glp-3 Is Required for Mitosis and Meiosis in the Caenorhabditis elegans Germ Line

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ABSTRACT

The germ line is the only tissue in Caenorhabditis elegans in which a stem cell population continues to divide mitotically throughout life; hence the cell cycles of the germ line and the soma are regulated differently. Here we report the genetic and phenotypic characterization of the glp-3 gene. In animals homozygous for each of five recessive loss-of-function alleles, germ cells in both hermaphrodites and males fail to progress through mitosis and meiosis, but somatic cells appear to divide normally. Germ cells in animals grown at 15°C appear by DAPI staining to be uniformly arrested at the G2/M transition with <20 germ cells per gonad on average, suggesting a checkpoint-mediated arrest. In contrast, germ cells in mutant animals grown at 25°C frequently proliferate slowly during adulthood, eventually forming small germ lines with several hundred germ cells. Nevertheless, cells in these small germ lines never undergo meiosis. Double mutant analysis with mutations in other genes affecting germ cell proliferation supports the idea that glp-3 may encode a gene product that is required for the mitotic and meiotic cell cycles in the C. elegans germ line.

CONTROL of cell proliferation is a crucial aspect of development. Proliferation of undifferentiated cells must occur early in development to generate appropriate numbers of cells for each tissue type; later, proliferation must slow down or cease when differentiated tissues are formed. In some tissues, stem cells remain capable of reentering the cell cycle throughout life; hence controls must exist to ensure they do not divide in an uncontrolled way that would result in a tumor. Therefore, both the initiation and cessation of the cell cycle must be tightly regulated during the development of multicellular organisms.

In Caenorhabditis elegans, the control of proliferation differs in somatic and germline tissues. Cells in somatic tissues divide a finite number of times during larval development before terminally differentiating or dying. In contrast, cell division in the germ line of both males and hermaphrodites continues throughout life: a population of mitotic stem cells at the distal end of the germ line divides to give rise to meiotic cells that form gametes in the proximal region. Maintenance of both the mitotic and meiotic cell cycles in the germ line is essential for continued fertility in both sexes. How does the germ line maintain proliferation when all other tissues have ceased dividing?

The best characterized control of germline proliferation is induction of mitosis by the distal tip cell (Kimble and White 1981). Three elements of the signal transduction pathway mediating that interaction have been identified: lag-2 appears to encode a signalling molecule expressed in the distal tip cell (Henderson et al. 1994; Tax et al. 1994), glp-1 encodes the germline-localized receptor (Austin and Kimble 1987, 1989; Yochem and Greenwald 1989; Crittenden et al. 1994), and lag-1 transduces the signal from the receptor to the nucleus in the germ line (Christensen et al. 1996; Roehl et al. 1996). Mutations have been identified in all these genes that cause a defect in the specification of the mitotic fate: after hatching, there are only one to two mitotic divisions in the germ line, followed by meiotic differentiation of all germline cells (a "Glp" phenotype) (Austin and Kimble 1987; Lambie and Kimble 1991).

The GLP-1 signal transduction pathway is used at other times during C. elegans development for cell-cell interactions that do not involve induction of mitosis (Schnabel 1996). Furthermore, this pathway is evolutionarily conserved, with homologues involved in cell-cell interactions in animals ranging from flies to humans (Artavanis-Tsakonas et al. 1995). Therefore, it is likely that within each tissue the pathway is modified to allow expression of target genes specific for that tissue. In the C. elegans germ line, neither these putative modifiers nor the target genes for mitotic proliferation have been identified. It is expected that mutations in genes that encode germline-specific positive modifiers of the GLP-1 pathway would cause a defect in promoting entry into mitosis, but would allow meiosis and gametogenesis. In contrast, mutations in target genes that are required for the cell cycle per se might cause an irreversible mitotic arrest or else result in defects in both the mitotic and meiotic cell cycles, thus preventing both proliferation and differentiation of the few germ cells that are present.
One gene that has been identified as being important for the germline cell cycle is glp-4 (Bean and Strome 1992). The glp-4 gene was originally identified as a temperature-sensitive allele, bn2 ts, that causes failure in both mitosis and meiosis in the germ line when grown continuously at the restrictive temperature, and that causes defective oogenesis when shifted to the restrictive temperature in adulthood (Bean and Strome 1992). Interestingly, three weaker alleles of glp-4 were identified in a screen for enhancers of a temperature-sensitive allele of glp-1 (Qiao et al. 1995), suggesting the possibility that glp-4 might be a target of GLP-1-mediated signaling.

In this paper, we focus on the glp-3 gene, which is required for cell cycle progression in the germ line. Like glp-4(bn2 ts), all five recessive mutations in glp-3 cause germ cells to fail in both mitosis and meiosis. glp-3 may be essential for the cell cycle in the germ line and thus may be a target of the GLP-1 signal-transduction pathway.

MATERIALS AND METHODS

Nematode strain maintenance: Worm strains were maintained on agar plates seeded with Escherichia coli strain OP50 (Brenner 1974). Growth was at 20°C unless otherwise indicated.

Strains used: All strains used are derivatives of the wild-type Bristol strain N2 (Brenner 1974). We used the following mutations, described in Hodgkin et al. (1988) or cited references:

LG I: fog-1(q253ts) (Barton and Kimble 1990), gld-1(q268) (Frangis et al. 1995), glp-4(bn2 ts) (Bean and Strome 1992)

LG II: glp-1(q231), glp-1(q175) (Austin and Strome 1997), gld-1(q43), gld-1(q56), gld-1(q71), gld-1(q363), gld-1(ts) (this paper), dpy-19(e1259 ts), unc-2(e729), unc-2(e1865), unc-32(e189), unc-36(e251), unc-36(e1855)

LG III: fem-3(q96) (Barton et al. 1987), dpy-20(e1282 ts)

Arrangements: e(t1III.V), dpy-16, dpy-17

Genetic mapping: The glp-3 locus was mapped on chromosome III by three-factor mapping (Table 1). Based on the mapping of glp-3 relative to smo-3 and unc-36, it is estimated to be ~0.02 map units to the right of smo-3, at position ~0.88 (Figure 2).

Mutagenesis: All alleles of glp-3 were obtained following EMS mutagenesis (Brenner 1974) in general screens for self-sterile mutants (Barton and Kimble 1990). S. Maples, P. Balaski and J. Kimble, unpublished results. Each allele was identified as a sterile in the F2 generation by dissection scope, and as lacking germ line proliferation by differential interference contrast (DIC) microscopy. The five alleles identified arose from ~28,000 haploid genomes screened, giving an isolation frequency of 2 × 10⁻⁶, similar to the isolation frequency of loss-of-function alleles of other genes identified in those screens (Barton and Kimble 1990). All alleles were backcrossed to the wild type at least five times.

Brood analysis: 1.4 animals of genotype glp-3(x)/+ smo-3(e991) unc-36(e251) were placed onto individual plates and transferred every day until an entire brood was laid. Progeny were transferred to individual plates as young larvae, then scored as sterile Glp-3 animals, fertile heterozygotes, or Sma Uncs over the next few days. Parent plates were also checked for dead embryos. In no case were dead embryos found. Two complete broods were scored for each allele. Brood sizes were normal. The average brood size for each allele, and the percentage of total progeny represented by self-sterile homozygotes for each allele was as follows: q145, 305 (25.2%); q156, 367 (24.6%); q171, 360 (24.7%); q363, 230 (24.8%); q56, 260 (25.2%).

Construction of double and triple mutants for epistasis analysis: fog-1(q253ts); glp-3(q145): glp-3(q145)/+ males were crossed to fog-1(q253ts) females (grown at 25°C). q253+/+: q145/+ cross-progeny were picked, and their self-progeny were screened for those that throw 100% sterile (Fog or Glp) progeny at 25°C. q253fog-1(q145)/+ females and q253fog-1(q145)/+ males were transferred to individual plates as young larvae, then scored, it was found that eight had a Glp-3 phenotype by DIC analysis: fog-1(q96gf); glp-3(q145): fem-3(q96gf) ts; dpy-20(e1282 ts) mutants: glp-3(q145)/+ males were crossed to fem-3(q96gf) ts; dpy-20(e1282 ts) hermaphrodites at 15°C. glp-3/+; fem-3 dpy-20/+ + cross-progeny were identified as animals that threw one-quarter Glp animals at 15°C; glp-3/+/+; fem-3 dpy-20 animals were allowed to lay some eggs at 15°C to maintain the strain before being transferred to 25°C. Those progeny grown at 25°C were scored by DIC optics for being either Glp-3 or Mag (a masculinized germ line, the fem-3 [gf] phenotype). Among 26 progeny scored, it was found that right had a Glp-3 phenotype by DIC optics and by DAPI-staining, indicating that glp-3 is epistatic to fem-3 (q96gf).

The phenotype of the double mutant was determined by shifting these animals to 25°C and determining the germ line phenotype of an entire brood. One hundred thirty-five (76.3%) of the progeny were Fog, whereas 42 (23.7%) were Glp-3.

glp-3(q145); fem-3(q96gf ts) dpy-20(e1282 ts) mutants: glp-3(q145)/+ males were crossed to fem-3(q96gf) ts; dpy-20(e1282 ts) hermaphrodites at 15°C. glp-3/+; fem-3 dpy-20/+ + cross-progeny were identified as animals that throw one-quarter Glp animals at 15°C; glp-3/+/+; fem-3 dpy-20 animals were allowed to lay some eggs at 15°C to maintain the strain before being transferred to 25°C. Those progeny grown at 25°C were scored by DIC optics for being either Glp-3 or Mag (a masculinized germ line, the fem-3 [gf] phenotype). Among 26 progeny scored, it was found that right had a Glp-3 phenotype by DIC optics and by DAPI-staining, indicating that glp-3 is epistatic to fem-3 (q96gf).

glp-3(q145) gld-1(q268); glp-3(q145) mutants: glp-3(q145)/+ mutants: glp-3(q145) unc-32(e189); glp-3(q145) ncl-1(e1865) unc-36(e251)/+ + hermaphrodites were allowed to self-fertilize at 25°C. Among the nongravid Dpy animals (which exclude recombinants between dpy-5 and gld-1) were 69 Dpy non-Uncs that had tumorous germ lines (presumably dpy-5 gld-1; gld-3 ncl-1 unc-36/+ + + and dpy-5 gld-1 animals) and 29 Dpy Uncs that were Glp-3 (presumably dpy-5 gld-1; gld-3 ncl-1 unc-36 animals).

glp-3(q145); glp-3(q175) mutants: glp-3(q145) unc-36(e251)/+ mutants: glp-3(q145) unc-36(e251) dpy-17(e164) unc-32(e189) glp-1(q175); him-5(e1490) males were mated to dpy-17(e164) unc-36(e251) hermaphrodites, and the F2, were screened for non-Dpy non-Unc recombinant cross progeny (of genotype either glp-3 unc-32 glp-1 dpy-17 unc-36 or unc-32 glp-1 dpy-17 unc-36). These recombinant animals were then tested for the presence of both glp-3 and glp-1 mutations by complementation.

glp-4(bn2 ts); glp-3(q145) mutants: glp-3(q145) unc-32(e189)+/+ males were crossed to glp-4(bn2 ts); smo-3(e991) unc-36(e251) hermaphrodites at 15°C. glp-4/++; glp-3 unc-32/sma-3 unc-36 progeny were allowed to self-fertilize to generate glp-4; glp-3 unc-32/sma-3 unc-36. Gravid heterozygotes grown at 15°C were transferred to 25°C and glp-4; glp-3 unc-32 progeny were scored.

Tests of glp-3 over a deficiency: glp-3(x)/+ males were mated to Unc hermaphrodites from strain BC4688 dpy-17(e164) dpy-17(e164) unc-32(e189); glp-3. (Dpy unc disturbance covers dpy-17 and dpy-17.) Non-Unc hermaphrodite cross-progeny were picked as L4s and screened by dissecting scope the following day for a sterile phenotype. These animals were picked and examined by DIC optics and by DAPI staining. Alternatively, glp-3(x)/+ males were mated into strain DA866 nD16/qC1 (dpy-19(e1259 ts) glp-1(q1339) ts) and examined for the presence of non-Dpy sterile Glp-3 progeny.

DAPI staining: Worms were washed briefly in M9 buffer, then fixed and stained in 300 μl of 0.2 μg/ml 2,4-diamidino-2-phenylindole (DAPI) in 95% ethanol. After ~10 min at room temperature, the worms were mounted on a slide in a drop of mounting medium (10% polyvinyl alcohol alcohol grade 51-05 [DuPont], 25% glycerol, 50 mM Tris pH 8.5, 5 mM sodium,
The development of the wild-type somatic gonad has been described in detail (Kimble and Ward 1988). We briefly review the structure of the adult hermaphrodite and male gonad and germ line for background information (see Figure 1). The hermaphrodite gonad consists of two U-shaped germline arms extending anteriorly and posteriorly away from the centrally located somatic gonad. Each germline arm has a distal-to-proximal axis relative to the central gonad, such that mitotic cells are found distally, and cells in various stages of meiosis and gametogenesis are found more proximally (Figure 1A). The mitosis in the distal region of each arm is induced by a single somatic cell, the distal tip cell, that caps each germ line arm (Kimble and White 1981). Cells enter meiosis ~20 cell diameters from the distal tip cell (Crittenden et al. 1994) and differentiate in the proximal region of the germ line (as sperm in L4, or as oocytes in adulthood). It should be noted that although we use the term "cells", germline nuclei are incompletely cellularized until the final stages of gametogenesis, hence the germ line is syncytial.

The male gonad is similar to that of hermaphrodites, but consists of only a single reflexed arm with a distal to proximal polarity relative to the somatic seminal vesicle and vas deferens in the posterior part of the animal (Figure 1B). Two somatic distal tip cells located at the distal end of the germ line are required to maintain the distal mitotic stem population, and cells in various stages of meiosis and gametogenesis are found more proximally. Spermatogenesis begins in L4 and continues throughout adulthood.

**RESULTS**

**Wild-type gonad and germline development:** The development of the wild-type somatic gonad has been described in detail (Kimble and Ward 1988). We briefly review the structure of the adult hermaphrodite and male gonad and germ line for background information (see Figure 1). The hermaphrodite gonad consists of two U-shaped germline arms extending anteriorly and posteriorly away from the centrally located somatic gonad. Each germline arm has a distal-to-proximal axis relative to the central gonad, such that mitotic cells are found distally, and cells in various stages of meiosis and gametogenesis are found more proximally (Figure 1A). The mitosis in the distal region of each arm is induced by a single somatic cell, the distal tip cell, that caps each germ line arm (Kimble and White 1981). Cells enter meiosis ~20 cell diameters from the distal tip cell (Crittenden et al. 1994) and differentiate in the proximal region of the germ line (as sperm in L4, or as oocytes in adulthood). It should be noted that although we use the term "cells", germline nuclei are incompletely cellularized until the final stages of gametogenesis, hence the germ line is syncytial.

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**Identification and characterization of glp-3:** To identify genes required for germline proliferation, we screened for recessive F2 steriles that failed to develop a germ line (see MATERIALS AND METHODS). Five alleles, q145, q156, q171, q363, and q456, define a new locus, glp-3. Each allele maps to the same approximate position between sma-3 and unc-36 on chromosome III and fails to complement the allele q145. More detailed three-factor mapping using q145 places glp-3 to the right of sma-3, at about position ~0.88 on chromosome III (Figure 2, Table 1). Figure 3 shows a comparison of the germline phenotypes of wild-type and glp-3 mutants. Wild-type adult hermaphrodites have a fully reflexed germ line that eventually produces >1000 mitotic and meiotic cells, mature sperm and oocytes (Figure 3A). In contrast, homozygous adults of all five alleles of glp-3 have germ lines with very few cells and no differentiated gametes (Figure 3B). Hence, germ cells fail to undergo...
both mitosis and meiosis. This phenotype is similar in males: whereas wild-type adults produce hundreds of germ cells that are found in mitosis and in various stages of spermatogenetic meiosis (Figure 3C), glp-3 males have very small reflexed germ lines and fail to generate sperm (Figure 3D). It is important to note that although there is a severe defect in germline proliferation in glp-3 mutants, no somatic proliferation defects are apparent. First, the size, shape and movement of the animals all appear to be normal, as if approximately the correct number of cells have been produced. Second, at least three other tissues that undergo postembryonic proliferation are unaffected by glp-3 mutations: the somatic gonad and male tail are morphologically correct, and the ventral nerve cord appears to function normally.

Wild-type and glp-3 animals were stained with DAPI to examine the number and morphology of germ line nuclei. Wild-type adults have >1000 germ line nuclei, and there is a range of nuclear morphologies along the distal to proximal axis, characteristic of cells in the mitotic cycle, in meiotic pachytene, in meiotic diakinesis, and fully differentiated sperm (Figure 4A). glp-3 adult hermaphrodites grown at 15° or 20° have only three to four germ line nuclei per gonad arm and these have a uniform morphology suggesting they are all arrested at the same stage of the cell cycle (Figure 4B). In these nuclei, chromosomes appear to be moderately condensed, as if at the G2/M transition. A similar phenotype is seen in glp-3 adult males (Figure 4C). This characteristic morphology resembles that of some of the distal mitotic nuclei in wild-type germ lines (compare Figure 4, B and C arrows with Figure 4D, arrow), as if the nuclei are arrested at a normally occurring stage of the cell cycle.

We found that glp-3 animals grown at 25° often have a larger number of germ cells than is seen in animals grown at 15° or 20° (Figure 4E). This observation might reflect faster germline growth at 25° than at 15° or 20°. Alternatively, it might reflect a tighter arrest of germ cell division at lower temperatures. To distinguish between these possibilities, we quantitated germline growth as a function of time for each allele at both 15° and 25°. glp-3 animals grown at either 15° or 25° were DAPI-stained at several time-points starting in L4, and the number of germ line nuclei per gonad arm was counted. Because the rate of development at 15° is approximately half that at 25° (HIRSH et al. 1976), animals picked at L4 were allowed to develop twice as long in adulthood at 15° as they were at 25° before they were stained, in order that similar developmental time-points could be compared. We found that at 15°, there were on average only six to 21 germ line nuclei per gonad after 4 days of adulthood. Furthermore, the number of germ line nuclei in glp-3 animals grown at 15° remained fairly constant or decreased by 12 days of adulthood (Table 2). In contrast, germline proliferation continued slowly in animals homozygous for any of the five alleles when grown at 25°: only a few germ line nuclei were present per gonad arm at L4, but by 6 days of adulthood some germ line arms had several hundred nuclei (Figure 4E, Table 2). We therefore conclude that there is a tighter arrest of germ cell growth at 15° than at 25°.

For all alleles grown at 25°, the amount of proliferation observed was highly variable; for example, some worms had several hundred germ line nuclei in one gonad arm, but only a few in the other. Most nuclei appeared abnormal in morphology and were frequently fragmented. Those arms with larger numbers of nuclei sometimes appeared reflexed, with nuclei that resembled early meiotic configurations near the bend of the germ line. Nevertheless, those germ lines never formed gametes, supporting the idea that glp-3 is required for meiosis. Homozygotes for q156 had significantly less proliferation than was seen in all the other alleles at 25°, perhaps indicating that it is a strong allele.

At both 15° and 25°, gonads from mutant L4 worms had on average fewer than 20 germ cells, by which time a wild-type worm has produced >500 germ cells, many of which have differentiated into sperm (KIMBLE and WHITE 1981). Since in the wild type there are more germ cells by the end of L1 than are seen in young adult glp-3 mutants, the glp-3 defect must occur soon after hatching.

**glp-3 mutations are loss-of-function**: Several lines of evidence are consistent with the idea that the glp-3 mu-

### TABLE 1

<table>
<thead>
<tr>
<th>Parental genotype*</th>
<th>Recombinant phenotype</th>
<th>Recombinant genotype</th>
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<td>Lon non-Unc</td>
<td>lon-1 glp-3/lon-1 ncl-1 unc-36</td>
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<td>Unc non-Glp</td>
<td>glp-3 ncl-1 unc-36/sma-3 <em>((\pm) ncl-1)</em> Unc-36</td>
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<td>Unc non-Glp</td>
<td>glp-3 ncl-1 unc-36/sma-4 <em>((\pm) ncl-1)</em> Unc-36</td>
<td>7/7</td>
</tr>
<tr>
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<td>glp-3 unc-36/sma-4 unc-36</td>
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</tr>
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</tr>
<tr>
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<td>Sma non-Unc</td>
<td>sma-3 glp-3/sma-3 unc-36</td>
<td>1/52</td>
</tr>
</tbody>
</table>

* glp-3 (q145) was used in all cases.

* *(\(\pm\) ncl-1)* indicates that the ncl-1 phenotype was not scored in these animals.
tations result in a reduction of gene activity. First, analysis of entire broods from glp-3 heterozygote hermaphrodites shows that all alleles of glp-3 are fully recessive and fully penetrant for the Glp-3 sterile phenotype (brood sizes were normal; see MATERIALS AND METHODS). Second, the alleles were picked up in screens for recessive steriles at a frequency of $\sim 2 \times 10^{-4}$ genomes, similar to the isolation frequency of loss-of-function alleles of other genes identified in those screens (BARTON and KIMBLE 1990). Third, when each allele was made transheterozygous to the deficiency slf127 at 20°, the phenotype was similar to that of the homozygous alleles: healthy but sterile adults with reduced germline proliferation (Table 3). Animals homozygous for two of the
alleles, q171 and q456, had similar numbers of germline nuclei when hemizygous as when homozygous, consistent with them being loss-of-function. Surprisingly, however, animals homozygous for three of the alleles (q145, q156, and q363) had significantly more germline nuclei when hemizygous than when homozygous ($P \leq 5 \times 10^{-5}$, Wilcoxon Rank Sum Test). To test the possibility that this increased level of proliferation resulted from haplo-insufficiency at another locus that is uncovered by sDf127, q145 was also placed in trans to nDf16, a second deficiency that only partially overlaps sDf127 (Figure 2). A similar level of increased proliferation was observed in the two different hemizygotes (Table 3), indicating that if such a haplo-insufficient locus exists, it is contained within the overlap of sDf127 and nDf16.

An alternative hypothesis is that the weaker phenotype observed in certain hemizygotes compared with the corresponding homozygotes reflects a difference in the nature of those alleles.

All pairwise combinations of trans-heterozygotes were made in an attempt to determine whether any of the alleles behaved like a deficiency (i.e., was likely to be a null allele). None of the alleles, when placed in trans to second allele, behaved like a deficiency placed in

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**Figure 4.**—DAPI-stained wild-type and glp-3 mutant germlines. (A) A wild-type (N2) adult hermaphrodite half-gonad stained with DAPI. The distal mitotic region is indicated by a small arrowhead, nuclei in pachytene by a short arrow, an oocyte nucleus by a long arrow, and sperm by a large arrowhead. (B) A glp-3 (q145) adult hermaphrodite half-gonad, from a worm grown at 15°. The three germline nuclei are indicated by arrows, and each has a morphology resembling a G2/M arrest. (C) A glp-3 (q145) adult male gonad from a worm grown at 15°. The six germline nuclei are indicated by arrows, and each has a morphology resembling a G2/M arrest. (D) A section of a wild-type distal germ line. The arrow indicates a germline nucleus resembling the morphology seen in glp-3 mutants grown at 15°. The arrowhead indicates a germline nucleus in metaphase. (E) A glp-3 (q145) half-gonad germ line from a 6-day adult animal grown at 25°. Note fairly extensive proliferation, but absence of gametes. This germ line is not reflected and has nuclei with variable morphologies. The arrowhead and arrow indicate the distal and proximal ends of the germ line, respectively. Bars, ≈20 μm.
in the wild type, the mitotic influence of the distal tip germline nuclei to the distal tip cells, which promote mitosis. We consider this unlikely for cells extends only glp-3 mutants might result from the proximity of the few alleles strongly suggests that lack of germ cell proliferation is the loss-of-function phenotype.

- For all alleles, some adults grown 12 days at 15° had at least one gonad arm with no identifiable germ nuclei. The percentage of animals with such arms ranged from 17 to 63%. Germ nuclei that were present almost always looked condensed or fragmented.
- Estimates of numbers of germ nuclei in the wild type (N2) are based on previous counts done at 20° (Kimble and White 1981). The corresponding numbers at 15° and 25° would be ~50% lower or higher, respectively, than those at 20°.
- The full genotype of these animals was glp-3(q363)/dpy-19(e1259 ts).
- These germ lines were highly variable in both nuclear morphology and in the numbers of nuclei/gonad arm. The averages given are based on rough estimates of numbers of nuclei when numbers were > ~200. The ranges in the number of nuclei/gonad arm for these timepoints were as follows: q145 = 0→150; q171 = 0→350; q363 = 0→1000; q456 = 0→1000. Pachytenic nuclei were often seen near the bend or the most proximal region of these arms, however sperm were never formed.
- Some nuclei had pachytenic-like structure.

**TABLE 2**

<table>
<thead>
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<th>Genotype</th>
<th>14</th>
<th>4-day adult</th>
<th>12-day adult*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
<td>4-day adult</td>
<td>12-day adult*</td>
</tr>
<tr>
<td>N2*</td>
<td>~500</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>glp-3(q456)</td>
<td>7.8 ± 1.4 (14)</td>
<td>5.6 ± 0.9 (14)</td>
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<tr>
<td>glp-3(q156)</td>
<td>7.8 ± 1.1 (11)</td>
<td>7.4 ± 2.0 (8)</td>
<td>3.8 ± 2.7 (11)</td>
</tr>
<tr>
<td>glp-3(q171)</td>
<td>16 ± 5.3 (10)</td>
<td>21 ± 7.4 (9)</td>
<td>12 ± 6.6 (8)</td>
</tr>
<tr>
<td>glp-3(q363f)</td>
<td>7.5 ± 1.2 (15)</td>
<td>8.9 ± 1.7 (11)</td>
<td>3.9 ± 1.8 (13)</td>
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<tr>
<td>glp-3(q456)</td>
<td>8.7 ± 1.3 (15)</td>
<td>7.9 ± 1.7 (16)</td>
<td>2.1 ± 1.9 (10)</td>
</tr>
</tbody>
</table>

Animals were grown at the indicated temperature to the indicated age and DAPI-stained; germ nuclei were counted using epifluorescence optics. Number of animals tested in parentheses.

* For all alleles, some adults grown 12 days at 15° had at least one gonad arm with no identifiable germ nuclei. The percentage of animals with such arms ranged from 17 to 63%. Germ nuclei that were present almost always looked condensed or fragmented.

**TABLE 3**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average no. of nuclei per gonad</th>
</tr>
</thead>
<tbody>
<tr>
<td>glp-3(q145)</td>
<td>5.8 ± 1.1 (15)</td>
</tr>
<tr>
<td>glp-3(q156)</td>
<td>7.0 ± 1.8 (20)</td>
</tr>
<tr>
<td>glp-3(q171)</td>
<td>38 ± 14 (22)</td>
</tr>
<tr>
<td>glp-3(q363f)</td>
<td>17 ± 16 (19)</td>
</tr>
<tr>
<td>glp-3(q456)</td>
<td>13 ± 6.5 (17)</td>
</tr>
<tr>
<td>glp-3(q456)/dpy-19(e1259 ts)</td>
<td>16 ± 6.8 (24)</td>
</tr>
<tr>
<td>glp-3(q156)/dpy-19(e1259 ts)</td>
<td>17 ± 2.5 (9)</td>
</tr>
<tr>
<td>glp-3(q171)/dpy-19(e1259 ts)</td>
<td>17 ± 6.2 (24)</td>
</tr>
<tr>
<td>glp-3(q363)/dpy-19(e1259 ts)</td>
<td>35 ± 17 (27)</td>
</tr>
<tr>
<td>glp-3(q456)/dpy-19(e1259 ts)</td>
<td>16 ± 6.0 (27)</td>
</tr>
</tbody>
</table>

All animals were grown at 20°, DAPI-stained as 1- to 2-day-old adults, and germ nuclei were counted using epifluorescence optics. Number of animals tested in parentheses.

* In all cases, glp-3(q363) was linked to dpy-19(e1259 ts).

Significantly different by the Wilcoxon Rank Sum Test, P < 5 x 10^{-3} (Freund 1988).

cell (Crittenden et al. 1994), yet the larger germlines formed in glp-3 mutants grown at 25° occasionally have several hundred germ cells, so that some cells are much further than 20 cell diameters from the distal tip cell; nevertheless none of these cells undergo meiosis. Second, glp-3 glp-1 (d) double mutants, which cannot transduce the mitosis-promoting signal from the distal tip cell, also usually fail to undergo meiosis. As described above, the germline-localized GLP-1 receptor is required for distal germ line mitosis, so that in 100% of glp-1 (d) mutants germ cells fail to proliferate mitotically and instead they differentiate meiotically to form ~16–32 sperm (Austin and Kimble 1987). When we examined DAPI-stained glp-3(q145) glp-1 (d) double mutants, we found that 61% had no detectable germline nuclei, 35% had a few germline nuclei per arm that resembled those seen in homozygous glp-3 mutants, and only 4% of the double mutants had some sperm, ranging from eight to 20 sperm/animal (Table 4). In those animals with no detectable germline nuclei, we hypothesize either that the morphology of those nuclei was indistinguishable from that of somatic nuclei, or else that the nuclei had degraded. We considered the possibility that germ cells in the double mutants formed sperm in larvae that then degraded by adulthood, as has been observed in some glp-1 single mutants (Austin and Kimble...
### TABLE 4

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>glp-3(q145)</td>
<td>Glp-3</td>
</tr>
<tr>
<td>glp-1(q268)</td>
<td>Tum*</td>
</tr>
<tr>
<td>glp-3(q145)</td>
<td>Glp-3</td>
</tr>
<tr>
<td>unc-32(e189) glp-1(q175g)</td>
<td>100% → sperm</td>
</tr>
<tr>
<td>glp-3(q145) unc-32(e189) glp-1(q175g)</td>
<td>61% → nuclei</td>
</tr>
<tr>
<td>fem-3(q96 gf ts) dpy-20(e1282 ts)</td>
<td>Mog*</td>
</tr>
<tr>
<td>glp-3(q145); fem-3(q96 gf ts) dpy-20(e1282 ts)</td>
<td>Glp-3</td>
</tr>
<tr>
<td>fog-1(q253 ts)</td>
<td>Fog*</td>
</tr>
<tr>
<td>fog-1(q253 ts); glp-3(q145)</td>
<td>Glp-3</td>
</tr>
</tbody>
</table>

Double mutants were made as described in MATERIALS AND METHODS. Animals were examined by DAPI staining as adults. All animals were grown at 20° except is strains, which were grown at 25°. *Tum indicates a tumorous germ line phenotype. Mog indicates a masculinized germ line phenotype (no oocytes are made). Fog indicates a feminized germ line phenotype (no sperm are made).

For unc-32 glp-1, n = 12. For glp-3 unc-32 glp-1, n = 104.

1987). However, examination of double mutant L4 larvae showed no increase in the number of animals with sperm over the number seen in adulthood (data not shown), arguing against the idea that sperm are first formed and then degraded. We conclude that the majority of glp-3 glp-1 (g) animals are unable to undergo meiosis, and therefore that proximity to the distal tip cells cannot fully explain the failure in meiosis in glp-3 mutants.

**gld-1; glp-3 double mutants:** If glp-3 is a gene that is essential for the mitotic and meiotic cell cycles in the germ line, then glp-3 mutations would be expected to be epistatic to mutations that affect the pattern of cell fates in the germ line. Strong loss-of-function and null alleles of gld-1 cause germ cells that have entered meiosis during oogenesis to return to a mitotic cell cycle, forming ectopic tumors in the proximal region of the germ line (FRANCIS et al. 1995). We made a double mutant with a strong loss-of-function allele of gld-1, (q268), and glp-3(q145). Of 29 double mutant animals examined, all resembled glp-3 when examined by DIC microscopy. This result is consistent with the hypothesis that glp-3 is required for the mitotic cell cycle of germ cells.

**The proliferation defect in glp-3 mutants is independent of the sexual fate of the germ cells:** Because both male and hermaphrodite glp-3 mutants have a germline proliferation defect, it appears unlikely that the sexual fate of the germ cells plays a role in the defect. However, it was possible that the cell cycle arrest in glp-3 mutants resulted either from the incorrect specification of the male sexual fate, (which is normally the first fate of germ cells in the hermaphrodite, and the only fate in the male) or from the simultaneous activation of both male and female fates in a single cell (an intersexual fate). To test these hypotheses, double mutants were made with the mutations fog-l(q253 ts) and fem-3(q96 gf ts) that prevent either the male or female fates, respectively, from being specified. glp-3 was found to be epistatic to both mutations at their restrictive temperature of 25° (Table 4). Hence, the defect in glp-3 germine proliferation does not appear to be related to the sexual identity of the germ cells.

**gfp-4 (bn2 ts); glp-3 double mutants resemble either single mutant:** The gfp-4(bn2 ts) mutation has a similar phenotype at 25° to that of glp-3 at 25°: germ cells divide very slowly over time and fail to make gametes. Furthermore, germ cells have a similar appearance in the two mutants, and double mutant analysis with gfp-4 and gfp-1 suggests that gfp-4, like glp-3, is also required for meiosis (BEANAN and STROME 1992). The similarity of the gfp-4(bn2 ts) and glp-3 phenotypes suggested that they might function as part of the same process. Therefore, we examined the phenotype of the double mutant grown at 25° for possible synergistic effects. DAPI-staining showed that the nuclei in the gfp-4(bn2 ts); glp-3(q145) double mutant resembled those in either single mutant. Measurement of germline growth showed that in the double mutant, the germ line continued to divide slowly at 25°, although more slowly than in either single mutant (Table 5). Hence, there may be some partial redundancy of function between glp-3 and gfp-4, or they may function in the same process.

**DISCUSSION**

Recessive mutations in the glp-3 gene cause failure in both proliferation and differentiation in the *C. elegans* germ line. In contrast to glp-1, a regulatory gene required to specify the mitotic fate, glp-3 is required for progression through both mitotic and meiotic cell cycles in the germ line: the number of germ cells formed is small, and no meiosis or gametogenesis occurs. At 15°, glp-3 germ cells in all five alleles arrest with a uniform terminal phenotype of partially condensed chromosomes, suggesting a checkpoint-mediated arrest. All five mutations specifically affect germ cells; no defects in somatic cell division are observed. Furthermore, glp-3 mutations have the same effect on germline proliferation in both sexes and are unaffected by mutations that affect the sexual fate of the germ line. Hence, glp-3 may be a gene that is fundamental to progression through the cell cycle in the germ line of *C. elegans*.

**glp-3 is required for mitosis and meiosis in the *C. elegans* germ line:** Several lines of evidence suggest that glp-3 is important for both the mitotic and meiotic cell cycles in the germ line. First, germ cells in glp-3 mutants fail to proliferate mitotically at 15° and 20°. At these
temperatures, nuclei arrest with a uniform terminal phenotype: the chromosomes appear partially condensed, resembling an arrest at the G2/M transition. Second, glp-3 is epistatic to gld-1(q268), a mutation that causes germ cells undergoing oogenesis to revert from meiosis to mitosis, resulting in ectopic proliferation in the proximal region of the germ line (Francis et al. 1995). Third, germ cells in glp-3 mutants fail to progress through meiosis, even at 25\(^\circ\), when several hundred mitotic germ cells can be formed. Fourth, in glp-3 glp-1(\(q\)) double mutants, 96% of gonads scored failed to make sperm, whereas in glp-1(\(q\)) single mutants, 0% of gonads failed to make sperm. The fact that most double mutant animals do not make sperm indicates that the defect in progression through meiosis in glp-3 is not simply due to promotion of the mitotic fate by the glp-1 signal-transduction pathway (although it remains possible that the failure to execute meiosis is a secondary defect of the mitotic failure). Finally, double mutants between glp-3 and mutations that either feminize (\(fog\)) or masculinize (\(fem-3g\)) the germ line resemble glp-3 single mutants, indicating that sexual fate does not affect the requirement for glp-3. In summary, glp-3 appears to be fundamentally required for the cell cycle in the germ line under all circumstances tested.

**The nature of the glp-3 mutations:** The glp-3 mutations fit some of the standard criteria for being loss-of-function, in that they are completely recessive, and were isolated at a typical loss-of-function frequency. Hemizygotes for the \(q_{171}\) and \(q_{456}\) alleles had essentially the same phenotype as that of the corresponding homozygotes, consistent with these alleles being simple loss-of-function mutations. In contrast, hemizygotes for \(q_{145}\), \(q_{156}\), and \(q_{363}\) had two to three times more germ cells than were observed in homozygotes. Hence, these alleles may have some recessive antimorphic activity caused by interaction of the mutant glp-3 gene product with another gene product that is required for the germline cell cycle. Alternatively, these alleles might be partial loss-of-function mutations in a gene that has both inhibitory and stimulatory effects on the cell cycle in the germ line: if we isolated alleles that inactivate the cell-cycle stimulatory but not the inhibitory functions of glp-3, it might be expected that in hemizygotes, where the inhibitory function is reduced relative to that in homozygotes, slightly more germline proliferation would occur. A similar model has been used to explain the phenotype of hemizygotes for certain partial loss-of-function alleles of \(let-23\) and \(mek-2\) (Aroian and Sternberg 1991; Church et al. 1995). Molecular identification of the lesions in glp-3 mutants may shed light on these possibilities. Nevertheless, we conclude that because the phenotype for all alleles is qualitatively the same, the primary wild-type function of glp-3 is to allow progression through the cell cycle in the germ line.

**The nature of the cell cycle arrest in glp-3 mutants:** glp-3 mutants grown at 15\(^\circ\) or 20\(^\circ\) have germ cells that arrest with partially condensed chromatin, as if in mitotic prophase. Such a uniform arrest phenotype suggests that these mutants fail to execute some cell cycle function that is monitored by a checkpoint, causing an arrest before mitosis. Interestingly, all five alleles show increased proliferation at 25\(^\circ\) compared to 15\(^\circ\) and 20\(^\circ\). While this may indicate that all alleles are simply partial loss-of-function lesions that have residual activity at 25\(^\circ\), a second possibility is that wild-type glp-3 might function in an inherently cold-sensitive process, such as microtubule assembly, so that even null alleles might result in a stronger phenotype at colder temperatures. An involvement of glp-3 in microtubule assembly might explain the apparent arrest in G2/M, since mutations might cause a defect in mitotic spindle formation. A third possible explanation of the cold-sensitive phenotype is that mutations in glp-3 might cause a germline cell cycle arrest that is mediated by an inherently temperature-sensitive checkpoint. Hence, at 25\(^\circ\), cells might bypass the checkpoint despite complete absence of glp-3 activity. This latter possibility is supported by the fact that the germline nuclei grown at 25\(^\circ\) often appear distorted or fragmented, as if they have progressed through the cell cycle in an abnormal way.

**The germline specificity of glp-3 function:** Why do the glp-3 mutations specifically affect the germ line and not somatic cell divisions? A simple hypothesis is that glp-3 may be specifically required for the germline cell
cycle and has no somatic function. Alternatively, glp-3 may be required for the cell cycle in many or most somatic tissues, but we have isolated alleles with germ-line-specific effects. By this latter hypothesis, it is necessary to argue either that the germline and somatic functions of glp-3 are independently mutable, and therefore qualitatively different, or else that the germ line is more sensitive to lower levels of glp-3 than is the soma. We discount the possibility that the germline-specific phenotype simply reflects maternal rescue of somatic functions: the glp-3 defect is apparent soon after hatching in the germ line, at a time when many somatic postembryonic cell divisions (e.g., in the somatic gonad, ventral nerve cord, and male tail) have yet to occur. Hence, it is expected that those somatic divisions would be affected as strongly as the germ cell divisions if rescuing maternal product were gone at hatching. However, there is no obvious somatic defect associated with glp-3 mutations, even when hemizygous.

Comparison with glp-4 (bn2 ts): Of the other C. elegans mutants that affect germ line proliferation, glp-4 (bn2 ts) mutants grown at 25° are the most similar to glp-3 mutants. In both cases, germ cells divide very slowly and fail to differentiate meiotically. Furthermore, like the glp-3 glp-1 (b) double mutant, some of the nuclei in the glp-4; glp-1 (b) double mutant become undetectable by DAPI staining (BEANAN and STROME 1992). We found that the glp-4 (bn2 ts); glp-3 double mutant grown at 25° is qualitatively similar to either single mutant; no new phenotypes were observed. Quantification of the germ cells in the double mutant shows that there may be a synergistic effect on the reduction of proliferation in older animals, supporting the possibility that the two genes may function in the same process in the cell cycle. Since some alleles of glp-4 were isolated as enhancers of a glp-1 (ts) mutation (QIAO et al. 1995), it is possible that these two genes may interact with the GLP-1 pathway in some way, perhaps as downstream targets. Interestingly, glp-4 also affects oogenesis: alleles isolated as enhancers of glp-1 (ts) have abnormal oocytes, as does glp-4 (bn2 ts) when it is shifted to the restrictive temperature in adulthood. If glp-3 and glp-4 really function in the same process, it might be that weaker alleles of glp-3 would also affect oogenesis.

The requirement for glp-3 in mitosis and meiosis: Because glp-3 mutants fail to undergo both mitosis and meiosis in the germ line, it may be that glp-3 is required for both cell cycles. Precedent for such a dual requirement in mitosis and meiosis comes from cell cycle genes that have been identified in the yeasts S. cerevisiae and S. pombe. For example, the key regulatory kinase controlling passage through the cell cycle, p34, is encoded by the cdc28 or cdc2 genes of these two organisms, respectively. Mutations in the cdc28 and cdc2 genes have arrest points at both the G1/S transition and the G2/M transition during the mitotic cycle (HARTWELL et al. 1973; NURSE and BISSETT 1981; PIGGOTT et al. 1982). In meiosis, cdc28 is required for exit from pachytene and for meiosis II (SHUSTER and BYERS 1989) and cdc2 is required for premeiotic DNA synthesis and for meiosis II. Many other cell division cycle mutants identified by their mitotic phenotype in S. cerevisiae also have meiotic phenotypes (SCHILD and BYERS 1978; SHUSTER and BYERS 1989; WEBER and BYERS 1992). Therefore, many genes required for cell cycle progression are required in both mitosis and meiosis. It is possible that glp-3 represents such a gene in C. elegans; however it is important to note that glp-3 mutants are not affected in somatic mitotic cycles. Therefore, it may be that another gene serves the function of glp-3 in the soma of C. elegans. An example of such redundancy is found in Drosophila, where there is a cdc25 homologue, twine, that functions in the germ line (ALPHEY et al. 1992), while a second cdc25 homologue, string, has somatic functions (EDGAR and O'FARRELL 1990).

Finally, it remains possible that the primary function of glp-3 is in mitosis, and that there is no direct requirement for glp-3 in meiosis. In support of this possibility is the fact that 4% of the animals in the glp-3 glp-1 (b) double mutant are able to form sperm. Although this may simply represent some leakiness in a glp-3 meiotic defect, there are several ways that a failure in the mitotic cycle could indirectly cause a failure to complete meiosis. For example, it may be that germ cells are unable to enter the meiotic cycle from the point of their mitotic arrest. In yeast, cells make the decision to enter meiosis at Start, early in G1 of the mitotic cycle (PRINGLE and HARTWELL 1981). If germ cells in glp-3 mutants arrest in G2 or M, they may not be able to reach the stage where they would normally choose to enter meiosis. Alternatively, it may be that some germ cells in the double mutants do attempt to enter meiosis but have usually suffered some irreversible damage at the mitotic arrest point, which prevents them from successfully completing meiosis. Perhaps attempting to enter meiosis in the presence of such damage could result in degradation of the nuclei, explaining why germ cells frequently become undetectable in a glp-3 glp-1 (b) double mutant. Hence, the few germ cells that are able to differentiate and form sperm in the glp-3 glp-1 (b) double mutant may represent those that have completed the mitotic cell cycle with relatively little damage and thus were able to complete one round of meiosis. Molecular analysis of the glp-3 gene may help resolve this issue, as well as help determine whether glp-3 function is truly germ-line-specific, and what its role is in the progression of the germline mitotic and meiotic cell cycles.

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LITERATURE CITED


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