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gon-4, a Cell Lineage Regulator Required for Gonadogenesis in *Caenorhabditis elegans*

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The *gon-4* gene is required for gonadogenesis in the nematode *Caenorhabditis elegans.* Normally, two precursor cells, Z1 and Z4, follow a reproducible pattern of cell divisions to generate the mature somatic gonadal structures (e.g., uterus in hermaphrodites, vas deferens in males). In contrast, in *gon-4* mutants, the Z1/Z4 cell lineages are variably aborted in both hermaphrodites and males: Z1 and Z4 divide much later than normal and subsequent divisions are either absent or severely delayed. In *gon-4* adults, normal somatic gonadal structures are never observed, and germ-line and vulval tissues, which depend on somatic gonadal cues for their development, are also aberrant. In contrast, nongonadal tissues and the timing of other developmental events (e.g., molts) appear to be normal in *gon-4* mutants. The *gon-4* alleles are predicted to be strong loss-of-function or null alleles by both genetic and molecular criteria. We have cloned *gon-4* in an attempt to learn how it regulates gonadogenesis. The *gon-4* gene encodes a novel, acidic protein. A GON-4::GFP fusion protein, which rescues a *gon-4* mutant to fertility, is expressed in somatic gonadal cells during early gonadal development. Furthermore, this fusion protein is nuclear. We conclude that *gon-4* is a regulator of the early lineage of Z1 and Z4 and suggest that it is a part of a genetic program common to the regulation of both hermaphrodite and male gonadogenesis.

Key Words: **cell lineage; organogenesis; *Caenorhabditis elegans;* gonad; *gon-4;* cell cycle.**

INTRODUCTION

During organogenesis, cell divisions, migrations, and fates must be tightly controlled to generate tissues and complex organs. Regulators of the cell cycle, cell motility, and differentiation are thought to be under a variety of tissue-specific, organ-specific, and stage-specific controls to coordinate these processes and generate functional organs. Although a few master regulators of organ identity have been identified, such as *pax-6* (Quiring *et al.*, 1994) and *pha-4* (Kalb *et al.*, 1998; Mango *et al.*, 1994), their roles in organogenesis are not well understood.

We have begun to investigate the genetic control of gonadogenesis in the nematode *Caenorhabditis elegans.* The *C. elegans* gonad is composed of multiple tissues and substructures, and it is sexually dimorphic. XX hermaphrodites possess a symmetrical bilobed gonad, whereas XO males possess an asymmetric single-lobed gonad. (A hermaphrodite is essentially a female that makes sperm transiently before embarking on oogenesis.) Despite the complexity of the *C. elegans* gonad, its development has been defined at the level of single cells (Kimble and Hirsh, 1979; Newman *et al.*, 1996; Sulston *et al.*, 1983). Therefore, controls of gonadogenesis can be analyzed with great precision, and this organ should serve as an excellent model for dissecting regulatory networks that generate a complex organ.

Figure 1 illustrates the major features of gonadogenesis relevant to this paper; details of sex-specific events and structures are mentioned where necessary under Results

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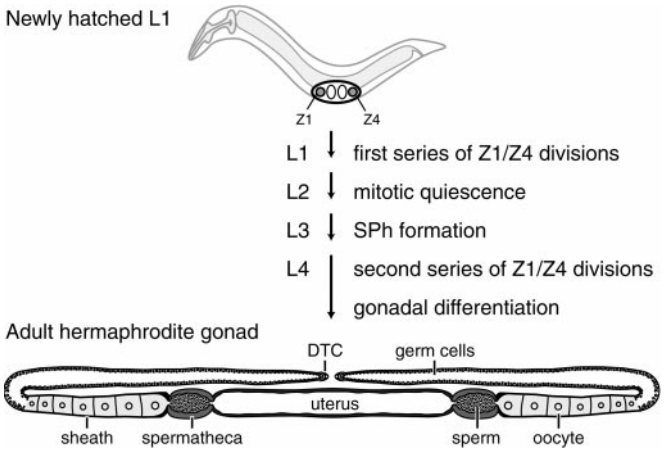


FIG. 1. Main features of gonadogenesis, illustrated in the *C. elegans* hermaphrodite. In the embryo, two somatic gonadal progenitor cells, Z1 and Z4 (gray), and two germ-line progenitor cells, Z2 and Z3 (white), assemble into a gonadal primordium, which is illustrated in a newly hatched L1. During L1 and early L2, Z1 and Z4 generate 12 descendants. During the rest of L2, the Z1/Z4 descendants are mitotically quiescent. In early L3, somatic gonad primordium (SPh) forms. During the rest of L3 and early L4, a second series of divisions generates 143 somatic gonadal cells. Finally, the somatic gonadal tissues differentiate in the later part of L4. A similar rhythm of events is observed in male gonadogenesis. The hermaphrodite somatic structures form a complex epithelial tube with a large central uterus, two spermathecae, and two sheaths or oviducts. The regulatory DTCs (distal tip cells) reside at the distal ends of the germ-line tubes; the anchor cell is not shown, because it joins the uterus after vulval induction (Newman *et al.*, 1996). For a recent review, see Hubbard and Greenstein (2000).

or elsewhere (reviewed in Hall *et al.*, 1999; Hubbard and Greenstein, 2000). Gonadogenesis begins in the embryo with assembly of a four-celled gonadal primordium (Sulston *et al.*, 1983). After the animal hatches from its eggshell, the four progenitor cells begin to divide. The two somatic gonadal precursor cells, Z1 and Z4, follow an almost invariant lineage to generate the mature somatic gonadal tissues and three regulatory cells, while the two germ-line precursor cells, Z2 and Z3, follow a variable pattern of divisions to generate the complete germ line (Kimble and Hirsh, 1979). The rhythm of the Z1/Z4 cell divisions is similar in the two sexes: early divisions in L1 and early L2 are followed by a period of mitotic quiescence; later divisions occur during L3 and early L4. The Z1/Z4 divisions generate a total of 143 (hermaphrodite) or 56 (male) somatic gonadal cells that differentiate into sex-specific structures during the later part of L4. In hermaphrodites, the adult gonad possesses two germ-line tubes or "arms" plus somatic structures that include a central uterus, two spermathecae, and two sheaths that house the germ line (Fig. 1, bottom); embryos exit from the uterus through a vulva (not shown in Fig. 1). The adult male gonad consists of a single germ-line arm with a somatic seminal vesicle and vas deferens extending posteriorly into the cloaca.

The early Z1/Z4 divisions generate three regulatory cells that play critical roles during gonadogenesis (Kimble, 1981; Kimble and White, 1981). In hermaphrodites, two distal tip cells (DTCs), one at the end of each germ-line tube, stimulate germ-line growth and direct extension of the growing arm of germ-line tissue; in the absence of the DTCs, the germ line consists of only a few sperm and does not acquire its normal U shape (Kimble and White, 1981). The third hermaphrodite regulatory cell, the anchor cell, is required for induction of both the vulva and the uterine π-cell fates (Kimble, 1981; Newman *et al.*, 1995, 1996). In the male, two DTCs promote germ-line growth at one end of the developing gonad, and a linker cell directs elongation at the other end (Kimble and White, 1981).

Previous genetic analyses have identified genes that control various aspects of *C. elegans* gonadal development. Of particular importance to this work are genes that control development of the somatic gonad (for a review, see Hubbard and Greenstein, 2000). Most genes identified as regulators of specific events during gonadogenesis also control similar events in nongonadal tissues. For example, *lin-12*, a Notch-like receptor, is required for specifying at least two somatic gonadal fates as well as various nongonadal fates (Greenwald *et al.*, 1983; Newman *et al.*, 1995). Similarly, *unc-5*, a netrin receptor, is required for guiding migration of the elongating germ-line tube as well as for directing neuronal migrations (Hedgecock *et al.*, 1990; Leung-Hagesteijn *et al.*, 1992). Other genes appear to be gonad-specific. For example, *gon-1* controls gonadal morphogenesis, but has no apparent effect on nongonadal tissues (Blelloch and Kimble, 1999; Blelloch *et al.*, 1999). Perhaps most relevant to this work is *gon-2*, a gene required for the onset of the postembryonic cell divisions of all four gonadal progenitor cells (Sun and Lambie, 1997). Therefore, the genetic program controlling development of the gonad involves both gonad-specific and more general regulators that act together to coordinate development of this complex organ.

Here we report the genetic and molecular characterization of *gon-4*, a gene that is part of the genetic program common to development of both hermaphrodite and male gonads. The *gon-4* gene is required for the normal Z1/Z4 cell lineage and for development of germ-line and vulval tissues. In contrast, *gon-4* activity is apparently not necessary for development of nongonadal tissues. The *gon-4* gene encodes a novel nuclear protein that appears to be expressed in somatic gonadal cells during early gonadogenesis and in germ-line tissues much later during L4 and adulthood. We conclude that *gon-4* is a regulator of the Z1/Z4 cell lineage and provides a link between controls of organ identity and the cell cycle machinery in the developing gonad.

MATERIALS AND METHODS

Nematode Strains and Maintenance

Nematode strains were derivatives of the wild-type Bristol strain N2 and were maintained as described (Brenner, 1974). Strain constructions and genetic analyses were done according to standard methods (Hodgkin *et al.*, 1988). Growth was at 20°C unless noted otherwise. The *glp-1(q224); gon-4* double mutants were grown at 25°C, the restrictive temperature for *glp-1*. The following mutations, deficiencies, rearrangement, and GFP-marked chromosomes were described by Hodgkin (Hodgkin, 1997; Hodgkin *et al.*, 1988) or cited references.

The mutations used were *LGIII, glp-1(q224ts); LGIV, unc-24(e132), gon-1(e1254), elt-1(zu180), gon-4(q519, q558, q597,* and *e2575*) (this paper), *unc-43(e408), dpy-20(e1282ts);* and *LGV, him-5(e1490).*

The deficiencies used were *mDf7(IV)* and *eDf19(IV).*

The rearrangement used was *nTI[unc(n754dm) let](IV;V).*

The GFP-marked chromosomes used were *qIs19(V)* (Blelloch *et al.*, 1999) and *qIs42(IV). qIs42* carries an integration of an extrachromosomal array containing pRF4 (*rol-6(su1006sd)*) and a *pha-4::GFP* promoter construct (Kalb *et al.*, 1998) on chromosome *IV*. To score the hermaphrodite gonadal sheath cells, we used an extrachromosomal array containing *plim-7::GFP* (Hall *et al.*, 1999).

To obtain *gon-4* males, spontaneous *gon-4/qIs42* heterozygous males were crossed to *gon-4/qIs42* hermaphrodites to propagate male progeny; *gon-4* males were also obtained from the strain *gon-4(x)/qIs42; him-5(e1490).*

Isolation of gon-4 Alleles and Genetic Mapping

All *gon-4* alleles were obtained after EMS mutagenesis (Brenner, 1974), and all were outcrossed to N2 at least five times. Specifically, L4 hermaphrodites were mutagenized, F1 progeny picked to individual plates at 25°C, and F2 progeny screened for gonadogenesis (Gon) mutants. Six *gon-4* alleles (*q519, q521, q526, q558, q597,* and *q642*) were isolated from 16,258 haploid genomes at a frequency of 3.7×10^{-4}, consistent with a loss-of-function character (Anderson, 1995). Two of these alleles (*q521* and *q526*) were subsequently lost. *gon-4(e2575)* was isolated in a separate screen. The *gon-4* mutations were shown to be allelic by both map position and failure to complement *gon-4(q519)* or *gon-4(e2575).*

Three-factor mapping with *gon-4(e2575)* placed *gon-4* 0.01 map units to the left of *unc-43*, at position 4.55. Specifically, 2 of 40 Unc non-Elt recombinants from *gon-4(e2575)/elt-1(zu180) unc-43(e408)* contain *gon-4(e2575).* Three-factor mapping data have been submitted to ACeDB.

Analysis of Phenotype

All progeny of at least five *gon-4(x)/qIs42* hermaphrodites were scored as fertile, Gon, or larval lethal (*x* is *q519, q558, q597,* or *e2575*). For all broods, approximately 25% were larval lethal, the *qIs42* homozygous phenotype. The remaining 75% developed to adulthood. All GFP-negative animals had a sterile Gon phenotype, demonstrating complete penetrance (32%; $n = 4287$). Percentages of larval lethality and Gon sterility were similar for all four alleles. All of the *gon-4(x)/qIs42* heterozygotes, identified by GFP expression in the pharynx, were fertile. Therefore, all four *gon-4* alleles are recessive and fully penetrant. The brood sizes of *gon-4(x)/qIs42* heterozygotes were similar to that of *+/qIs42* (not shown).

Phenotypes of *gon-4* homozygotes were scored using Nomarski differential interference contrast microscopy. Both hermaphrodites and males were scored for extent of arm extension as well as the size and composition of the adult germ line; hermaphrodites were further scored for vulval morphology and males for tail formation.

Microscopy

Microscopy was performed using a Zeiss Axioscope fitted with standard epifluorescence filters and Nomarski differential interference contrast optics. For image acquisition a Kontron Electronik ProgRes 3008 scanning camera was used to import images into Adobe PhotoShop. Confocal imaging was done on a Bio-Rad MRC-1024 laser scanning confocal microscope. Images were processed with Adobe PhotoShop software.

Cell Lineage Analysis

Cell lineages were followed as described (Sulston and Horvitz, 1977). L1 *gon-4* homozygotes were recognized among the progeny of *gon-4(x)/qIs42* parents by the absence of GFP. In some animals, one germ cell precursor (either Z2 or Z3) was removed by laser ablation to reduce the number of germ cells and thereby simplify analysis of the gonadal lineage. Ablations were done as described (Bargmann and Avery, 1995; Sulston and White, 1980) using a Micropoint Ablation Laser System. The adult phenotype of each lineaged animal was determined by returning each lineaged animal to a petri plate once the lineage could no longer be followed and allowing it to develop to adulthood before recording the adult structures.

gon-4 Rescue

Germ-line transformation was achieved as described (Mello and Fire, 1995). To identify the *gon-4* gene, each of six cosmids (W01E12, K08F4, T14G10, K04D7, and C24F3) was injected at 10 μg/ml with pRF4 [*rol-6(su1006sd)*] at 80 μg/ml into *gon-4(e2575) dpy-20/unc-24 gon-1* hermaphrodites. Fertile *dpy-20* animals with small broods of >90% Gon animals and no Unc Dpy progeny were scored as positive for rescue. Rescued lines were maintained; however, the lines gave rise to a large percentage of rolling Gon animals (>80%) and a small percentage of rolling fertile animals (1–10%). This low level of fertility may be due to the phenomenon of gene silencing (Kelly and Fire, 1998; Kelly *et al.*, 1997). Two overlapping cosmids (K04D7 and T14G10) were further tested by injecting *gon-4(e2575) dpy-20/unc-24 gon-1* animals with complex mixtures of DNA: either cosmid at 10 μg/ml cut with *Fsp*I, 2 μg/ml pRF4 cut with *Sca*I, and 100 μg/ml N2 genomic DNA cut with *Pvu*II. Finally, a 10-kb gel-purified fragment of K04D7, which contains one predicted transcript K04D7.5 (GenBank Accession No. 269664), was tested as a complex mixture. These experiments identified K04D7.5 as *gon-4.* To generate *gon-4::GFP*, a complex mixture of linearized DNAs [10 μg/ml pLF103 (*gon-4::GFP,* see below for construction), 2 μg/ml pRF4, and 100 μg/ml N2 genomic DNA] was injected into N2 hermaphrodites to create the *qEx453* extrachromosomal array. To assay for *gon-4* rescue, *gon-4(q519)/+*; *qEx453* hermaphrodites were generated by standard crosses; a rescued line was obtained that produced a small percentage of fertile rolling progeny (between 1 and 20%); the remainder of the progeny were either Gon Rol (approximately 75–90%) or Gon (1–10%).

RNA-Mediated Interference (RNAi)

RNAi was performed essentially as described (Fire *et al.*, 1998): 3 or 5 mg/ml RNA corresponding to a 1-kb portion of each predicted, full-length transcript on cosmid K04D7 was injected into N2 gonads.

SL1 Race and cDNA Analyses

Eleven incomplete *gon-4* cDNAs are present in the *C. elegans* EST database: yk11e8, yk11h11, yk14g4, yk5e8, yk33e3, yk353a9, yk428d3, yk295b12, yk348a4, yk152g4, and yk18e5. One of these, yk353a9 (kindly provided by Yuji Kohara, National Institute of Genetics, Mishima, Japan), was sequenced in full to confirm the intron/exon boundaries of the predicted transcript K04D7.5. The 5′ end of the *gon-4* transcript was determined by RT-PCR amplification (using an SL1 primer for the 5′ end and nested primers corresponding to sequences from yk353a9), gel purification, and sequencing of the PCR product. This PCR product combined with the cDNA sequence defines the full-length *gon-4* transcript. Sequence comparison of the 5′ and 3′ sequence reads from the above cDNAs suggests that all of the cDNAs listed correspond to a single transcript.

RNA Analysis

RNA was extracted using RNA Isolator solution and protocol (Genosys Biotechnologies, Inc.) from wild-type N2 Bristol worms raised at 20°C. Poly(A)$^+$ RNA was isolated using an mRNA Separator kit (Clontech Laboratories, Inc). RNA was run on a glyoxal gel at 65 V for 3 h and then hybridized according to Gallegos *et al.* (1998). The Northern blot was probed with two separate 1-kb DNA probes corresponding to the 5′ and 3′ ends of *gon-4*, respectively.

Sequence Analysis of gon-4 Mutants

Mutant lesions were determined by sequencing PCR products, as described by Kaltenboeck *et al.* (1992). For each mutant, three PCRs were used to amplify *gon-4* genomic DNA from individual animals. The entire genomic region was sequenced for all alleles. Any sequence change was verified using at least one independently derived PCR product.

Construction of GON-4::GFP

An 8511-bp genomic fragment containing 3409 bp of 5′ flanking region and the entire *gon-4* coding region (minus the stop) was PCR amplified using Expand High Fidelity PCR (Boehringer Mannheim) from cosmid K04D7 and cloned into pBlueScript II KS(−) (Stratagene) to generate pLF100. Next, a 902-bp fragment encoding GFP was PCR amplified from pPD95.77 (provided by Andy Fire) and cloned into plasmid pLF-100. Finally, the *gon–4* 3′UTR and 3′ flanking region (2064 bp) were PCR amplified from cosmid K04D7 and cloned into the plasmid containing the 5′ *gon-4* PCR product and GFP to create pLF103, which we call *gon-4::GFP*.

Bioinformatics

Cosmid positions were obtained from the *C. elegans* Genome Consortium (Wilson *et al.*, 1994) and viewed in ACeDB (see Waterston *et al.*, 1997). DNAStar programs were used for routine sequence analysis (viewing restriction sites, displaying open reading frames, translating nucleotide sequence, etc.). Database searches were performed using BLAST (Altschul *et al.*, 1990).

RESULTS

Identification and Mapping of gon-4 Mutations

The *gon-4* gene was identified by seven independently isolated mutations that cause severe defects in gonadogenesis; these *gon-4* mutations were isolated at a frequency typical for loss-of-function mutations after EMS mutagenesis in *C. elegans* (see Materials and Methods). All *gon-4* alleles mapped to linkage group *IV* and failed to complement the canonical allele, *gon-4(q519)* (see Materials and Methods). The four well-characterized *gon-4* alleles (*q519*, *q558*, *q597*, and *e2575*) are recessive and fully penetrant for sterility; the remaining alleles appeared similar, but were not as rigorously tested.

The Terminal gon-4 Phenotype

Wild-type gonads are highly organized structures of defined shape and size (Figs. 1, 2A, and 2D) (Kimble and Hirsh, 1979). In contrast, *gon-4* mutants possess grossly malformed gonads of variable shape and size (Figs. 2B, 2C, and 2E). The description below is compiled from the examination of mutants homozygous for any of four *gon-4* alleles of equivalent strength (see Fig. 3). All *gon-4* homozygotes are sterile: *gon-4* hermaphrodites produce no self-progeny ($n >$ 1000), and *gon-4* males produce no cross-progeny ($n = 20$). The most defective *gon-4* gonad consists of only a few somatic gonadal cells and a small clump of undifferentiated germ cells or sperm (Figs. 2B and 2E). Less defective *gon-4* gonads possess increasing amounts of tissue, but somatic gonadal structures (e.g., uterus, spermatheca, vas deferens, and seminal vesicle) never form (Fig. 2C).

The germ line develops to varying extents in *gon-4* mutants. Most *gon-4* hermaphrodites contain only undifferentiated germ cells (85%, $n = 1329$), whereas the rest produce some gametes (sperm, deformed oocytes, normal-looking oocytes either alone or in combination with sperm). Similarly, *gon-4* males can possess either undifferentiated germ cells or sperm. Finally, the size of the germ-line tissue varies from as few as five undifferentiated germ cells (6%, $n = 1329$) to gonad arms full of germ-line tissue and central bulges of hundreds of undifferentiated germ cells. To ask whether *gon-4* germ cells were capable of differentiation, we examined *glp-1; gon-4* double mutants. In *glp-1* mutants, all germ cells differentiate as sperm (Austin and Kimble, 1987). We found that all *glp-1; gon-4(q519)* double mutants similarly produced sperm. Therefore, *gon-4* is not required for spermatogenesis per se, suggesting that the defects in gamete differentiation may be a secondary consequence of somatic gonadal defects.

The vulva develops to varying extents in *gon-4* mutants. Some animals are vulvaless, others have a protruding vulva, and rare animals possess multiple vulvae. These vulval

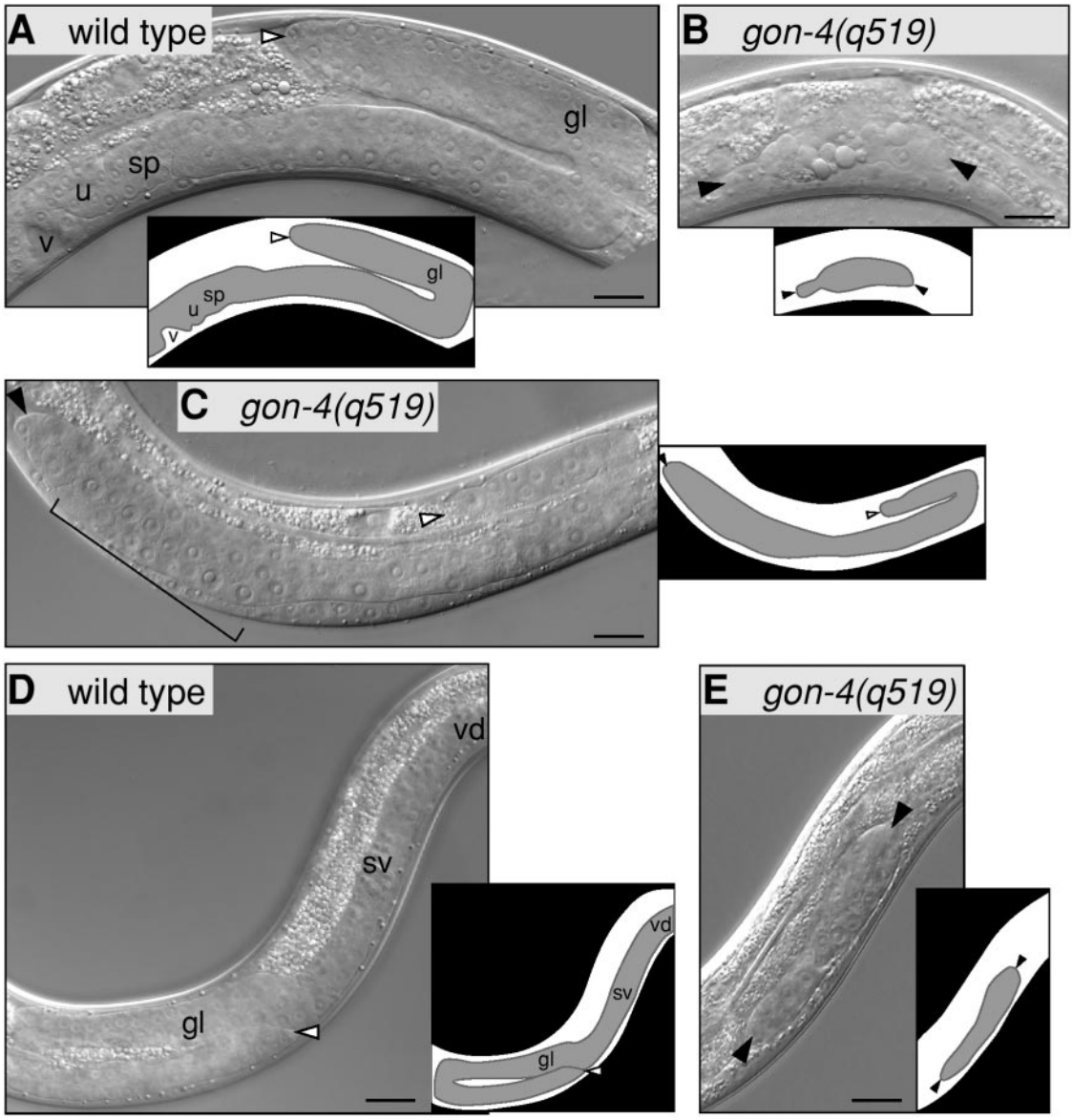


FIG. 2. The *gon-4* gene is required for gonadogenesis in both sexes. Nomarski micrographs with adjacent diagrams, lateral views. Magnification is the same in all photos. All animals are L4; all mutants are *gon-4(q519)* homozygotes. (A) Wild-type hermaphrodite. v, vulva; u, uterus; sp, spermatheca; open arrowhead, distal tip cell; gl, germ-line tube. (B) *gon-4* hermaphrodite with severe gonadal defect: gonad is small and misshapen. Arrowheads, anterior and posterior boundaries of gonadal tissue. (C) *gon-4* hermaphrodite with mild gonadal defect: one germ-line tube forms (open arrowhead at distal end), but no somatic gonadal structures are made. Arrowhead, anterior limit of gonadal tissue; bracket, central region where somatic gonadal structures and vulva should have formed. (D) Wild-type male. sv, seminal vesicle; vd, vas deferens; gl, germ-line tube; open arrowhead, distal end. (E) *gon-4* male: gonad is smaller and aberrantly shaped. Arrowheads indicate anterior and posterior boundaries of gonadal tissue. Scale bars, 10 μm.

abnormalities may be a secondary consequence of defects in the somatic gonad (see Discussion).

In sum, *gon-4* homozygotes are defective in the somatic gonad, germ line, and vulva. Other tissues appear to be unaffected: overall body size, body shape, and male tail morphology were indistinguishable from the wild type (n = 40). Furthermore, *gon-4* mutant males exhibited normal mating behavior, and *gon-4* mutant hermaphrodites moved normally until adulthood. As adults, their movement sometimes became awkward, which can be most simply explained by the presence of an abnormally shaped mass of gonadal tissue in the center of the animal.

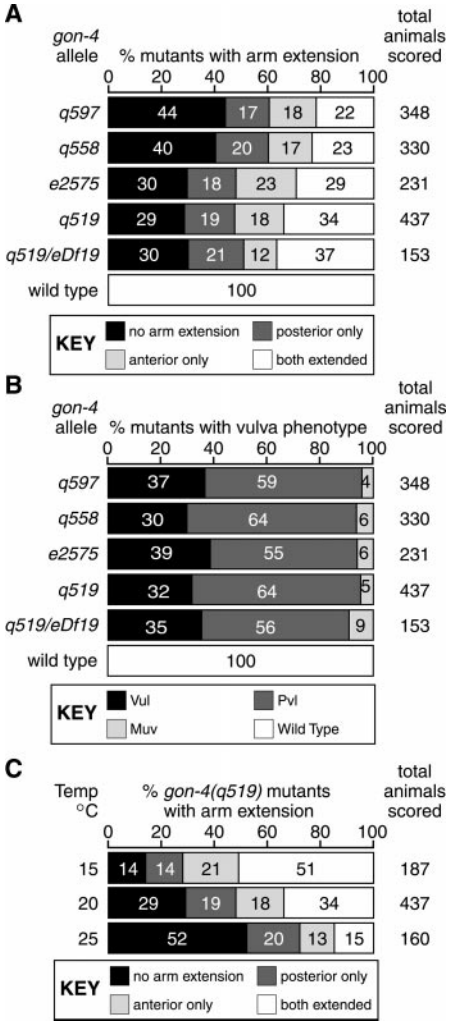


FIG. 3. Allelic strength and temperature sensitivity of *gon-4* mutants. (A) Variability in gonadal arm extension used to compare strengths of *gon-4* alleles. An arm was scored as extended when significant elongation, which was clearly led by a distal tip cell, was observed. (B) Variability in vulval development was used to compare strengths of *gon-4* alleles. Black, vulvaless (Vul); dark gray, protruding vulva (Pvl); light gray, multiple vulvae (Muv), white, normal vulva. (C) The *gon-4* phenotype is temperature-sensitive. Percentage of mutants with severe defects was greater at 25°C than at 15°C, although mutants were sterile at all three temperatures. Similar data were obtained for *gon-4(q597), gon-4(q558),* and *gon-4(e2575)* homozygotes. A similar phenomenon was also noted for vulval development.

Therefore, the *gon-4* defects appear to be confined to the gonad and vulva.

The gon-4 Alleles Are Equivalent in Strength

To assess the relative strengths of four *gon-4* alleles (*q519, q558, q597,* and *e2575*), we compared homozygotes for the variability of two specific defects: arm extension and vulval development (Fig. 3). Arm extension provides an excellent measure of DTC function. In wild-type hermaphrodites, two DTCs control the formation of two gonadal arms; in contrast, in *gon-4* mutants, the number of arms varied from 0 to 2 (Fig. 3A). Nonetheless, all four *gon-4* alleles exhibited a similar variability, suggesting that they are of equivalent strength. An examination of vulval morphology in mutants homozygous for these same four alleles yielded similar results (Fig. 3B). Finally, when *gon-4(q519)* was placed in *trans* to a deficiency, *eDf19,* the range of defects did not change significantly from that of the homozygote (Figs. 3A and 3B), suggesting that these alleles may be strong loss-of-function or null mutations.

To assess the effect of growth temperature on the *gon-4* phenotype, we compared arm extension in four *gon-4* mutants (*q519, q558, q597,* and *e2575*) grown at 15, 20, and 25°C. All *gon-4* homozygotes were sterile at all three temperatures, but all were more severe at higher temperatures (Fig. 3C for *q519;* data not shown for *q558, q597,* and *e2575*). Similar temperature effects were observed for vulval development (data not shown). Since these mutations are likely to eliminate *gon-4* activity (see below, Fig. 6E), it appears that loss of *gon-4* activity renders arm extension and vulval development sensitive to temperature.

Somatic Gonad Cell Divisions Are Delayed in gon-4 Mutants

To gain insight into the underlying cellular defect in *gon-4* mutant gonads, we examined early gonadogenesis in *gon-4* homozygotes. All *gon-4* mutants possessed a morphologically normal four-celled gonadal primordium at hatching (not shown). In addition, markers of the somatic gonadal precursors [*pes-1::GFP* (Hope, 1994) and *lag-2::GFP* (Blelloch *et al.,* 1999)] were expressed as normal in Z1 and Z4 (not shown). Therefore, Z1 and Z4 appear to be specified correctly.

The early cell lineage of Z1 and Z4 was examined in *gon-4(q519)* hermaphrodites (n = 10), *gon-4(q519)* males (n = 6), and *gon-4(q519)/gon-4(q558)* males (n = 8). Virtually all *gon-4* Z1/Z4 lineages showed a severe, but variable, delay of cell divisions (Fig. 4). In wild-type animals, Z1 and Z4 divide during L1 and divide within an hour or so of each other (Figs. 4A and 4C). In contrast, in *gon-4* mutants, they divided much later, if at all, and Z1 often lost synchrony with Z4 (Figs. 4B and 4D). The cleavage planes and sizes of progeny appeared normal despite the delay in cell divisions. In contrast to Z1 and Z4, the germ-line precursor cells, Z2 and Z3, began cell division at their normal time in *gon-4* mutants. Furthermore, the germ-line cells divided at regular intervals of about 3–4 h during the first two larval stages (n = 10, data not shown), which is typical of the wild type. Therefore, the *gon-4* defect affects the somatic gonadal lineage, but not the germ-line divisions during early gonadogenesis.

To look in more detail at cell divisions in *gon-4* mutants,

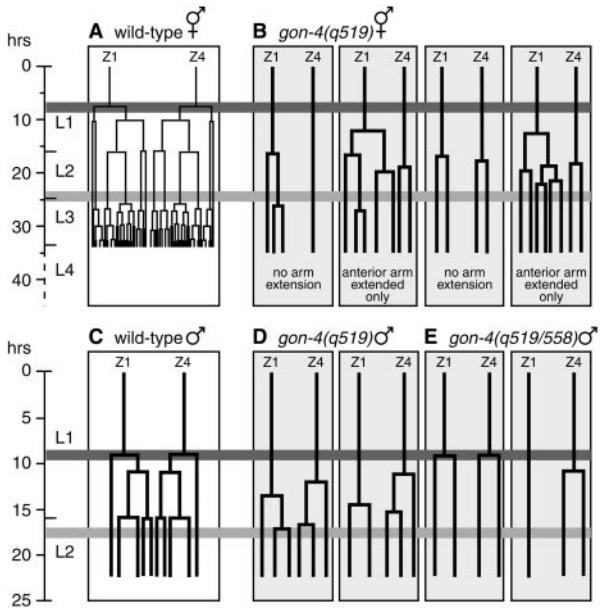


FIG. 4. Cell lineage defects in *gon-4* hermaphrodites and males. Vertical lines represent cells; horizontal lines represent cell divisions; anterior daughters are to the left; posterior daughters are to the right. Dark gray bar marks time of first cell division in wild-type animals; light gray bar marks the approximate time of formation of the somatic gonad primordium. In wild type, cell divisions are coordinated with progression through the larval stages, L1–L4, as shown by time scale to left. Hrs, hours at 20°C. (A, B) Z1/Z4 lineages from L1 through L3. (A) Wild-type hermaphrodite. (B) Four *gon-4(q519)* hermaphrodites. These lineages are representative; others are not shown (see text). (C–E) Z1/Z4 lineages from L1 through L2. (C) Wild-type male. (D) Two *gon-4(q519)* males. (E) Two *gon-4(q519)/gon-4(q558)* males.

we examined the time from nuclear breakdown to reformation of daughter cell nuclei. We found that this part of the cell cycle took 20–30 min for both somatic gonadal and germ-line cell divisions in both wild-type and *gon-4* mutant gonads (n = 22 cells in *gon-4(q519)* and *gon-4(q558)* mutants; n = 6 cells in wild type). Therefore, progression through this part of the cell cycle was normal in *gon-4* mutants.

Given the dramatic delay in Z1/Z4 cell divisions, we examined two nongonadal lineages for cell division defects and various nongonadal characters for developmental delays. The M cell lineage in hermaphrodites (n = 10) and the B cell lineage in males (n = 14) were followed during the first two larval stages; no defect in timing, orientation of cleavage planes, or pattern of divisions was observed. Furthermore, the L1–L3 molts occurred at the same time in wild-type and *gon-4* mutant larvae (n = 12), lateral alae acquired their normal stage-specific characteristics, and entry and exit from the dauer larval stage was unaffected. Therefore, as predicted from the apparently normal morphology of nongonadal structures when assayed at a gross level (see above), we found no nongonadal defects in *gon-4* mutants.

Other Steps of Gonadogenesis Are Not Delayed in gon-4 Mutants

Given the dramatic delay in Z1/Z4 cell divisions, we next asked whether other events in gonadogenesis were similarly delayed. First, we assayed formation of the somatic gonadal primordium (SP) in hermaphrodites and males. The somatic gonadal primordium forms during gonadogenesis and serves as an anlage for formation of the mature somatic gonadal structures (e.g., uterus in hermaphrodites, vas deferens in males) (Kimble and Hirsh, 1979) (Fig. 1). Second, we examined the differentiation of somatic gonadal tissues in L4 hermaphrodites.

In wild-type hermaphrodites, 10 descendants of Z1 and Z4 coalesce toward the center of the gonad to form the hermaphrodite somatic gonadal primordium; this rearrangement occurs at the L2/L3 stage and results in the separation of the germ-line tissue into anterior and posterior lobes (Kimble and Hirsh, 1979). In the lineaged *gon-4(q519)* hermaphrodites, the somatic gonadal cells, though dramatically fewer in number than wild type, moved centrally at the correct stage (n = 10). However, separate regions of germ-line tissue were never established, perhaps as a result of an insufficient number of somatic gonadal cells. In wild-type males, 8 descendants of Z1 and Z4 rearrange to the anterior of the developing gonad to form the male somatic gonad primordium; this rearrangement occurs at the L1/L2 stage (Kimble and Hirsh, 1979). In lineaged *gon-4* males, appropriate somatic gonadal cells moved toward the anterior at the proper time (data not shown) (n = 6). Thus, wild-type somatic gonadal primordia were not made in either sex—too few cells were present, but movements reminiscent of SP formation were observed in both sexes.

In wild-type hermaphrodites, the somatic gonadal tissues differentiate during the L4 stage. Thus, *lim-7::GFP* is expressed in the hermaphrodite gonadal sheath during L4 and is maintained throughout adulthood (Hall *et al.*, 1999) (Fig. 5A). In *gon-4* mutants, *lim-7::GFP* is expressed in a small number of cells at the same time during L4 and throughout adulthood (n = 50 worms) (Fig. 5B). Similarly, MH27, a marker of spermathecal differentiation (Francis and Waterston, 1991), was expressed as normal in L4 *gon-4* mutants in a few cells that were likely to be spermathecal (not shown). Therefore, differentiation of somatic gonadal tissues appears to occur at the normal developmental stage in *gon-4* mutants.

gon-4 Encodes a Novel Acidic Protein

We cloned *gon-4* by finely mapping *gon-4* on the genetic map (Fig. 6A), identifying a cosmid, K04D7, that rescues *gon-4(q519)* homozygotes to fertility (Fig. 6B), and then identifying a transcript, K04D7.5, that yields a *gon-4* phe-

notype when tested by RNA-mediated interference (Fig. 6C). Identification of *gon-4* was confirmed by mutant rescue with a 10-kb (*Pvu*II) restriction fragment predicted to contain only K04D7.5. The *gon-4* mRNA is predicted to be 4035 nt, consistent with the size of a single 4.1-kb band detected on a Northern blot (Fig. 7A); the exon/intron boundaries predicted by Gene Finder were confirmed by sequencing a cDNA, yk353a9, and comparing it with the genomic sequence (The *C. elegans* Sequencing Consortium, 1998); the 5′ end of the *gon-4* transcript was determined using PCR with SL1 and an internal primer on single-stranded cDNA (see Materials and Methods). We conclude that the *gon-4* transcript is *trans*-spliced to SL1 and that it is assembled from 13 exons (Fig. 6D).

Analysis of the *gon-4* coding region revealed a single open reading frame encoding a protein of 1338 amino acids with a predicted p*I* of 4.4 (Fig. 7B). Searches of GenBank and EMBL databases with protein, nucleic acid, and translated nucleic acid sequences identified no protein with significant similarity to this predicted protein. Thus, the *gon-4* locus encodes a novel protein. PSORT analysis of the amino acid sequence predicted five nuclear localization signals (Fig. 7B, underlines). The only other notable feature of GON-4 is the extraordinary number of glutamic acid and aspartic acid residues, which together comprise 21% of the 1338 amino acids (Fig. 7B, red).

We sequenced the entire *gon-4* coding region from the four *gon-4* alleles that had been characterized in some depth. In each allele, we identified a single nucleotide substitution predicted to introduce a stop codon into the ORF (Fig. 6E). These nonsense mutations are consistent with comparison of variable characters in the four alleles (Fig. 3), which suggested them to be equivalent in strength and loss-of-function mutations.

gon-4::GFP Expression

To ask where *gon-4* is expressed, we constructed a *gon-4::GFP* transgene in which a GFP coding sequence was inserted into a *gon-4*-rescuing genomic fragment (Fig. 8A). This *gon-4::GFP* translational fusion rescues *gon-4(q519)* mutants to fertility when incorporated into the extrachromosomal array *qEx453*. A stable rescued line of *gon-4(q519); qEx453* was examined for GFP expression. Nine other rescuing lines also exhibited the same pattern (data not shown). GON-4::GFP was found in the nucleus, consistent with PSORT predictions (see above) (Figs. 7B, 8C, and 8D). Furthermore, GON-4::GFP was found in the somatic gonadal precursor cells, Z1 and Z4, and their descendants from L1 (Fig. 8B) until the early L3 stage (not shown). Expression was weak, but reproducible. During L4, GFP was observed in four nuclei of developing vulva, but not in other nongonadal cells (not shown).

Intriguingly, GON-4::GFP was detected in a scattering of germ-line nuclei of rescued animals. In contrast to the somatic gonadal expression, which occurred during early gonadogenesis, germ-line expression started in the second half of L4 and continued in adulthood (Fig. 8D). Preliminary results with polyclonal antibodies raised to the C-terminal portion of GON-4 are consistent with expression of GON-4 in the nuclei of dissected germ lines in mid- to late L4s and adults (not shown). These same antibodies have not proven useful with whole mounts of early larvae, which require a different protocol for antibody staining. Expression of the *gon-4::GFP* transgene was silenced in both somatic gonad and germ line, a phenomenon that is more typical of genes expressed in the *C. elegans* germ line (Kelly and Fire, 1998; Kelly *et al.*, 1997; Mello and Fire, 1995; Okkema *et al.*, 1993).

Attempts to Identify a Germ-Line Function for gon-4

To explore the functional importance of the germ-line expression of *gon-4*, we did three experiments. First, we asked whether a mutant *gon-4* germ line might somehow result in somatic gonadal defects. To test this idea, we ablated the two germ-line precursor cells during early L1 in *gon-4* mutants. In wild-type animals, such an ablation has no effect on development of the somatic gonad (Kimble and White, 1981). Similarly, in *gon-4* mutants, ablation of the germ line did not alter Z1/Z4 development: the typical delay in cell divisions was observed. Second, we used RNA-mediated interference to ask if the *gon-4* gene products present in the adult germ line might be required maternally for development of the next generation; however, *gon-4(RNAi)* progeny showed only gonadal defects similar to those of bona fide *gon-4* mutants; no embryonic or larval lethality was seen (not shown). Finally, we asked if the site of GON-4 protein in the germ line might correlate with the cell cycle stage. Perhaps GON-4 has a function in controlling germ-line cell divisions during adulthood. If true, this may shed light on the *gon-4* control of Z1/Z4 cell lineages. To explore this idea, we double-stained with anti-GON-4 antibodies and anti-phosphohistone H3 antibodies, which detect chromosomes in metaphase (Ajiro *et al.*, 1996). We found that some germ-line nuclei stained with both antibodies, whereas other nuclei stained with either one or the other of the antibodies (not shown). Therefore, we were unable to demonstrate any specificity of GON-4 staining with respect to the stage of the cell cycle. The role of *gon-4* in the adult germ line remains a mystery.

DISCUSSION

In this paper, we introduce the *gon-4* locus, a gene critical for gonadogenesis in *C. elegans*. The *gon-4* mutations are likely to be null. The *gon-4* mutant phenotype is organ-specific, affecting both somatic and germ-line tissues of the gonad and one associated structure, the vulva. In contrast, nongonadal tissues appear to develop normally in *gon-4* mutants. In the following discussion, we address ideas about the functions of *gon-4* and how its activity may integrate the development of multiple tissues and structures in the gonad.

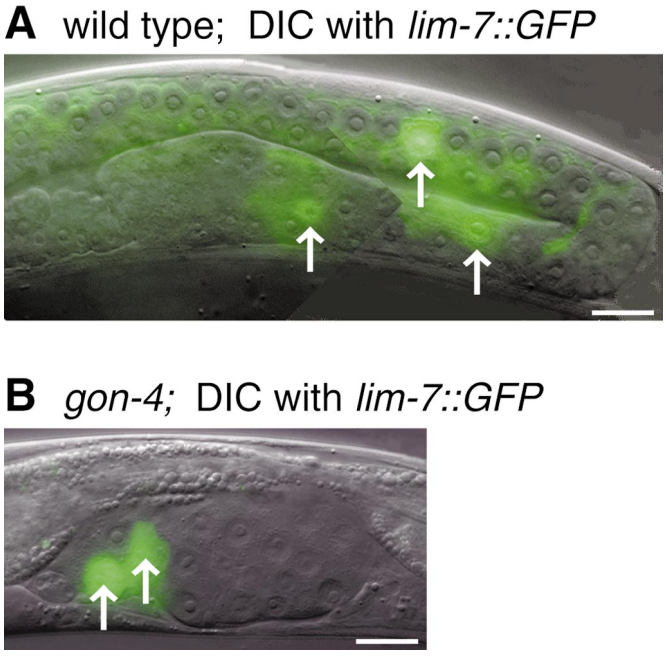


FIG. 5. Sheath cells differentiate at the correct time in *gon-4* mutants. A marker of sheath cell differentiation, *lim-7::GFP* (Hall *et al.*, 1999), is shown in a Nomarski micrograph overlaid with GFP fluorescence. (A) *lim-7::GFP* expression in a wild-type L4 hermaphrodite. (B) *lim-7::GFP* expression in a *gon-4(q519)* L4 hermaphrodite. White arrows indicate nuclei of expressing cells. Photos are to same scale. Scale bars, 10 μm.

gon-4 Regulates Cell Divisions in the Developing Somatic Gonad

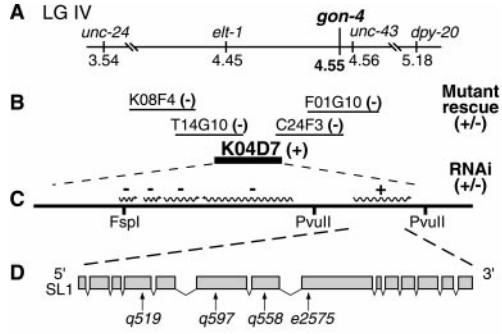
In wild-type animals, the somatic gonadal blast cells, Z1 and Z4, follow an essentially invariant pattern of cell divisions (Kimble and Hirsh, 1979). These cell divisions are part of a temporal program of gonadogenesis, consisting of cell divisions, migrations, and differentiation (see Fig. 1). In *gon-4* mutants, Z1 and Z4 appear to be specified correctly, but their cell divisions are variably delayed and aborted. These lineage defects result in a loss of adult tissues and a failure to generate regulatory cells that are essential for morphogenesis, germ cell proliferation, and vulval development. In an attempt to reveal nongonadal defects in *gon-4* mutants, we examined the cell lineages of two nongonadal blast cells in *gon-4* mutants, the temporal progression of other developmental events (e.g., molts), and the overall growth and morphology of the animal (see Results). However, we found no defects in nongonadal tissues (other than the vulva, see below). We conclude that *gon-4* is a regulator of the Z1/Z4 lineage and that *gon-4* has little or no role outside of the gonad.

Despite the *gon-4* lineage defects, other aspects of somatic gonadal development appear to occur normally. For example, cells migrate at the appropriate time in both sexes in an apparent attempt to form the somatic gonad primordium. Furthermore, markers of gonadal differentiation are expressed at the normal time in *gon-4* mutants. We conclude that progression through the normal pattern of Z1/Z4 cell divisions can be uncoupled from progression through the morphogenetic and differentiation steps of gonadogenesis.

A Role for gon-4 in the Germ Line?

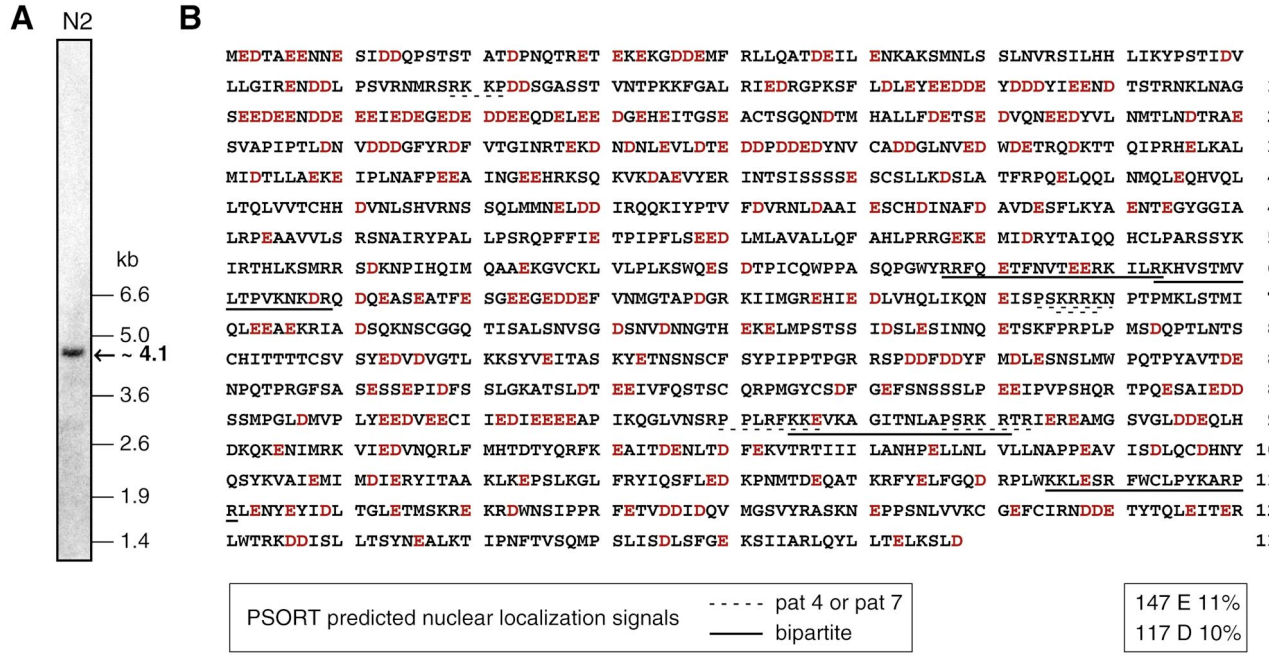
In wild-type adults, over 1000 germ-line cells are organized into a reproducible pattern of cell fates in an elongated tube. In contrast, the *gon-4* mutant has a smaller germ line, and germ-line differentiation is either abolished or defective. What role does *gon-4* activity play in the germ line? Unlike the *gon-4* somatic gonad, the cell divisions of the germ-line progenitor cells are indistinguishable from wild type during early larval development. Moreover, the *gon-4* gene does not appear to be expressed in the germ line until just before adulthood. Finally, the *gon-4* germ line produced mature sperm when the proliferation signal was removed in a *glp-1; gon-4* double mutant. Therefore, *gon-4* is not essential for early germ-line cell divisions or for spermatogenesis.

The simplest explanation for the *gon-4* germ-line defects is that they are a secondary consequence of defects in the somatic gonad. In wild-type, the somatic distal tip cell is



E	Allele	Mutation	Nucleotide	Amino Acid	Location
	gon-4(q519)	STOP	T->A	Tyr	228
	gon-4(q597)	STOP	C->T	Gln	432
	gon-4(q558)	STOP	C->T	Gln	618
	gon-4(e2575)	STOP	T->G	Tyr	970

FIG. 6. Molecular cloning of the *gon-4* gene. (A) Genetic map, region of chromosome *IV*. Three-factor and deficiency mapping places *gon-4* at position 4.55. (B) Cosmids in *gon-4* region. Cosmid KO4D7 (thick line) rescues *gon-4* mutants to fertility (+); neighboring cosmids fail to rescue *gon-4* (−). (C) Five transcripts (wavy lines) predicted for cosmid KO4D7 are numbered from left to right. RNAi directed against KO4D7.5 yields the *gon-4* phenotype (+); RNAi directed against the others did not (−). (D) Exon/intron structure of *gon-4* transcript, as determined by SL1-directed PCR for the 5′ end and comparison of cDNA and genomic sequences for the remainder. (E) Molecular basis of *gon-4* mutations.



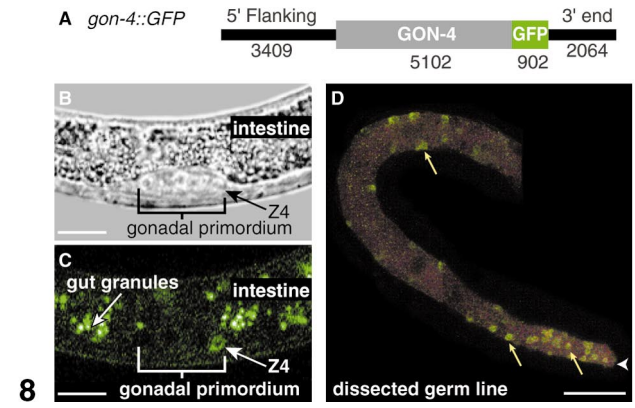


FIG. 7. The *gon-4* RNA and protein products. (A) Northern blot of mixed-stage poly(A)$^+$ RNAs probed with two 1-kb fragments of *gon-4* cDNA. A single major transcript of 4.1 kb is detected (arrow). (B) The GON-4 protein is composed of 1338 amino acids of which 21% are either aspartic acid (D) or glutamic acid (E). The PSORT program predicts that GON-4 is nuclear localized. Different nuclear localization signals (NLS) are underlined: solid line, bipartite NLS; dashed lines, pat4 or pat7 NLS.

FIG. 8. The *gon-4::GFP* transgene is expressed in Z1 and Z4 and in the germ line of adults. (A) Structure of *gon-4::GFP*. The construct included the entire 10.6-kb fragment of genomic DNA that rescues *gon-4* to fertility plus a 902-bp insert encoding GFP in frame at the C-terminus. (B, C) *gon-4(q519); qEx453* L1 hermaphrodite. The birefringent intestine extends throughout the animal; the four-celled gonadal primordium (bracket) lies midventrally. Expression is weak but reproducible. Z4, arrow. (B) Nomarski differential interference contrast micrograph. (C) Fluorescent image of same animal shown in B: the intestine is full of autofluorescent gut granules; GFP is localized to the Z4 nucleus; Z1 is out of the plane of focus. (D) GON-4 protein is present in nuclei of the adult germ line. Due to the low level of GFP expression in the adult gonad anti-GFP antibodies were used to enhance the expression pattern. Yellow arrows indicate some of the cells showing staining; arrowhead indicates distal end. A similar pattern was obtained with polyclonal anti-GON-4 antibodies (not shown). Scale bars, 20 μm.

essential for germ-line proliferation (Kimble and White, 1981), and sheath/spermathecal cells are critical for robust germ-line proliferation and normal differentiation (McCarter *et al.*, 1997). Most *gon-4* mutants possess either one or no DTCs and fewer than normal sheath and spermathecal cells. Therefore, the germ-line defects may simply reflect the absence of these somatic gonadal cells. However, given the expression of *gon-4* in germ-line nuclei during late L4 and adulthood, we surmise that *gon-4* may have some later role in germ-line development as well.

gon-4 and Development of the Vulva

The vulva is a specialization of the body wall created for egg-laying. The vulva is induced by a regulatory cell in the somatic gonad, called the anchor cell (Kimble, 1981); furthermore, vulval morphology requires attachment to the body wall by uterine cells (Newman *et al.*, 1996). We suggest that the *gon-4* vulval defects are, at least in part, a secondary consequence of somatic gonadal defects. The *gon-4* mutants with no vulva are likely to lack an anchor cell and those with a protruding vulva are likely to lack some of the uterine cells required for proper attachment of the vulva to the body wall. The rare class of *gon-4* mutants with two vulvas might be explained by a failure in lateral signaling, which normally ensures the production of a single anchor cell (Greenwald *et al.*, 1983; Kimble, 1981; Seydoux and Greenwald, 1989). Therefore, all three classes of *gon-4* vulval defects can be explained by defects in the somatic gonad. However, a more direct role in vulval development is consistent with the expression of *gon-4* in the developing vulval cells and cannot be excluded. Experiments to address this possibility are beyond the scope of this work, but provide an important avenue for future studies.

GON-4 May Act Cell Autonomously

The GON-4 protein is present in the somatic gonadal progenitor cells, Z1 and Z4, and their progeny during the first three larval stages. Therefore, GON-4 is expressed in the proper place and at the correct time to control Z1/Z4 cell divisions in a cell-autonomous manner. Although mosaic analysis is essential for any definitive statement regarding the site of gene function, the tools are not yet available for mosaic analysis of *gon-4:* no free duplication is available for the *gon-4* region and *gon-4* extrachromosomal arrays rescue poorly and are subject to gene silencing. In an attempt to gain some insight into the site of *gon-4* function, we showed that the Z1/Z4 cell divisions are defective in *gon-4* mutants from which the germ line had been surgically removed. Therefore, *gon-4* is likely to act either in Z1 and Z4 themselves or in some nongonadal tissue. The GON-4 protein is nuclear and bears no signal sequence or any motif suggestive of cell signaling. Therefore, the simplest model is that *gon-4* controls the Z1/Z4 lineages in a cell-autonomous manner.

How Does gon-4 Regulate Z1 and Z4 Cell Divisions?

The *gon-4* gene promotes cell divisions of the somatic gonadal precursor cells, Z1 and Z4. How does it do this? Is there some clue in other genes controlling these divisions? Although numerous genes that are required for Z1 and Z4 cell divisions have been identified, none of those genes is specific to these precursor cells. One rather large class of such genes includes *lin-5* and *lin-6* (Albertson *et al.*, 1978), *cul-1* (Kipreos *et al.*, 1996), *cki-1* (Hong *et al.*, 1998), *ncc-1* (Boxem *et al.*, 1999), and *stu-7/air-2* (Woollard and Hodgkin, 1999). These genes are required for all postembryonic cell divisions and encode known components of the cell cycle machinery that are used in virtually all cells. They are therefore not Z1/Z4-specific lineage regulators. A much smaller class, represented by a single gene called *gon-2*, is critical for cell divisions of both Z1 and Z4 as well as cell divisions of the germ-line precursor cells, Z2 and Z3 (Sun and Lambie, 1997). Although the *gon-2* and *gon-4* phenotypes are similar, their defects are distinct and we do not yet know if they are in the same pathway.

The *gon-4* gene encodes a novel protein: database searches have failed to identify any homolog. The prediction of five nuclear localization signals is supported by localization of GON-4::GFP to nuclei (this work) and the presence of endogenous GON-4 in nuclei (L. Friedman, unpublished). This localization suggests a nuclear function. One remarkable characteristic of the GON-4 amino acid sequence is its acidity: 277/1388 amino acids are either glutamic acid or aspartic acid and its predicted p*I* is 4.4. Given the novelty of the GON-4 protein, its molecular function is clearly not known. However, one reasonable guess, which is based on its acidity and nuclear location, is that GON-4 may control the expression of genes that drive the cell cycle, perhaps at the transcriptional level.

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