strains obtained were used for further outcrossing, mapping, and complementation tests.

div mutants were mapped to linkage groups by crossing *ts* males from *him* strains and MT3751 (*dpy-5* I; *rol-6* II; *unc-32* III) hermaphrodites, or MT464 (*unc-5* IV; *dyp-11* V; *lon-2* X) hermaphrodites. Broods from outcross hermaphrodites were examined for exclusion of the *ts* mutations by a homozygous marker chromosome, indicating linkage. To map *div-1,* Dpy non-Unc and Unc non-Dpy recombinant progeny from a *div-1(or148*ts*)/dpy-17 unc-50* strain were picked. Of 18 Dpy non-Unc, 16 picked up *div-1(or148*ts*),* and 1 out of 26 Unc non-Dpy recombinants picked up *div-1(or148*ts*).* These data position *div-1* at approximately +2.08 map units (mu) on chromosome III, just to the left of *unc-50.* To map *div-2,* Dpy non-Daf, and Daf non-Dpy recombinants from a *div-2(or182*ts*)/daf-7 dpy-1* strain were picked. Of 40 Dpy non-Daf, 13 picked up *div-2(or182*ts*),* while 10 out of 28 Daf non-Dpy picked up *div-2(or182*ts*).* This positions *div-2* at approximately −23.7 mu on chromosome III.

For complementation tests, males from a *ts; him* strain and *ts* hermaphrodites from a non-*him* strain were mated at 15°C. L4 hermaphrodite outcross progeny were grown at 25°C to determine if they produced embryos that hatched (complementation) or that failed to hatch (noncomplementation). Due either to their close proximity to where our *div* alleles map, or due to their similarity in phenotype, complementation tests were done between *div(or148*ts*)* and *zyg-7(b187*ts*),* *div(or148*ts*)* and *emb-34(g62*ts*),* and *div(or234*ts*)* and *emb-13(g6*ts*).* Outcrossed (F1) progeny from both *div(or148*ts*); him-8* and *zyg-7,* and *div(or234*ts*); him-8* and *emb-13* crosses produced 100% viable embryos at 25°C (234/234 and 315/315, respectively), indicating that these alleles complement. Outcrossed (F1) progeny from *div(or148*ts*); him-8* and *emb-34* crosses produced 45% (553/1229) viable embryos at 25°C. Both *div(or148ts)* and *emb*-34 alone make 100% dead embryos at 25°C (0/214 and 0/187, respectively). The early phenotype of *emb-34(g62*ts*)* also shows a P1 delay, although there are also earlier severe chromosomal segregation defects not observed in any of our *div-1* alleles (data not shown). We tentatively conclude that *div(or148ts)* and *emb*-34 complement, and that the dead embryos seen in the progeny of F1s in the complementation test indicate an interaction between the *div-1* and *emb-34* gene products.

Microscopy and Immunocytochemistry

Light microscopy was done with a Zeiss Axioskop equipped with Nomarski DIC optics and epifluorescence. Intestinal cells were identified both using polarized light microscopy to score for the presence of birefringent gut granules in living terminally differentiated embryos and by incubating fixed terminally differentiated embryos with the antibody J126, followed by a fluorescently labeled secondary antibody (Bowerman *et al.,* 1992b). Pharyngeal cells were detected by staining fixed, terminally differentiated embryos with the monoclonal antibody 9.2.1, specific for pharyngeal myosin (Miller *et al.,* 1983; Bowerman *et al.,* 1992a). Hypodermal cells were identified based on their morphological appearance in the light microscope using Nomarski DIC optics: they have characteristically flattened cell shapes with relatively large smooth nuclei containing a single prominent nucleolus. For immunocytochemical localization of P-granules and of the SKN-1 protein, embryo fixation and staining protocols were used as described (Shelton *et al.,* 1999; Bowerman *et al.,* 1993). Antibodies recognizing P-granules (OIC1D4) were a gift from Susan Strome (Strome and Wood, 1983).

To measure the cell cycle timing of the first three embryonic cell divisions of *div* and wild-type embryos (Figs. 2 and 3), time-lapse digital movies were made using a Dage-MTI Ve1000 digital camera linked to the Scion Image program run on a Macintosh PowerPC. All measurements were done at room temperature. The length of interphase of a particular blastomere was established as the time elapsed from the end of cytokinesis of its mother cell and the beginning of its nuclear envelope breakdown (NEB). The time that a blastomere spent in mitosis was measured from the beginning of NEB to the end of cytokinesis. The total cell cycle times for each blastomere (shown in Fig. 3) are the sums of the times it spent in interphase and mitosis. All the timing landmarks used for the measurement of cell cycle timing were based on the morphological criteria using Nomarski optics and time-lapse videos.

To calculate the volumes of sister blastomeres, the diameter of each blastomere at the beginning of interphase (end of cytokinesis of parent cell) was measured, and the volume was calculated assuming spherical dimensions, as in Tabara *et al.* (1999).

Imaging of wild-type embryos carrying the *pie-1*::GFP construct, of *div-1(or27); pie-1*::GFP embryos, and of fixed embryos stained with the antibody recognizing P-granules was done on a Bio-Rad 1024 confocal microscope, using a 60X oil immersion lens. Collections were made of 1 mm step z-series for embryos at 4-cell, 8-cell, 12-cell stages of development, and Bio-Rad software was used to project each z-series into two-dimensional images. Images of wild-type *pie-1::GFP* and *div-1(or27); pie-1*::GFP 4-cell embryos were taken during an interval of time that corresponds to 56–64% the duration of EMS interphase. Images of the same embryos at the 8-cell stage correspond to 29–33% of C interphase. Confocal and Nomarski images were assembled into figures using Adobe Photoshop (version 5.0).

div-1 Cloning and RNA-Mediated Gene Interference (RNAi)

To clone *div-1,* RNA interference (Fire *et al.,* 1998), germline transformation with genomic DNA to rescue the mutant phenotype (Mello *et al.,* 1991), and DNA sequencing were done.

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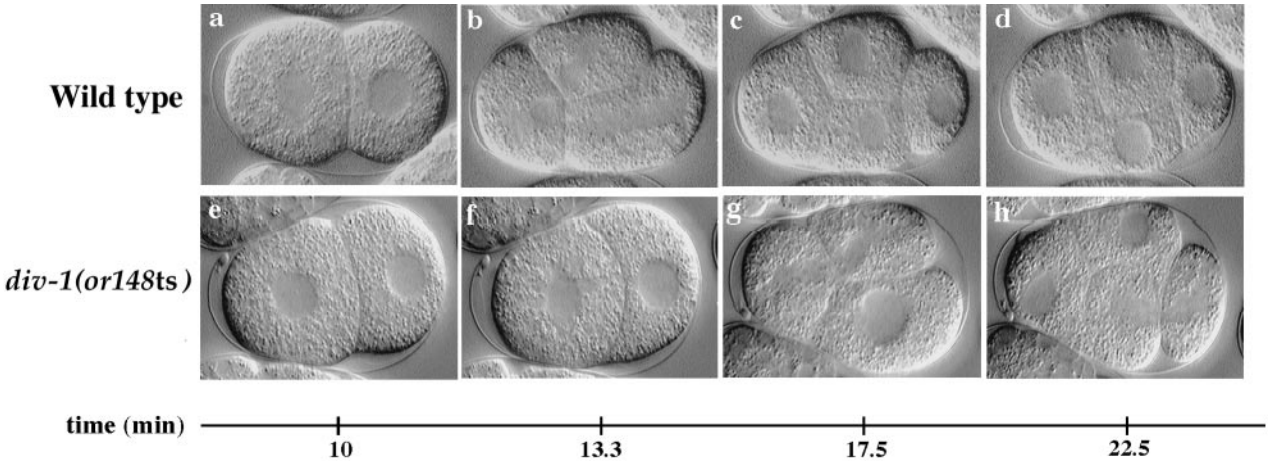

FIG. 2. Delayed cell divisions in *div* mutant embryos. Nomarski photomicrographs of early wild-type and *div-1(or148*ts*)* embryos undergoing the second round of mitotic division. All embryos are shown with anterior to the left and dorsal up. Time in minutes is indicated, beginning with the end of P_0 cytokinesis. Observations of all embryos (wild-type and *div*) were made at room temperature. To obtain *div-1* mutant embryos, hermaphrodite mutant mothers were shifted to 25°C overnight.

Inserts from lambda phage cDNA clones (provided by Dr. Yuji Kohara at the National Institute of Genetics, Mishima, Japan), corresponding to genes near *unc-50* were PCR-amplified. Double-stranded RNA was synthesized from these PCR products and microinjected into the gonads of young adult wild-type hermaphrodites. All embryos failed to hatch 24 h after worms were injected with RNA from a nearly full-length 2.12-kb cDNA clone, yk464h5, corresponding to the predicted gene R01H10.1. In RNAi mutant embryos, the P_1 division was delayed, and a prominent 3-cell stage observed. In addition, after germline transformation using the yeast artificial chromosome Y70G10 which includes R01H10.1, one of seven transmitting lines containing this YAC rescued the mutant phenotype of *div-1(or148*ts*)* embryos when animals were grown at the restrictive temperature of 25°C. To confirm the gene identity by sequence, genomic *div-1* DNA fragments from *lin-2* mutants (the strain we used for mutagenesis) were PCR-amplified and compared to those for each of the three *div-1* alleles. PCR bands were excised from gel, and DNA was isolated using the GeneClean II Kit (Bio 101, LaJolla, CA) and cloned into pGEM-T (Promega, Madison, WI). Three separate PCR amplifications and sequencing reactions were performed for the *lin-2* strain and the three *div-1* alleles. Each completed sequence contig contained sequences from approximately 500 bp upstream of the initiation codon, and approximately 260 bp downstream of the stop codon (approximately a total of 4127 bases sequenced for each allele). All sequencing was done at the University of Oregon DNA Sequencing Facility, using an ABI 377 Prism automated fluorescent sequencer. In all three *div* alleles, a unique lesion, not present in either the wild-type or the parental *lin-2* strain, was found in the R01H10.1 coding sequences. RNAi to reduce the function of the DNA polymerase α-primase complex primase subunits C and D (phage clones yk232d4-cosmid W02D9.1 and yk141c6-cosmid F58A4.4, respectively), and the A subunit of the DNA polymerase δ complex (yk461c5-cosmid F10C2.4), was done as described above for yk464h5/*div-1*.

RESULTS

Delayed Cell Divisions in div Mutant Embryos

In genetic screens for *C. elegans* embryonic-lethal mutants with cell fate patterning defects (see Materials and Methods), we identified two recessive, nonconditional, maternal-effect alleles of a gene we call *div-1* (Table 1). Subsequently, we began genetic screens for heat-sensitive, embryonic-lethal *C. elegans* mutants with defects in cell division (see Materials and Methods). We examined the early cleavages in about 700 conditional mutants and found 40 that resemble *div-1* mutants in having delayed embryonic cell divisions (Table 1; Fig. 3). These mutations define at least 10 different loci based on genetic mapping data and complementation tests (Materials and Methods), including two *ts* alleles of *div-1, or148*ts and *or345*ts (Table 1). Like *div-1* mutants (see below), some alleles undergo terminal differentiation despite of their delayed cell divisions but frequently fail to produce intestinal and pharyngeal cells. However, many of the conditional *div* mutants we have isolated exhibit more severe cell division defects, arresting early in development with large undifferentiated cells (data not shown). Mutants in a third class differentiate well, making roughly normal numbers of intestinal and pharyngeal cells, but exhibit variable defects in elongation of the initially oval embryo into a long, thin larva (data not shown).

In wild-type embryos, the posterior-most 2-cell stage blastomere P_1 is well into mitosis by the time the anterior blastomere AB finishes dividing. Thus only a very brief 3-cell stage occurs during normal development (Fig. 2b). In *div* mutant embryos, an increased time interval between the divisions of P_1 and AB results in a prominent and

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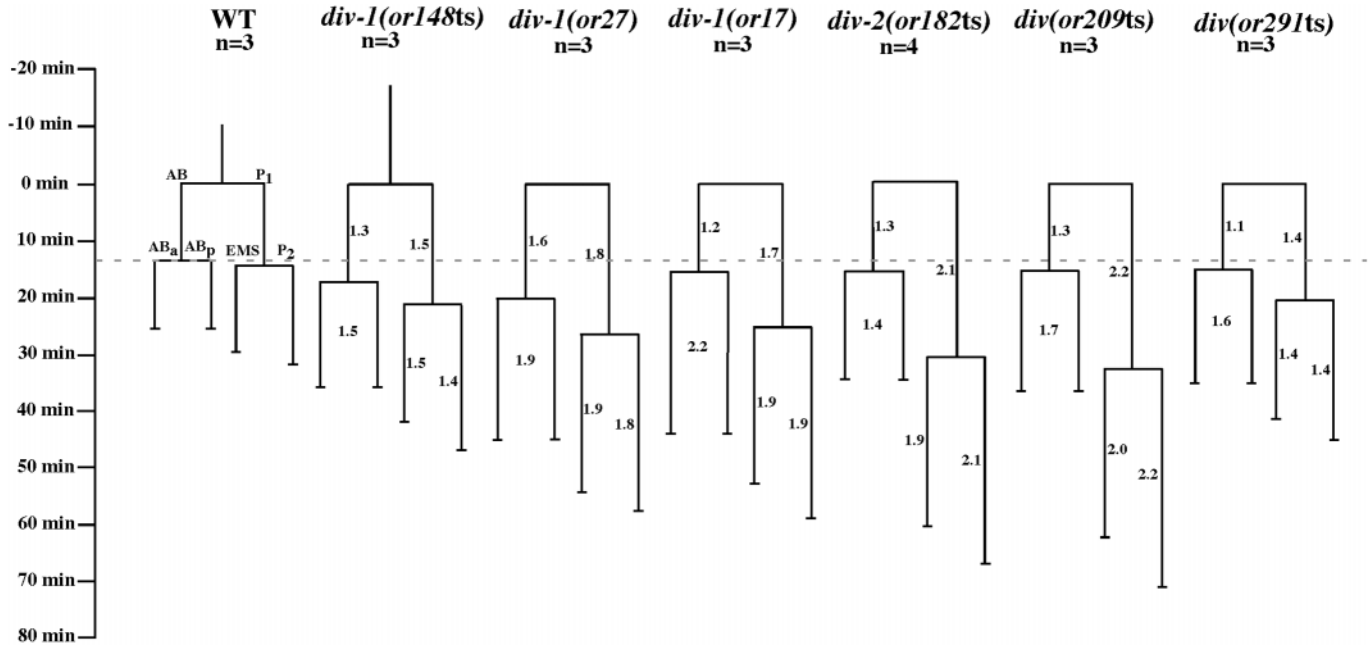

FIG. 3. The cell lineages of wild-type and *div* mutant embryos. Horizontal lines indicate the time of cell division, while the vertical lines indicate the duration of the cell cycle for each blastomere during the second and third rounds of embryonic cell division (time = 0 min is the beginning of the 2-cell stage to the 8-cell stage divisions). The time from the appearance of the maternal pronucleus (shortly after the completion of meiosis II using DIC optics), to P_0 nuclear envelope breakdown, is shown for wild-type (n = 4) and *div-1* (*or148*ts) mutant embryos (n = 5). The numbers next to each vertical line represent the increase in length of time relative to a wild-type value of 1. For example 1.3 means that a cycle is 1.3 times longer than in a wild-type embryo. The average cell cycle times of three or four embryos are diagrammed for each mutant and for wild-type. Cell cycle times for each blastomere were calculated as the time spent in interphase and mitosis; see Materials and Methods for details.

persistent 3-cell stage (Figs. 2g and 3). Moreover, the divisions of all blastomeres are delayed in *div* mutant embryos at least through the first three cell cycles, including the time prior to the division of P_0 in at least *div-1(or148*ts*)* (Fig. 3). To more precisely define the delays, we measured the lengths of interphase and mitosis in early blastomeres (see Materials and Methods). While the duration of mitosis appears normal or nearly normal in *div-1* and *div*(*or209*) mutants, the duration of interphase is substantially increased (Table 2). As the cell cycles in early *C. elegans* embryos consist only of DNA replication and mitosis, with no gap phases (Edgar and McGhee, 1988; Azzaria and McGhee, 1992), the delayed divisions in *div* mutant embryos may reflect defects in DNA replication (see below).

div-1 Mutant Embryos Fail to Produce Intestinal and Pharyngeal Cells

Homozygous mutant *div-1* hermaphrodites produce embryos that differentiate well, but they invariably fail to hatch and frequently fail to produce intestinal and pharyngeal cells. For example, mutant embryos from homozygous *div-1(or17)* mothers fail to hatch (1283/1283 embryos), and also fail to make both intestinal cells (185/190 embryos) and pharyngeal muscle cells (347/376 embryos; see Materials and Methods). Similarly, mutant embryos produced by homozygous *div-1(or27)* mothers do not hatch (5824/5824 embryos) and fail to produce both intestinal cells and pharyngeal muscle cells (142/148, and 25/74 embryos failed to make these cell types, respectively).

Instead of making pharynx and intestine, terminally differentiated *div-1* mutant embryos produce large numbers of epidermal or skin cells (called hypodermal cells in *C. elegans*). Based on morphological criteria using Nomarski DIC optics (see Materials and Methods), large numbers of hypodermal cells are produced, not only on the surface of *div-1* mutant embryos but also internally (data not shown). Thus terminally differentiated *div-1* mutant embryos resemble *skn-1* mutants, which also lack pharynx and intestine and instead produce excess skin (Bowerman *et al.*, 1992a). The *skn-1* gene encodes a transcription regulator that specifies the fate of EMS, the ventral-most blastomere in a 4-cell stage embryo (Bowerman *et al.*, 1992a; Blackwell *et al.*, 1994; see Fig. 1). Homozygous *skn-1* mutant mothers produce embryos that differentiate but lack the endoderm and mesoderm normally produced by EMS. Instead, both EMS daughters in *skn-1* mutant embryos produce hypoder-

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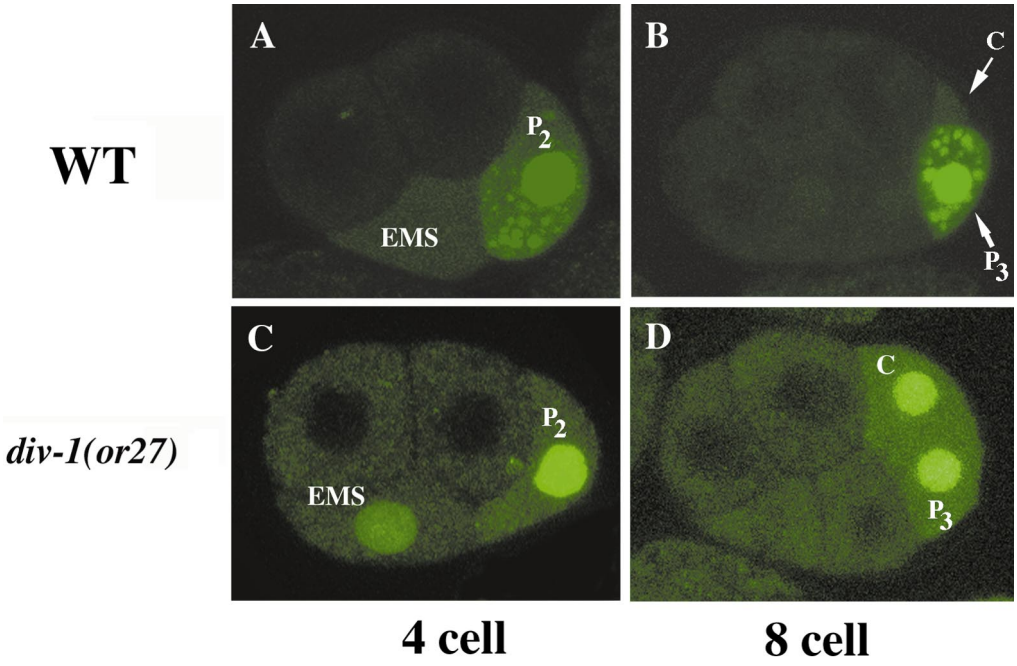

FIG. 4. PIE-1 is mislocalized to EMS and to C in *div-1* mutant embryos. (A, B) Laser scanning confocal images of PIE-1::GFP localized to the nuclei of P_2 (A) and of P_3 (B), and to P-granules in both, in 4-cell and 8-cell stage wild-type embryos, respectively (see Materials and Methods). PIE-1 is present only at low levels and transiently in the cytoplasms of EMS and C. (C, D) PIE-1::GFP expression in *div-1(or27)* mutant embryos at the 4- and 8-cell stages. PIE-1 is mislocalized to EMS and C. Although P_1 and P_2 no longer divide as asymmetrically in *div-1* mutant embryos as in wild-type, the daughters are labeled based on their relative division times and their relative positions in the embryo. The micrographs in A and C, and in B and D, were taken at equivalent stages for wild-type and mutant embryos (see Materials and Methods).

mis and body wall muscle, a fate similar to the that of C, a daughter of P_2 (Bowerman *et al.*, 1992a).

Mislocalization of PIE-1 Is Responsible for the Loss of Intestine and Pharynx in div-1 Mutant Embryos

Because terminally differentiated *div-1* mutants resemble *skn-1* mutants, we examined the expression of SKN-1 protein in *div-1* mutant embryos. We found that, as in wild type (Bowerman *et al.*, 1993; see Fig. 1), SKN-1 accumulates to readily detectable levels only in the posterior-most 2-cell stage blastomere P_1 and its immediate descendants (data not shown). As SKN-1 expression appears normal, we next asked if an antagonist of SKN-1, a CCCH finger protein called PIE-1, might be mislocalized in *div-1* mutant embryos, preventing the SKN-1-dependent specification of pharynx and intestine. In a wild-type embryo at the 4-cell stage, PIE-1 accumulates to high levels in the nucleus of P_2

TABLE 2
Duration of Interphase and Mitosis in WT and *div-1* Embryos[a]

	AB			$AB_{a/p}$			P_1			EMS			P_2		
	WT	*div-1*[b]	*div (or209)*	WT	*div-1*	*div (or209)*	WT	*div-1*	*div (or209)*	WT	*div-1*	*div (or209)*	WT	*div-1*	*div (or209)*
Interphase	9.3	12.3	11.8	9.8	16.3	17.7	11.0	17.8	28.8	11.4	18.3	25.9	13.9	22.7	34.3
Mitosis	3.8	4.7	5.7	3.1	3.1	3.6	3.8	3.6	3.8	3.6	4.1	4.6	3.8	2.8	4.8

[a] Numbers represent the time (in minutes) a particular blastomere spends in either interphase or mitosis. Duration of time of a blastomere in interphase or mitosis is taken as the average for three embryos, for both wild-type and *div-1* mutants.
[b] The *div-1* allele used for these analyses is *or148*ts.

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but can be detected only transiently at low levels in EMS (Mello *et al.*, 1996; Tenenhaus *et al.*, 1998; Reese *et al.*, 2000; Figs. 1 and 4A). At the same time, SKN-1 accumulates to high levels in the nuclei of both P_2 and EMS (Bowerman *et al.*, 1993; Fig. 1). In P_2, PIE-1 prevents SKN-1 function, thereby limiting to EMS the specification of pharyngeal and intestinal cell fates (Mello *et al.*, 1992). In *pie-1* mutants, SKN-1 is free to specify EMS-like identity in both P_2 and EMS, leading to excess pharynx and intestine. In *pie-1; skn-1* double mutants both P_2 and EMS fail to make pharynx and intestine, instead producing hypodermis and body wall muscle, as in *skn-1* mutants (Bowerman *et al.*, 1992a; Mello *et al.*, 1992). We constructed a *div-1 pie-1* double mutant strain and observed in most double mutant embryos large clumps of pharyngeal cells (in 79/90 embryos) and intestinal cells (in 138/142 embryos; see Materials and Methods), in contrast to the absence of any intestinal and pharyngeal cells in most *div-1* mutant embryos (see above). Thus *div-1 pie-1* double mutants resemble *pie-1* mutant embryos, suggesting that the SKN-1 protein is functional in *div-1* mutants but blocked by PIE-1 activity. Consistent with this hypothesis, we detected abnormally high levels in EMS of a PIE-1::GFP fusion protein in *div-1(or17)* mutant embryos (Fig. 4C). Although the levels of PIE-1 in EMS are lower than in P_2 in *div-1 pie-1* double mutant embryos, our analysis suggests that the levels of ectopic PIE-1 are sufficient to block SKN-1 function in most *div-1* mutant embryos. Mislocalization of PIE-1 becomes more severe in later divisions. For example, PIE-1 is more equally distributed when P_2 divides (Fig. 4D). Apparently the mechanisms that regulate the asymmetric distributions of PIE-1 cannot compensate for the delayed cell divisions in these mutants, suggesting that PIE-1 regulation may not be tightly coordinated with the cell cycle during early development in *C. elegans*.

Additional Cell Polarity Defects in div-1 Mutant Embryos

The mislocalization of PIE-1 prompted us to ask whether other elements of asymmetry might also be defective in *div-1* mutant embryos. During our analysis of PIE-1::GFP distribution, we noticed that the daughter cells produced by early cell divisions in *div-1* mutant embryos appeared more similar in size than in wild-type. We therefore compared the sizes of sister blastomeres quantitatively by measuring cell radii immediately after birth to calculate their volumes in wild-type and mutant embryos (Table 3, see Materials and Methods). We found that the early cell divisions in *div-1* mutant embryos generate daughter cells of nearly equal sizes, with the loss in asymmetry more pronounced during the division of P_1 and P_2 than in P_0. Although the daughters of P_1 and P_2 are of equal size in *div-1* mutant embryos, they nevertheless retain a wild-type sequence of asynchronous cell division times (Figs. 2 and 3).

We also examined the localization of cytoplasmic ribonucleoprotein particles called P-granules to germline precursors in early embryos. In wild-type embryos, P-granules are actively segregated to germline precursors during each of the four asymmetric cell divisions that produce the germline founder P_4 (Strome and Wood, 1983; Hird *et al.*, 1996). Although P-granules in *div-1* mutant embryos are localized properly at the 1-cell stage (data not shown), and at the 2-cell stage (Fig. 5D), they are partially mislocalized at the 4- and 12-cell stages to the nuclei of EMS and C, respectively (Figs. 5E and 5F). P-granules are further mislocalized to D, C_a, and C_p in *div-1* mutants (data not shown). As with the PIE-1 and size asymmetries, these P-granule segregation defects become more pronounced as development proceeds.

div-1 Encodes a DNA Polymerase α-Primase Subunit

To understand the molecular basis for the delayed cell divisions in *div* mutant embryos, we cloned *div-1*. Using meiotic recombination, we mapped *div-1* to a position about 0.29 mu to the left of *unc-50* on chromosome III (Fig. 6A; see Materials and Methods). Reducing with RNA interference the function of one gene in this region, R01H10.1, resulted in embryonic lethality, with delayed divisions and a Skn-1-like terminal phenotype indistinguishable from *div-1* mutant embryos (data not shown). Moreover, a YAC clone of genomic DNA that includes R01H10.1 rescued the *div-1* mutant phenotype after germline transformation (see Materials and Methods). To confirm the gene identity, we sequenced R01H10.1 DNA from *div-1* mutants and identified a unique lesion in all three alleles (Fig. 6A).

BLAST searches indicate that R01H10.1 encodes the *C. elegans* DNA polymerase α-primase subunit B (hereafter referred to as the B subunit; Fig. 6B). The *C. elegans* B subunit is 44% similar (24% identical) to the human 70-kDa B subunit, and 42% similar (23% identical) to Pol12p, the B subunit of the α-primase complex from *Saccharomyces cerevisiae*. The B subunit is essential in *S. cerevisiae* (Foiani *et al.*, 1994). The human homolog interacts with both the catalytic A subunit of DNA polymerase α-primase complex and with SV40 T antigen, serving as a tether between these two proteins, and thus promoting formation of a DNA replication initiation complex during SV40 T antigen-mediated viral DNA replication (Collins *et al.*, 1993). In both yeast and human cells, the B subunit undergoes cell cycle-regulated phosphorylation (Fioani *et al.*, 1995; Nasheuer *et al.*, 1991), leading to speculation that it might be a target of cell cycle regulators.

To determine if reducing the function of other *C. elegans* DNA replication genes also results in a delayed division phenotype, we used RNA interference to block the function of three other genes. We microinjected double-stranded RNAs corresponding to DNA polymerase α-primase subunit C and D genes and to the catalytic A subunit gene of the DNA polymerase δ complex into the germline of adult wild-type worms (see Materials and Methods). In each case we observed delayed early cell divisions and prominent

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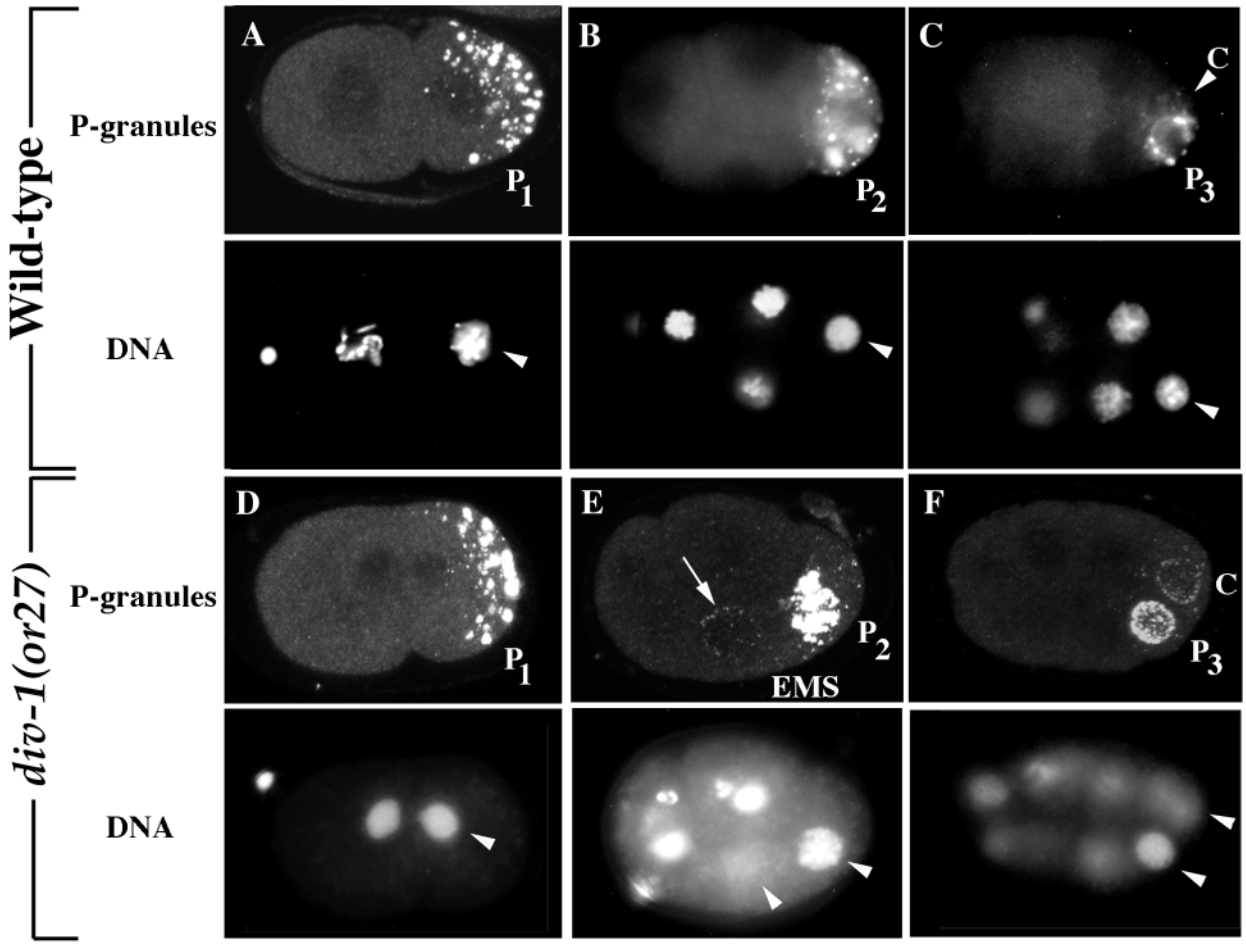

FIG. 5. P-granules are mislocalized in *div-1* mutant embryos. (A,D,E) Laser scanning confocal images, and (B,C) fluorescent photographs of P-granule staining in wild-type (A–C) and *div-1* mutant embryos (D–F). P-granules are normally localized in *div-1* embryos at the 2-cell stage (D), but they are ectopically localized around the nuclei of EMS (arrow in E), and C (F) at the 6- and 12-cell stages. DAPI photographs showing DNA staining are shown below each embryo for both wild-type and *div-1* mutant embryos (arrowheads point to the P_1 nucleus and nuclei of its descendants).

3-cell stages resembling those in *div* mutant embryos (Figs. 7A and B; and data not shown). PIE-1 is also mislocalized to lower levels in both EMS and C in embryos produced by blocking the function (by RNAi) of the catalytic A subunit of the DNA polymerase δ complex (data not shown). In contrast to the loss of asymmetry in early *div-1* embryos, the divisions of at least some the P_1 descendants in early embryos produced by RNAi with the catalytic A subunit of the δ polymerase DNA polymerase complex are still asymmetric (Table 3).

In addition to the delayed division phenotype, misshapen daughter cell nuclei often remain in close proximity to the site of cytokinesis termination in δ-pol RNAi embryos instead of moving toward the center of the daughter cells and becoming spherical, as in wild-type embryos (Fig. 7C). We also observed multinucleated daughter cells in these RNAi embryos (data not shown), and they invariably arrested without fully differentiating (Fig. 7D). These phenotypes appear likely to reflect defects in chromosome segregation, presumably due to incomplete DNA replication. While even an early premature stop codon in the second exon of *div-1(or17)* does not appear to prevent but only delays cell division (see above), we conclude that reducing the function of other DNA replication genes can prevent successful cell division. As noted in Table 1, several *div* mutants exhibit similar defects. While we have not directly examined chromosome segregation in these mutants, it seems likely that in some *div* mutants mitosis is defective due to incomplete DNA replication. Consistent with this possibility, segregation defects have been reported in budding yeast mutants that fail to replicate DNA but nevertheless undergo mitosis (Toyn *et al.*, 1995).

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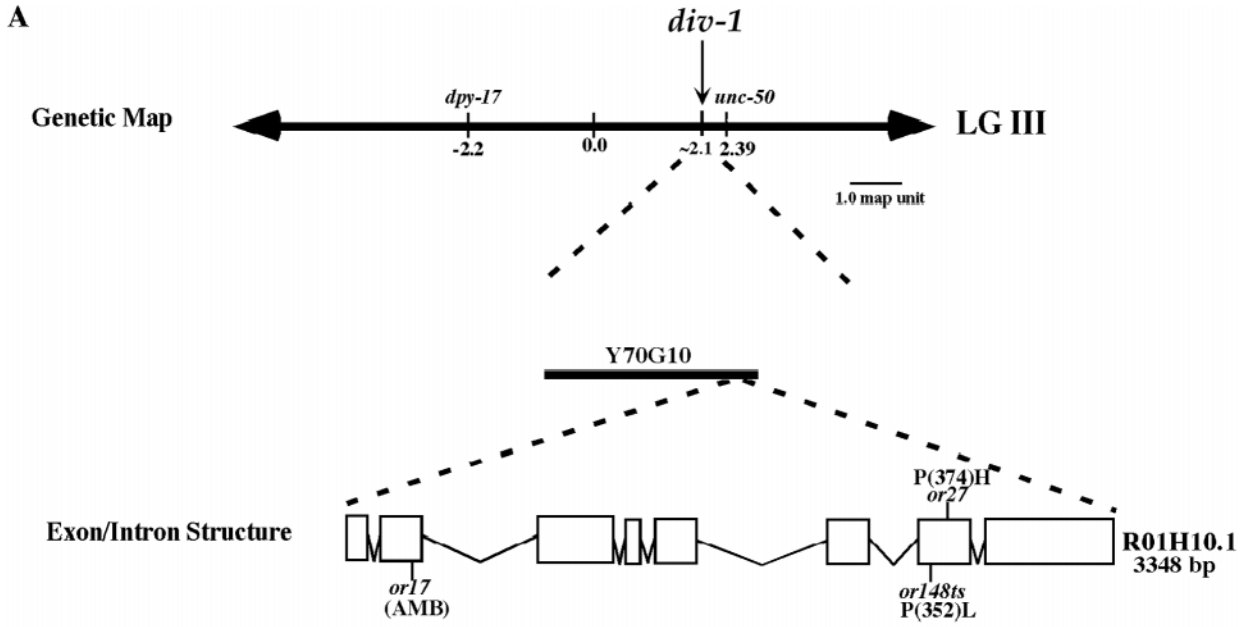

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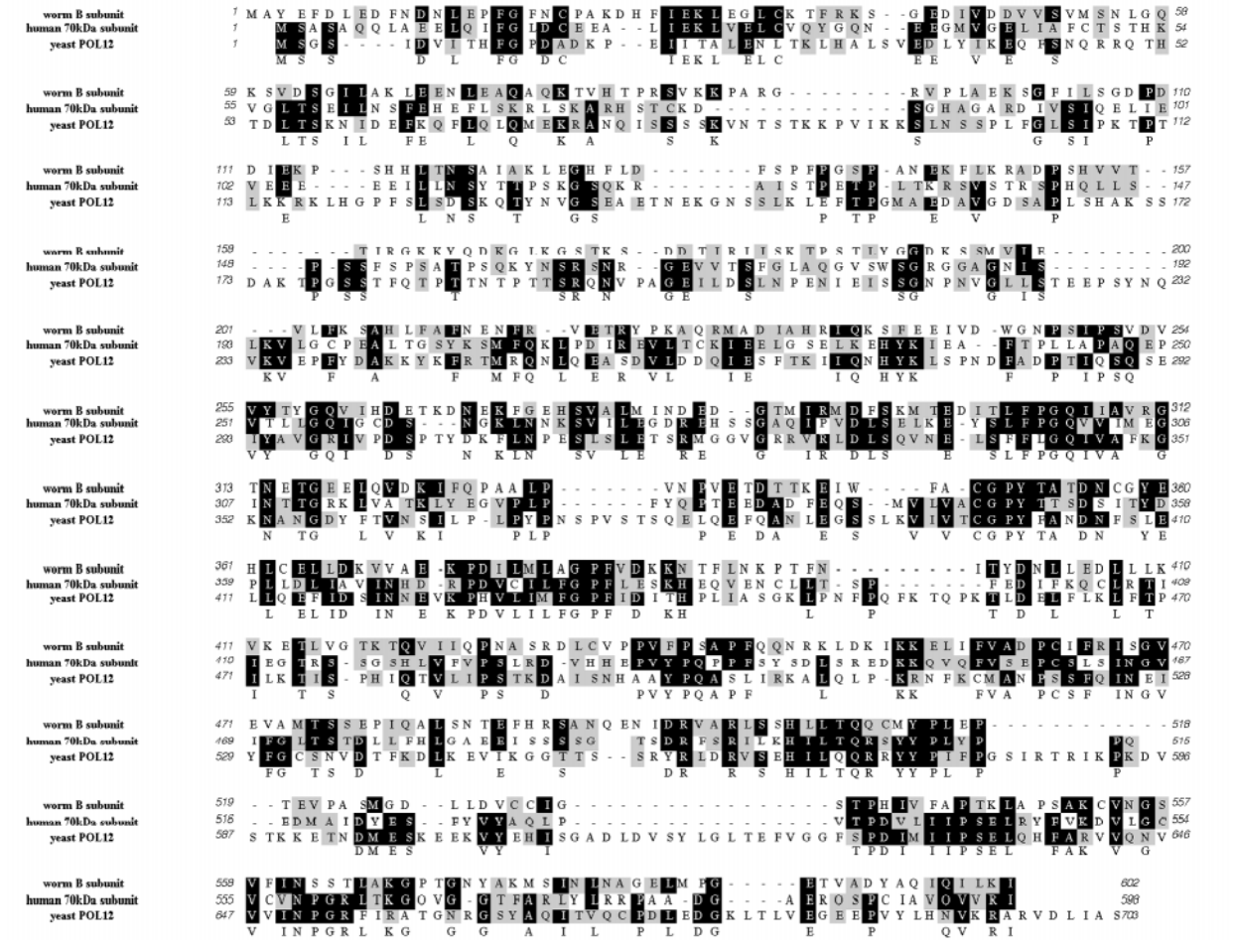

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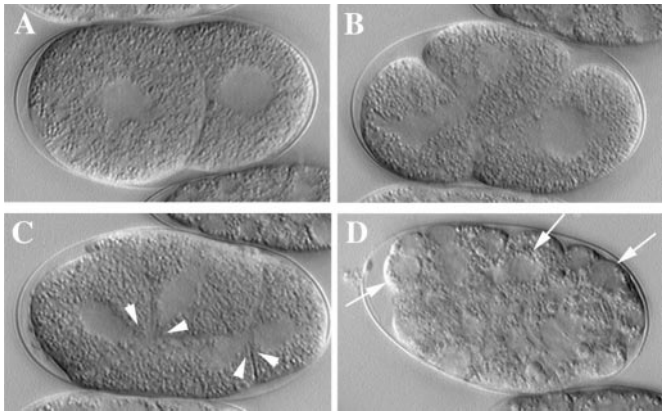

FIG. 7. δ DNA polymerase A subunit RNAi embryos. (A,B,C) Nomarski micrographs of two-, three-, and four-cell RNAi embryos collected approximately 25.5 h after RNA injection and showing the delayed division phenotype similar to that of *div* mutant embryos. Arrowheads indicate where nuclei appear to remain attached to the sites where cytokinesis terminated, suggestive of chromosome segregation defects. (D) Nomarski micrograph of the terminal stage δ DNA polymerase A subunit RNAi embryos collected between 35 and 40 h postinjection. Arrows point to abnormally large and undifferentiated cells in these arrested embryos.

TABLE 3

Loss of Asymmetry during Early Cleavages in *div-1*, and Catalytic Subunit A δ Polymerase RNAi Mutant Embryos

	Ratio of sister blastomeres/volumes[a]		
Blastomeres	Wild-type (*n*)	*div-1(or27)* (*n*)	δ-pol (RNAi) (*n*)
AB/P_1	2.1 (3)	1.8 (3)	2.2 (5)
EMS/P_2	2.4 (3)	1.0 (3)	1.7 (7)
MS/E	1.9 (2)	1.0 (4)	0.9 (6)
C/P_3	2.7 (4)	1.0 (4)	2.3 (6)

[a] Ratios were calculated by dividing the volumes of anterior to posterior sister blastomeres (see Materials and Methods).

DISCUSSION

C. elegans DNA Replication Mutants

From a collection of about 700 conditional embryonic-lethal mutants, we identified 40 delayed division (*div*) mutants that define at least 10 genes, 7 of which are represented by single conditional alleles. The first few cell divisions in these mutants remain asynchronous but are delayed, in some cases by more than twofold relative to wild type. Some *div* mutants differentiate well despite the delays but exhibit patterning defects, others differentiate well but fail to fully elongate or hatch, and still others arrest early in development with large, undifferentiated and apparently aneuploid cells. We conclude that a substantial number of genes in *C. elegans* can mutate to produce embryonic-lethal, delayed division mutant phenotypes.

We suspect that many of the genes we have identified will prove to encode components of the DNA replication machineries used during early embryogenesis in *C. elegans*. We base this prediction on our identification of *div-1* as a DNA Primase subunit B gene, on our demonstration that reducing the function of other *C. elegans* DNA replication genes also results in a delayed division phenotype, and on the relatively large number of genes needed to encode the subunits that make up the DNA replication machinery in a eukaryotic cell (Chervitz *et al.*, 1998). It may prove possible to identify genes not previously known to function in DNA replication because of their identification as *div* loci in *C. elegans*. It also is perhaps likely that mutational inactivation of processes other than DNA replication, including, for example, nucleotide metabolism, can result in a delayed division phenotype. Genome-wide surveys using RNA interference to identify essential *C. elegans* genes should soon make it possible to rapidly determine the molecular identity of most *div* loci. This collection of mutants provides a foundation for genetic and molecular studies of DNA replication and nucleotide metabolism in a developing animal embryo. In particular, the availability of conditional mutants may facilitate efforts to more completely define DNA replication factors through genetic screens for enhancer and suppressor loci.

An Embryonic Cell Cycle Checkpoint for DNA Replication?

Embryonic cells in some *div* mutants appear to attempt mitosis without completing DNA replication, resulting in severe cell division defects. However, our analysis of *div-1* mutant embryos suggests that in less severely defective mutants an embryonic cell cycle checkpoint may delay the

FIG. 6. Molecular cloning of *div-1*. (A) *div-1* maps at approximately +2.1 mu, on LGIII (left of *unc-50). div-1* mutants are rescued by YAC Y70G10. All three alleles of *div-1* have lesions in the predicted gene R01H10.1, as shown above and below the exon/intron diagram. (B) Alignment of the predicted *C. elegans* α DNA polymerase-primase B subunit with B subunits from other organisms. *C. elegans* B subunit is 44% similar (24% identical) to the human 70-kDa B subunit, and is 42% similar (23% identical) to Pol12p, the B subunit of the α DNA polymerase-primase complex from *Saccharomyces cerevisiae*. The consensus line is the last sequence line. Identical and similar amino acids are shown in dark and light shades, respectively.

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onset of mitosis until the completion of DNA replication. In *div-1* and in other *div* mutants, embryonic cell divisions are delayed but otherwise appear normal. This is true even of our strongest *div-1* allele, *or17,* with a premature stop codon in the second exon. As we observe only a delay in DNA replication in *div-1* mutants rather than an absolute block, our alleles may not be null. Alternatively, some DNA replication components may not be essential for DNA replication per se but necessary for the rapid completion of DNA synthesis that normally occurs in early embryonic cells. As the delay in cell division occurs almost entirely during interphase, we conclude that in *div-1* mutants the onset of mitosis is delayed sufficiently to compensate for a longer S phase. Indeed, the embryonic lethality of *div-1* mutants appears to be due not to defects in cell division but rather to defects in pattern formation. Perhaps the cell cycle machineries in rapidly dividing, early embryonic cells are better equipped to compensate for changes in the rate of DNA replication than are the developmental programs that specify founder cell fates.

Developmental Regulation of the Cell Cycle in Early C. elegans Embryos

Our results indicate that developmental regulators in the early *C. elegans* embryo cannot tolerate significant delays in cell division. However, they do not address whether the different cell cycle times of early embryonic cells are important for specifying founder cell fates, or if the differences in division time are important for other developmental processes, such as the proper positioning of early embryonic cells. Whatever their role in early development, the different cell cycle times of early embryonic cells in *C. elegans* appear to result from the differential regulation of DNA replication rates (see Introduction). Presumably developmental regulators target components of the DNA replication machinery to modulate the rate of replication in early embryonic cells.

One candidate for a regulator of DNA replication is a predicted ser/thr kinase encoded by the *C. elegans* gene *par-4* (Watts *et al.*, 2000), one of six maternally expressed *par* genes (reviewed in Rose and Kemphues, 1998). Inactivation of any one *par* gene results in synchronous, or nearly synchronous, cell divisions in the early embryo. However, the most specific timing defects occur in *par-4* mutants. Unlike other *par* mutants, the first division is unequal, as in wild-type, and mitotic spindle orientations at the 2-cell stage also are roughly normal in *par-4* mutants. Nevertheless, mutations in *par-4* do result in other defects, as cytoplasmic P-granules fail to localize posteriorly (Kemphues *et al.*, 1988). Thus *par-4* must have a more general role in establishing anterior-posterior polarity, in addition to any role it may have in regulating the timing of cell division. The putative ser/thr PAR-4 kinase is present uniformly throughout the cytoplasmic cortex of all early embryonic cells, but its targets remain unknown. Intriguingly PAR-4 interacts with the *C. elegans* replication factor C in a yeast two-hybrid assay (D. Hurd and K. Kemphues, personal communication), providing some support for *par-4* having a role in regulating the timing of cell division.

Proper Cell Division Timing Is Required to Specify Founder Cell Fates in Early C. elegans Embryos

We have shown that in addition to their delayed cell divisions, *div-1* mutant embryos also exhibit significant losses of cell polarity during what are normally highly asymmetric cell divisions. The polarity defects in early *div-1* mutant embryos include a failure to limit the expression and function of PIE-1 to germline precursors, a loss of sister cell size asymmetries, and a failure to segregate cytoplasmic P-granules to germline precursors. Delayed cell divisions are the first defect we have observed in *div-1* mutant embryos, while all of the polarity defects become more severe as development proceeds. Based on these observations, and because *div-1* encodes a DNA replication subunit, we conclude that the cell polarity defects are secondary consequences of delayed cell division. Thus the correct timing of cell division appears essential for properly executing the developmental programs that specify the fates of early embryonic cells. Perhaps this inability to tolerate delayed cell division reflects a lability of cell polarity maintenance mechanisms in the early embryo. Furthermore, it is possible that some of the polarity defects we have observed might be linked. For example the defects in PIE-1 and P-granule localization might be secondary to the loss of sister cell size asymmetries. Alternatively, each defect might reflect the sensitivity of a separate process that contributes to cell polarity. Finally, we emphasize that not all asymmetries are lost in *div-1* mutant embryos. In particular, early embryonic cells in *div-1* mutants retain the same sequence of asynchronous divisions times seen in wild-type embryos. Apparently the differential regulation of cell division timing can occur in large part independently of cell size and perhaps of other asymmetry-generating processes in the early embryo.

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