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The *sys-1* Gene and Sexual Dimorphism during Gonadogenesis in *Caenorhabditis elegans*

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In wild-type *Caenorhabditis elegans,* the hermaphrodite gonad is a symmetrical structure, whereas the male gonad is asymmetric. Two cellular processes are critical for the generation of these sexually dimorphic gonadal shapes during early larval development. First, regulatory "leader" cells that control tube extension and gonadal shape are generated. Second, the somatic gonadal precursor cells migrate and become rearranged to establish the adult pattern. In this paper, we introduce *sys-1,* a gene required for early organization of the hermaphrodite, but not the male, gonad. The *sys-1(q544)* allele behaves genetically as a strong loss-of-function mutant and putative null. All hermaphrodites that are homozygous for *sys-1(q544)* possess a grossly malformed gonad and are sterile; in contrast, *sys-1(q544)* males exhibit much later and only partially penetrant gonadal defects. The *sys-1(q544)* hermaphrodites exhibit two striking early gonadal defects. First, the cell lineages of Z1 and Z4, the somatic gonadal progenitor cells, produce extra cells during L2, but the regulatory cells that control gonadal shape are not generated. Second, somatic gonadal precursor cells do not cluster centrally during late L2, and the somatic gonadal primordium typical of hermaphrodites is not established. In contrast, the early male gonadal lineage is asymmetric as normal, the somatic gonadal primordium typical of males is established correctly, and the male adult gonadal structures can be normal. We conclude that the primary role of *sys-1* is to establish the shape and polarity of the hermaphrodite gonad.

Key Words: sys-1;* gonadogenesis; sexual dimorphism; *C. elegans.

INTRODUCTION

During organogenesis, multiple processes must be coordinated to generate a complex organ with a typical shape, size, and organization. At the cellular level, these processes include controls over cell proliferation and differentiation, as well as cell shape, polarity, and migration. At the tissue level, cells become organized into tubes or cell clusters, which themselves possess a defined shape and polarity. Although much is known about the regulatory molecules and signaling pathways that govern these cellular processes, little is known about how they function together to organize and shape organs during development.

We have focused on development of the gonad in the nematode *Caenorhabditis elegans* to investigate controls of organogenesis. This organ is sufficiently simple that it can be dissected genetically and analyzed at the level of individual cells. Nonetheless, the gonad is also sufficiently complex to embody organizational principles that may apply to more complex organs in vertebrates. During gonadogenesis, a four-celled primordium is assembled in the embryo; this primordium comprises two somatic gonadal progenitor cells and two germ-line progenitor cells (Sulston *et al.,* 1983). After the embryo hatches, the somatic gonadal precursor cells, called Z1 and Z4, follow either a hermaphrodite- or a male-specific program to produce a hermaphrodite or male gonad, respectively (Kimble and Hirsh, 1979). These two gonads share several common features: both are tubular structures with the germ-line tissue housed in elongate "arms" and somatic gonadal structures (e.g., uterus in hermaphrodites, vas deferens in males) that are essentially specialized epithelial tubes. Despite these similarities, the overall organization of the hermaphrodite gonad is strikingly different from that of the male gonad. The hermaphrodite gonad consists of two

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equivalent ovotestes that together constitute a symmetrical organ, whereas the male gonad is composed of a single testis and is asymmetrical.

We have begun to investigate the regulatory mechanisms that achieve the distinct organ shapes of the hermaphrodite and male gonads. Previous work delineated two cellular mechanisms essential for this process. The first is generation of regulatory cells that "lead" growing gonadal arms (Kimble and White, 1981). In hermaphrodites, two "leader" cells that are responsible for formation of the two ovotestes are born, whereas in males, only one leader cell is made, resulting in the single testis (Fig. 1, red cells). In hermaphrodites the two leader cells are called distal tip cells (DTCs) and in males the single leader cell is called a linker cell. When leader cells are removed by laser ablation, the gonadal arms do not extend and the developing gonad does not acquire its normal shape. A separate function of the DTCs is their control over germ-line proliferation (Kimble and White, 1981). This control, which relies on expression of the LAG-2 ligand by the DTCs (Henderson *et al.*, 1994; Tax *et al.*, 1994), plays little role in molding organ shape, but instead regulates the polarity of germ-line fates (e.g., mitosis/meiosis).

A second key step in controlling gonadal morphogenesis is the rearrangement of somatic gonadal precursor cells during early larval development (Kimble and Hirsh, 1979). In hermaphrodites, these cells coalesce at the center of the developing gonad to establish the somatic gonadal primordium specific for hermaphrodites (SPh) (Fig. 1, left column, early L3). SPh formation separates the germ-line tissue into equivalent anterior and posterior arms and places the somatic gonadal blast cells in their correct positions to generate symmetrical somatic gonadal structures. In males, the somatic gonadal precursor cells coalesce at the anterior tip of the developing gonad to establish the somatic gonadal primordium specific for males (SPm) (Fig. 1, right column, mid-L2 and early L3). This male rearrangement establishes the asymmetry of the male gonad. Hence, sex-specific cell rearrangements establish sex-specific somatic gonadal primordia in a process that is crucial for gonadal morphogenesis.

Several genes that govern leader cell migration have been identified. In particular, the *unc-5* and *unc-6* genes, among others, control the direction of leader cell elongation (reviewed in Antebi *et al.*, 1997; Hedgecock *et al.*, 1990), and the *gon-1* gene is essential for leader cell migration per se (Blelloch *et al.*, 1999). The *unc-6* gene encodes a laminin-related protein called netrin and *unc-5* encodes a netrin receptor (Ishii *et al.*, 1992; Leung-Hagesteijn *et al.*, 1992). Together these proteins guide the migrating DTC during its dorsal–ventral turn. The *gon-1* gene encodes a secreted metalloprotease; localization of this metalloprotease to the leader cell is essential for gonadal arm extension (Blelloch and Kimble, 1999). In contrast to the progress made in understanding leader cell function, relatively little is known about the controls that govern the generation of leader cells or the rearrangements of somatic gonadal precursor cells to form hermaphrodite- or male-specific somatic gonadal primordia.

Here we introduce *sys-1* (for symmetrical sisters), a gene required for generation of leader cells in hermaphrodites and also for SPh formation. Because *sys-1* mutants do, in fact, display minor defects during male gonadogenesis, we suggest that the *sys-1* gene is part of a common gonadogenesis program that has become specialized during evolution to govern hermaphrodite-specific morphogenesis.

MATERIALS AND METHODS

Strains

Animals were maintained using standard procedures (Brenner, 1974) and grown at 20°C unless otherwise noted. All strains used were derivatives of the Bristol strain N2 (Brenner, 1974). We used the following mutations (Riddle *et al.*, 1997): *LGI, fog-1(q187), ces-1(n703sd), unc-11(e47), dpy-5(e61), unc-13(e51), mec-8(e398), unc-29(e193* or *e1072), fog-3(q469* or *q470), lin-11(n566), srf-2(yj262), unc-75(e950); LGII, tra-2(e1095); LGV, egl-1(n2164d), him-5(e1490); LGX, xol-1(y9)* (Miller *et al.*, 1988). *qIs23* is an integration of *lag-2*::GFP on chromosome *I* that was used as a green balancer for *sys-1;* self-progeny from *sys-1/qIs23* heterozygotes were either glowing (GFP+) animals of genotype *qIs23*/+ or *qIs23/qIs23* or nonglowing (GFP−) *sys-1* homozygotes. *qIs48*, an integration of *myo-2*::GFP, *pes-10*::GFP, and *ges-1*::GFP on *hT2*, was similarly used as a green balancer. Extrachromosomal arrays (Ex) or integrants (Is) carrying markers include the following: *qIs19[lag-2::GFP] V, qEx87[lag-2::lacZ], evIn54[unc-5::lacZ],* and *leIs8 IV*.

Genetic Mapping

The *sys-1* locus was mapped near *fog-3* on *LGI* by three-factor mapping (Table 1). To further map *sys-1*, we used deficiencies generated to analyze *fog-3* (Ellis and Kimble, 1995) and found that *qDf5* balances *sys-1*, whereas *qDf14* removes *sys-1* (not shown).

Isolation of sys-1 Mutations

One allele, *sys-1(q7),* was isolated in an F2 screen for sterile mutations following an ethylmethane sulfonate (EMS) mutagenesis screen (Austin and Kimble, 1987). A second allele, *sys-1(q544),* was isolated in a noncomplementation screen (this work). To ask whether a null allele could be isolated by a screen for mutations that fail to complement *sys-1(q7),* we first showed that *sys-1(q7)/qDf14* animals are viable and sterile. We then mutagenized strain *ces-1; tra-2/mnC1; egl-1; xol-1* with 40 mM EMS. From the self-progeny of mutagenized L4 hermaphrodites, XX males of genotype *ces-1; tra-2; egl-1; xol-1* were mated singly to *unc-13 sys-1(q7)/unc-13 fog-3 lin-11* hermaphrodites. Cross-progeny were scored to find plates with approximately 1/4 non-Unc steriles. From such plates, non-Unc fertile siblings were placed individually onto plates to recover the new mutation from *sys-1*(new)*/unc-13 fog-3 lin-11* heterozygotes. *sys-1(q544)* was isolated after screening 5916 mutagenized chromosomes.

Brood Analysis

All progeny from a single hermaphrodite, *sys-1(x)/unc-29 fog-3*, were scored by DIC microscopy for gonadal arm extension, differ-

entiated somatic gonadal cells/tissues, germ cells, and vulva formation. Some animals exploded under the coverslip and could not be scored. For both *sys-1(q7)* and *sys-1(q544)*, the Sys phenotype was recessive, and the brood size of a heterozygote was comparable to wild type (average brood size ranged from 210 to 240 depending on allele). Dead embryos segregated at 1 and 6.5% for *q7* and *q544*, respectively.

Immunofluorescence

Antibody staining followed protocols of Albertson (1984) and Strome and Wood (1983). MH27, a monoclonal antibody that recognizes adherens junctions and highlights spermathecal tissue (Wood, 1988), was used at a 1:50 dilution. α-PGL-1, a polyclonal antibody that stains germ-line tissue (Kawasaki *et al.*, 1998), was used at a 1:10,000 dilution. Donkey anti-mouse FITC-conjugated and donkey anti-rabbit Cy3-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA) were used at 1:200 dilution. Propidium iodide, which stains DNA, was diluted to 10 μg/ml in the secondary antibody mixture (Orsulic and Peifer, 1997).

lacZ and Green Fluorescent Protein (GFP) Reporters

All markers were introduced into *sys-1* mutants by mating *sys-1(x)*/+ males into strains carrying the transgene; all transgenic arrays carried a *rol-6* marker. Rolling Sys animals were either stained for β-galactosidase according to Fire (1992) or observed using a Zeiss Axiophot microscope equipped with fluorescence.

lacZ markers. *leIs8* marks spermathecal cells, a subset of uterine cells, and rectal valve cells, while UL26 marks spermathecal cells and vulval D cells (Hope, 1991). *lin-3*::lacZ expresses in the anchor cell during L3 (Hill and Sternberg, 1992) and *unc-5*::lacZ marks DTCs from L3 lethargus through L4 (Su *et al.*, 2000). Animals expressing *unc-5*::lacZ were staged by vulval development as viewed by DAPI staining. *leIs8* and UL26 were examined in *sys-1(q7)* and *sys-1(q544)* adult animals, respectively. *lin-3*::lacZ and *unc-5*::lacZ expressions were examined in *sys-1(q544)* mutants at L3 and L4 stage, respectively.

GFP markers. *lim-7*::GFP marks hermaphrodite gonadal sheath cells (Hall *et al.*, 1999) and was examined in *sys-1(q544)* adults. *lag-2*::GFP is a good marker for Z1 and Z4 as well as hermaphrodite DTCs and the male linker cell (Blelloch *et al.*, 1999); it is also a weak marker for the hermaphrodite anchor cell and the male DTCs (Blelloch *et al.*, 1999; this work). *lag-2*::GFP was examined in both *q7* and *q544* adults. In addition, 39 self-progeny of *sys-1(q544)*/+; *lag-2*::GFP hermaphrodites were examined as L1s for expression in Z1 and Z4, rescued individually from the slide, and examined again for DTC expression in L3 when Sys mutants can be easily distinguished. All L1s expressed *lag-2*::GFP in at least Z1 or Z4 and 87% expressed it in both. The five L1s expressing *lag-2*::GFP in only Z1 or Z4 included one *sys-1(q544)* homozygote and four +/+ or *q544*/+ animals.

Cell Lineage and Laser Ablation

Cell lineages were done by standard methods (Sulston and Horvitz, 1977), and laser ablations were performed as described (Bargmann and Avery, 1995) using a Micropoint Ablation Laser System (Photonics Instruments, Inc., Arlington, IL). L1 *sys-1(q544)* homozygotes were identified among the progeny of heterozygotes carrying a green balancer (see above under strains) by their lack of GFP fluorescence. For lineaging, either Z2 or Z3 was ablated to reduce gonadal complexity. To determine cells capable of making anchor cells, either Z1.a/Z4.p or Z1.p/Z4.a were killed soon after they were born. To eliminate the entire gonad, all four gonadal precursors (Z1–Z4) were killed in early L1.

TABLE 1
Three-Factor Mapping of *sys-1*

Genotype	Recombinant	Segregation
sys-1(q7)/unc-11 dpy-5	Dpy non-Unc	0/8 Sys
	Unc non-Dpy	5/5 Sys
sys-1(q7)/dpy-5 unc-13	Dpy non-Unc	13/13 Sys
	Unc non-Dpy	0/13 Sys
sys-1(q544)/unc-13 fog-3	Unc non-Fog	13/13 Sys
	Fog non-Unc[a]	0/4 Sys
sys-1(q7)/fog-3 srf-2 unc-75	Unc non-Fog[b]	10/10 Sys
	Fog non-Unc[a]	0/14 Sys

[a] *sys-1(x)*/Balancer males were crossed into Fog recombinants to test for *sys-1* mutation on recombinant chromosome.

[b] Unc Sys progeny derived from Unc non-Fog recombinants were scored for the *srf-2* cuticular defect by staining with fluorescein soybean agglutinin (Ellis and Kimble, 1995): 16/20 carried the *srf-2* mutation.

Germ Cell Number

To assess the number of germ cells, we double-stained *sys-1(q544)* homozygotes with the germ-line-specific α-PGL-1 antibody (Kawasaki *et al.*, 1998) and MH27 to determine developmental stage. Timing of vulval divisions in *sys-1(q544)* mutants is the same as in wild type. Mid-L3 *sys-1* mutants contained 20 germ cells (±2, $n = 3$); late L3 mutants averaged 38 (±3; $n = 5$).

Electron Microscopy

Two each of wild type and *sys-1(q544)* L3 hermaphrodites were prepared for electron microscopy as described (Bargmann *et al.*, 1993) with modifications. After overnight fixation in 2% osmium tetroxide, an equal volume of 3% potassium ferricyanide was added and samples were set at room temperature for 50 min. Samples were then rinsed in water and embedded in 2% agarose. Small blocks containing the samples were cut and placed in 50% ethanol for 10 min. They were then stained with saturated uranyl acetate for 60 min at room temperature and again rinsed with water. Dehydration and infiltration were accomplished using a Pelco 3440 microwave oven (Giberson and Demaree, 1995). Micrographs were obtained at 60 kV with a Philips EM 120 transmission electron microscope.

Male Mating Assay

Males homozygous for *sys-1(q544)* were identified as nonglowing (GFP−) cross-progeny from a mating of hermaphrodites and males of the genotype *sys-1(q544)/qIs48*. Five *sys-1(q544)* males were placed on petri dishes with 3–5 females of genotype *fog-1 unc-11*. Among 60 males tested, we observed cross-progeny on two plates. In both cases, the cross-progeny were nonglowing and were

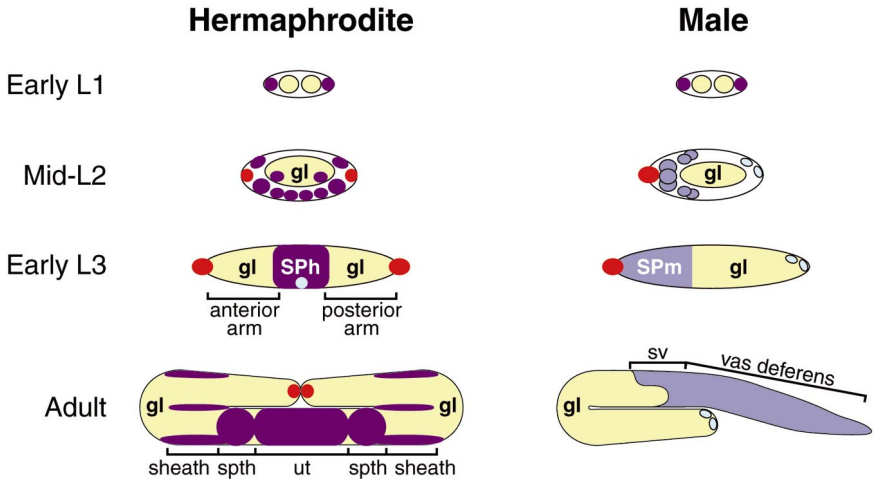

FIG. 1. Critical stages of gonadogenesis in wild-type *Caenorhabditis elegans.* Hermaphrodite, left column; male, right column. L1–L3, first to third larval stage. Anterior is to the left; ventral is down. Most somatic gonadal cells (blue), hermaphrodite DTCs (red), hermaphrodite anchor cell (pale blue), male leader cell (red), male DTCs (light blue), germ line cells (yellow). In newly hatched L1s, hermaphrodites and males possess identical-looking 4-celled gonad primordia that consist of two somatic gonadal and two germ-line precursor cells. The somatic gonadal precursors in each sex undergo a stereotyped pattern of divisions during L1 and L2 to make 12 cells in hermaphrodites and 10 in males. Rearrangement of somatic gonadal cells to form sex-specific somatic gonadal primordia (SP) establishes sexual dimorphism. The somatic gonadal primordium of the hermaphrodite (SPh) is symmetrical: somatic gonadal blast cells are clustered in the center with germ line flanking each side and leader cells, called DTCs, at each end. The DTCs control the shape of the hermaphrodite gonad and induce germ-line proliferation (Kimble and White, 1981). Cells in the SPh divide further in L3 and L4 to generate adult structures: sheath, spermathecae (spth), and uterus (ut). The somatic gonadal primordium of the male (SPm) is asymmetrical, with somatic gonadal blast cells at the anterior. The single leader cell, called the linker cell, is at the anterior tip of the male developing gonad and controls its extension and shape (Kimble and White, 1981). The two male DTCs are at the posterior end, where they stimulate germ-line mitosis (Kimble and White, 1981). Cells in the SPm divide further in L3 and L4 to generate adult structures: seminal vesicle (sv) and vas deferens.

themselves progeny tested to ensure segregation of *sys-1(q544)* mutants in the next generation to verify the genotype of the male.

RESULTS

sys-1 Mutant Hermaphrodites Possess Severely Malformed Gonads

The *sys-1* gene is defined by two recessive mutations that map near *fog-3* on chromosome *I* (see Materials and Methods). Hermaphrodites homozygous for either allele have severely malformed gonads and are 100% sterile. Although the *sys-1* alleles differ in strength (see below), they all have two general defects in hermaphrodite gonadogenesis. First, whereas wild-type hermaphrodites have two elongate gonadal arms (Fig. 2A), *sys-1* mutant hermaphrodites have little or no gonadal arm extension (Fig. 2B). In addition, no coherent somatic gonadal structures (e.g., uterus, spermatheca) are recognizable in adults; instead, the central region of the animal, in which uterus and spermathecae normally form, is filled with a mass of disorganized germline and somatic gonadal tissues (see below). In contrast, the gonads of wild-type and *sys-1* males are both J-shaped and asymmetrical (Figs. 2C and 2D). We conclude that the *sys-1* gene is critical for early morphogenesis of the hermaphrodite gonad, but not for that of the male.

Identification of a Strong Loss-of-Function sys-1 Allele

The severity of gonadal malformation differs for the two *sys-1* alleles. We quantitated this difference using the easily scorable feature of arm extension. Among all *sys-1(x)* homozygotes in a brood from a *sys-1(x)*/+ mother, 16% (n = 42) exhibited some gonadal arm extension in *sys-1(q7)* mutants, whereas 0% (n = 33) extended gonadal arms in *sys-1(q544)* mutants. We infer that *q7* is a weaker allele than *q544*.

Since *sys-1(q544)* had the more severe phenotype and since this allele was isolated in a noncomplementation screen that could have identified a null mutation (see Materials and Methods), we asked whether it behaved genetically like a null. To this end, we first placed *sys-1(q544)* in *trans* to a deficiency and found the phenotype of *sys-1(q544)* homozygotes to be equivalent to that of *sys-1(q544)/Df* animals. In addition, we examined *sys-1(q544)* or the deficiency in *trans* to the *sys-1(q7)* weak allele: arm extension was reduced to 8% in *sys-1(q7)/Df* animals (n = 18) and to 6% in *sys-1(q7)/sys-1(q544)*

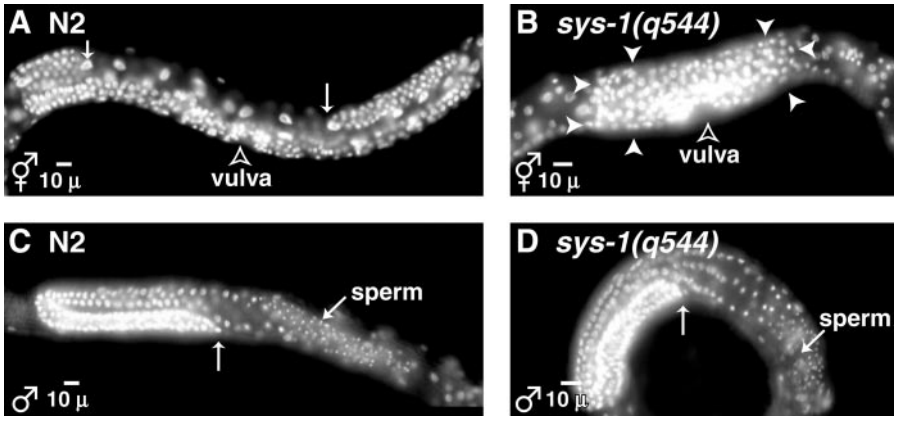

FIG. 2. *sys-1* is required for gonadogenesis in hermaphrodites, but not males. DAPI staining of wild-type and *sys-1(q544)* hermaphrodites and males. Anterior is left; ventral is down. (A) L4 wild-type hermaphrodite. Two tubular U-shaped gonadal arms extend from the central vulva (open arrowhead). Closed arrows, distal ends of gonadal arms. (B) L4 *sys-1(q544)* hermaphrodite. No gonadal arms are found, but instead the gonadal mass is oblate. Closed arrowheads, perimeter of gonadal mass; open arrowhead, developing vulva. (C) L4 wild-type male. One tubular J-shaped gonadal arm is present. Vertical arrow, distal end of testis; oblique arrow, mature sperm in proximal gonad. (D) L4 *sys-1(q544)* male. Single J-shaped gonad is similar to the wild-type. Arrows same as in (C).

animals ($n = 22$). Therefore, *sys-1(q544)* behaves like a deficiency in this genetic test and may be a null allele.

sys-1 Hermaphrodites Exhibit Cell Lineage Defects during Early Gonadogenesis

To learn the cellular basis of the gonadal defects in *sys-1* hermaphrodites, we followed the cell lineages of Z1 and Z4, the two somatic gonadal precursor cells, from hatching through early L3. In *sys-1(q544)* mutants, we observed the following lineage alterations (Fig. 3). First, *sys-1* mutants entered L1 lethargus with only four somatic gonadal cells compared with eight in the wild type ($n = 11$). This reflects a delay in cell division rather than a premature entry into lethargus, because all progeny from a *sys-1(q544)/+* hermaphrodite entered lethargus at the same time and only *sys-1* homozygotes had fewer cells than normal. This delay has been seen in mutants developing on a petri plate as well as those developing under a coverslip for lineage analysis. Second, *sys-1* mutants exhibited aberrant somatic gonadal divisions during L2. In wild-type, the Z1 cell lineage is asymmetric (Fig. 3, arrow): Z1.a divides once before L3,

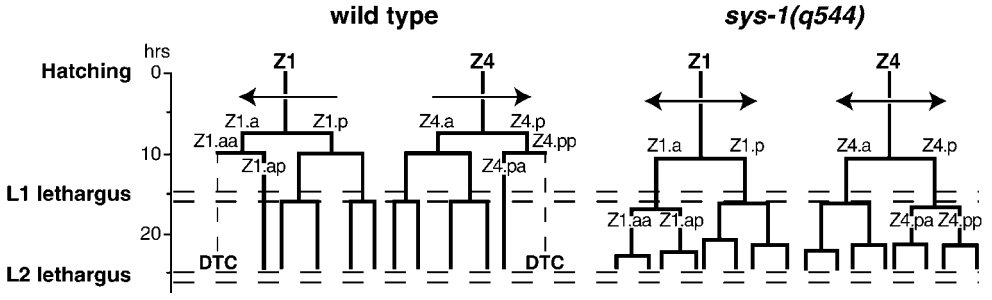

FIG. 3. Somatic gonadal cell lineages in wild-type and *sys-1* mutants. Z1/Z4 cell lineages in wild-type (left) and *sys-1(q544)* animals (right). Each vertical line represents a cell; each horizontal line represents a cell division, with anterior daughters on left and posterior daughters on right. Arrows denote asymmetry of Z1/Z4 lineages in wild-type or apparent symmetry in *sys-1* hermaphrodites. Time scale is shown at left in hours (20°) with important life cycle events noted. Cells are named for their mother and position with respect to cleavage plane. Although *sys-1* mutants enter L1 lethargus (dashed lines) at the same time as wild type, they have only four cells instead of eight. In wild-type gonads, Z1.aa and Z4.pp become DTCs and do not divide again; in *sys-1* mutants, these cells usually divide (see below for variability). In wild-type gonads, Z1.ap and Z4.pa join the SPh and continue divisions only in L3; in *sys-1* mutants, these cells usually divide precociously during L2. The variability of extra divisions is as follows: Z1.aa, Z4.pp, Z1.ap, and Z4.pa divided ($n = 2$); Z1.aa, Z4.pp, and Z4.pa ($n = 1$); Z4.pp and Z4.pa ($n = 1$); Z4.pp ($n = 1$); Z4.pa and Z1.ap ($n = 1$); and Z4.pa ($n = 1$).

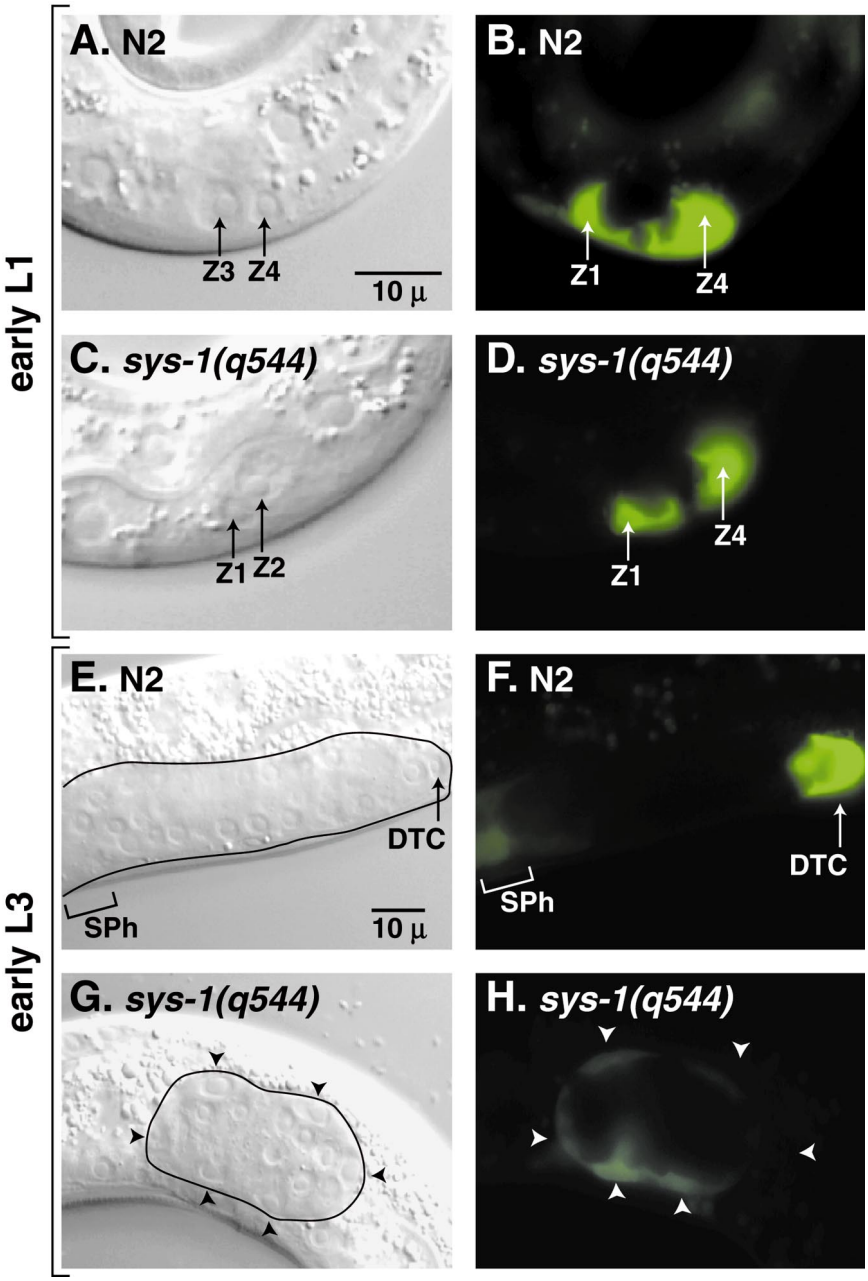

FIG. 4. Expression of *lag-2*::GFP in wild-type and *sys-1* developing gonads. Left, DIC; right, fluorescence. Anterior is left and ventral down in all pictures. All animals carry *qIs19[lag-2::GFP].* (A) Wild-type L1 hermaphrodite, at hatching. Arrows, Z3 and Z4 in gonad primordium. Z1 and Z2 are out of the plane of focus. Scale marker same for A–D. (B) Same animal as in (A), *lag-2*::GFP is expressed in Z1 and Z4 (arrows). (C) *sys-1(q544)* L1 hermaphrodite, at hatching. Arrows, Z1 and Z2 in gonad primordium; Z3 and Z4 are out of the plane of focus. (D) Same animal as (C), *lag-2*::GFP is expressed in Z1 and Z4, as in wild type. (E) Wild-type L3 hermaphrodite, SPh stage. Arrow, DTC at end of posterior arm; anterior arm not shown. Scale marker same for E and F. (F) Same animal as in (E) with intense *lag-2*::GFP expression in DTC (arrow). Weak expression is seen in somatic gonadal cells in SPh, perhaps due to low level expression or GFP perdurance. (G) *sys-1(q544)* L3 hermaphrodite, SPh stage. Somatic gonadal cells (arrowheads) are found along gonad perimeter. (H) Same animal as in (G), weak *lag-2*::GFP expression is observed (arrowheads), but no cells show intense expression like wild type (F). Frames (B) and (D) were exposed for 1 s. Frames (F) and (H) were exposed for 0.33 s due to the intense expression in the DTC. Micrographs were taken at 63×.

while Z1.p divides twice. In addition, Z1.a generates a DTC but not an anchor cell, while Z1.p generates an anchor cell, but not a DTC. The Z4 cell lineage is similarly asymmetric in wild type. In contrast, in *sys-1* mutants, the Z1.a and Z4.p divisions are aberrant and can approximate those of Z1.p and Z4.a. In the most extreme cases (2/7), the *sys-1* Z1.a and Z4.p cells divided twice to exactly duplicate the division pattern of Z1.p and Z4.a. In the remaining cases, at least one of the Z1.a or Z4.p daughters divided an extra time (Fig. 3, see legend for details). None of the *sys-1* mutants generated any cell with the morphology or function of a DTC. We conclude that the lineages of Z1 and Z4 are aberrant in *sys-1* mutants and, in the most extreme cases, appear to be rendered symmetrical (Fig. 3, double arrowheads).

sys-1 Hermaphrodites Do Not Express DTC Markers

The lack of morphologically typical DTCs in *sys-1* mutants and the lack of gonadal arms suggested that DTCs are not made. To confirm this conclusion, we used a set of DTC molecular markers. First was *lag-2*::GFP, which assays expression of the signal for germ-line proliferation. In wild-type hermaphrodites and males, *lag-2*::GFP is expressed strongly in Z1 and Z4 and their descendants during L1 (Figs. 4A and 4B; not shown). Similarly, in *sys-1(q544)* mutant hermaphrodites, *lag-2*::GFP was expressed normally in Z1 and Z4 (Figs. 4C and 4D), suggesting that they are correctly specified. This conclusion is further supported by the normal expression of another Z1/Z4 marker, *pes-1*::lacZ (Hope, 1994), in *sys-1* mutants (data not shown). By early L3, intense expression of *lag-2*::GFP is restricted to the DTCs in wild-type hermaphrodites; in addition, faint expression is detectable in the remaining somatic gonadal precursor cells (Figs. 4E and 4F). In L2 or later *sys-1(q544)* mutant hermaphrodites, the intense expression typical of DTCs was not observed (Figs. 4G, 4H, and 5) (n = 80). For the weaker allele, *sys-1(q7),* a fraction of animals showed intense *lag-2* expression in distally located cells during L2, as well as later, consistent with the partially penetrant arm extension observed with this allele (Fig. 5).

To assay expression of genes typical of leader cells, we used two other markers. In wild-type, *unc-5*::lacZ is first expressed during L3 lethargus in hermaphrodite DTCs (Su *et al.,* 2000; Fig. 6A), and *gon-1*::GFP is expressed from L2 through L4 (Blelloch and Kimble, 1999). In *sys-1* hermaphrodites, neither marker is observed (Fig. 6B; not shown). We conclude that no DTCs are generated in hermaphrodites homozygous for the strong loss-of-function *sys-1* allele.

Generation of Extra Anchor Cells in sys-1 Mutants

During examination of larval *sys-1(q544)* mutants by Nomarski and *lag-2*::GFP, we suspected that two anchor cells were present in some animals. For example, two neighboring cells were sometimes observed with the weak

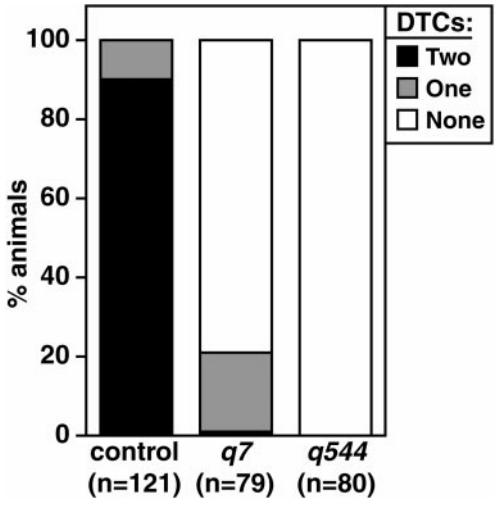

FIG. 5. Expression of *lag-2* reporters in wild-type and *sys-1* mutants. DTC number expressing *lag-2*::lacZ or *lag-2*::GFP was scored in adults: two DTCs, black; one DTC, gray; none, white. Control, wild-type and *sys-1(x)*/+ heterozygotes; *sys* mutants, *q7* and *q544* homozygotes (n = number of animals). Note that *lag-2* was expressed in only one DTC in some control animals. The fraction of mutants expressing *lag-2* reporters correlates well with the fraction of mutants exhibiting some arm extension: 16 and 0% exhibited gonadal arm extension in *sys-1(q7)* and *sys-1(q544)* homozygotes, respectively. Similarly, 21 and 0% exhibited *lag-2* expression in *sys-1(q7)* and *sys-1(q544)* homozygotes, respectively.

GFP expression typical of anchor cells. To further examine this possibility, we used a *lin-3*::lacZ marker, which highlights anchor cells (Hill and Sternberg, 1992). Among *sys-1* mutant hermaphrodite L3s, most (13/22) had two adjacent anchor cells (Fig. 7). Others possessed either one anchor cell as normal (5/22) or three (4/22).

The generation of a third anchor cell at low penetrance suggested the possibility that Z1.a and Z4.p might be capable of generating anchor cells. To test this idea, we laser ablated Z1.p and Z4.a in L1 *sys-1* mutants and scored later animals for vulval development. In all cases (n = 4), a vulva was induced (Fig. 8). Control experiments were done to ensure that Z1.p and Z4.a could also produce an anchor cell in *sys-1* mutants and that the somatic gonad was required for vulva formation (Fig. 8). We conclude that extra anchor cells are generated in *sys-1* mutants and that Z1.a and Z4.p are capable of generating them.

The Somatic Gonadal Primordium Does Not Assemble in sys-1 Hermaphrodites

In wild-type hermaphrodites, the somatic gonadal precursor cells coalesce in the center of the early L3 gonad and assume stereotyped positions in the SPh (Figs. 1, 4F, and 9A). As gonadogenesis proceeds, the central precursors generate the uterus, and flanking precursors generate ante-

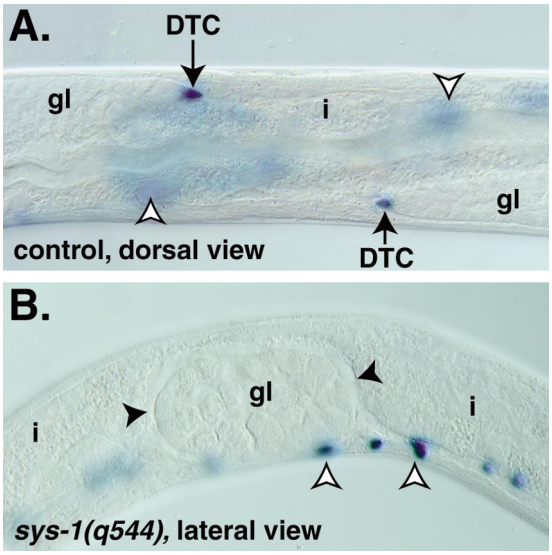

FIG. 6. Expression of *unc-5*::lacZ in wild-type and *sys-1(q544)* gonads. Anterior is left; ventral is down; gl, germ line; i, intestine. In wild type, *unc-5* expression in DTCs begins at L3 lethargus when they turn dorsally and it is maintained during L4 (Su *et al.*, 2000). *unc-5*::lacZ is detected in cells other than the DTCs, including the ventral nerve cord. (A) *sys-1(q544)/+* control at mid-L4 stage, dorsal view. Strong *unc-5*::lacZ expression is observed in both DTCs (arrows). Staining in the ventral nerve cord is out of focus (open arrowheads). (B) *sys-1(q544)* homozygote at mid-L4, lateral view. No *unc-5* expression is observed in the oval-shaped gonadal mass marked by closed arrowheads. Staining in the ventral nerve cord (open arrowheads) serves as a positive control. Micrographs were taken at 63×.

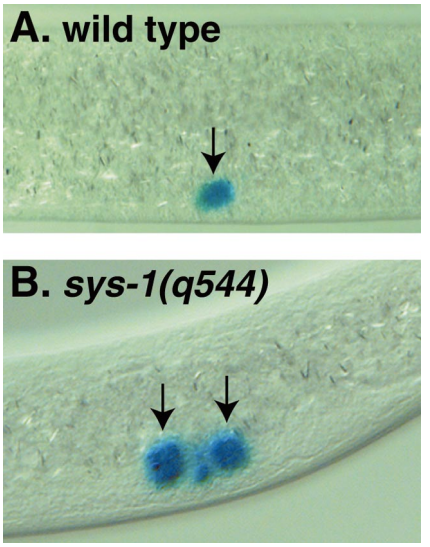

FIG. 7. Extra anchor cell in *sys-1* hermaphrodite. The presence of anchor cells was assayed using *lin-3*::lacZ (Hill and Sternberg, 1992). (A) Wild-type L3 hermaphrodite. One anchor cell is observed (arrow). (B) *sys-1(q544)* L3 hermaphrodite carrying a *lin-3*::lacZ transgene. Two anchor cells can be observed (arrows).

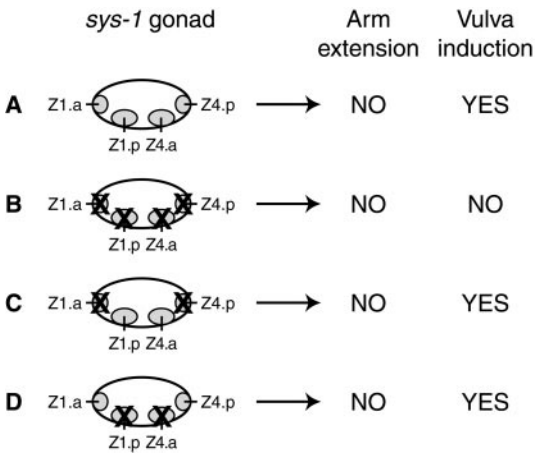

FIG. 8. Z1.a and Z4.p can induce vulva formation in *sys-1* hermaphrodites. Left column, *sys-1* gonad showing cells of developing gonad; cells ablated are marked with an X. Right columns, arm extension and vulval induction reflect presence of functional DTCs and anchor cells, respectively. (A) Unoperated *sys-1(q544)* mutant. (B) Entire somatic gonad ablated in *sys-1(q544)* mutant. The failure in vulval induction indicates that vulval development is gonad-dependent in *sys-1* mutants as in wild-type ($n = 5$). (C) Z1.a and Z4.p ablated in *sys-1(q544)* mutants. Vulval induction indicates that the normal AC precursors can produce an AC ($n = 4$). (D) Z1.p and Z4.a ablated in *sys-1(q544)* mutants. Vulval induction indicates that the remaining somatic gonadal precursor cells, Z1.a and Z4.p, which in wild type cannot produce an anchor cell, can nevertheless produce an anchor cell in *sys-1* mutants ($n = 4$).

rior and posterior spermathecae plus sheaths (Fig. 1). In contrast, in *sys-1* mutants, somatic gonadal cells do not coalesce centrally, but instead assume positions along the periphery of an oblate L3 gonad (Figs. 4H and 9B). In L4, the somatic gonadal cells can still be found along the perimeter of the gonadal mass (not shown). Therefore, the dramatic reorganization of somatic gonadal cells in wild-type gonadogenesis, which forms the SPh and serves to establish the prepattern of the adult hermaphrodite gonad, does not occur in *sys-1* mutants.

We next examined the somatic gonadal precursor cells in *sys-1(q544)* L3 larvae by thin-section electron microscopy. Two *sys-1* animals were examined in transverse sections and two in longitudinal sections. Consistent with the view at the light microscopic level, the somatic gonadal precursors form either a complete ring or a nearly complete ring of cells that surround the germ line (Fig. 10). We also examined the somatic gonadal cells in wild-type L3 hermaphrodite gonads by thin-section EM; these did not show the epithelial ring, but instead had a cluster of somatic gonadal blast cells between two germ-line arms as expected (not shown).

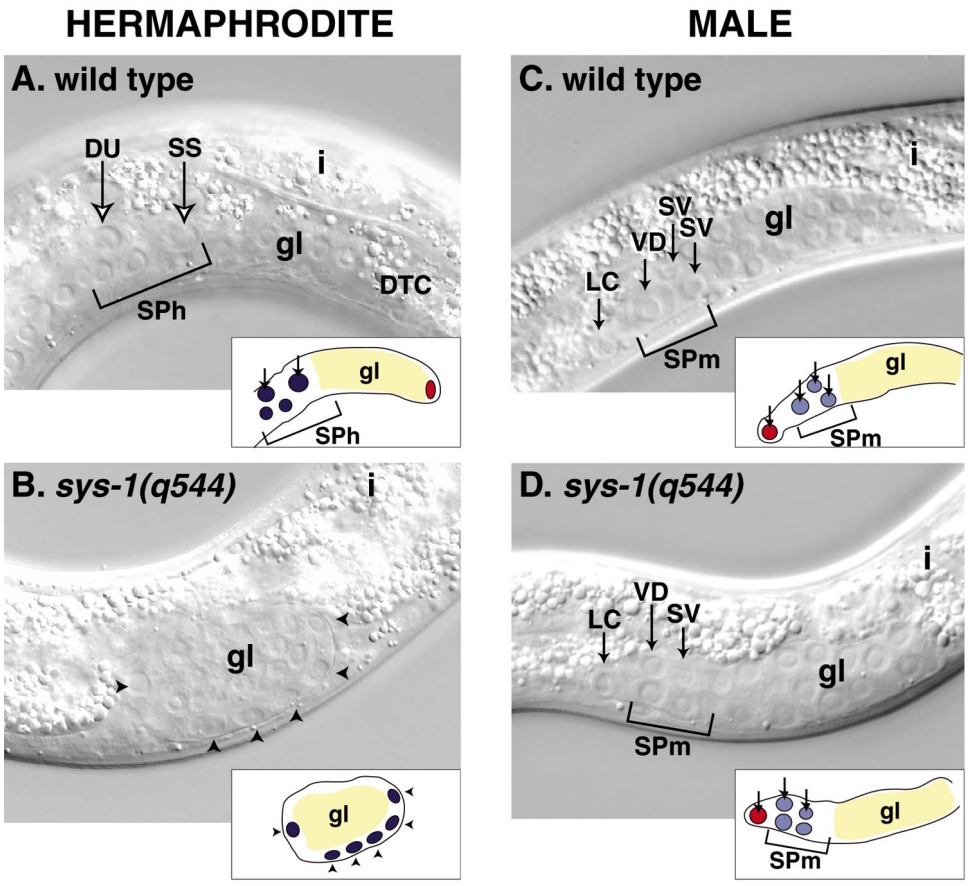

FIG. 9. Somatic gonadal primordia in hermaphrodites and males. Nomarski DIC micrographs, with diagrams inset. Anterior is left; ventral is down; gl, germ line; i, intestine. In diagrams, red, DTC in hermaphrodites and linker cell (LC) in males; blue, other somatic gonadal cells; yellow, germ cells. (A) Wild-type hermaphrodite gonad, early L3. Somatic gonadal cells in SPh are at center of organ (DU, dorsal uterus precursor; SS, sheath/spermathecal precursor); germ-line cells span between SPh and DTC, which is out of the plane of focus. The two smaller cells in the SPh diagram that lack arrows are ventral uterine precursors. Anterior arm not visible. (B) *sys-1(q544)* hermaphrodite gonad, early L3. In sharp contrast to the wild-type gonad, somatic gonadal cells (arrowheads) resemble cuboidal epithelial cells and are positioned along the periphery of the gonad, while germ cells remain in the center. Gonadal arm extension does not occur. (C) Wild-type male gonad, mid-L2. The LC and other somatic gonadal blast cells (VD, vas deferens precursor; SV, seminal vesicle precursor) reside at the anterior end of developing gonad, with germ line and DTCs more posterior. The complete SPm is composed of three VD and four SV precursors; those not shown are out of focus. (D) *sys-1(q544)* male gonad, mid-L2. The SPm is morphologically wild-type. In the SPm, two VD precursors are visible (larger blue cells in inset) and two SV precursors are visible (smaller blue cells in inset). Micrographs were taken at 63×.

Why Does the SPh Not Form in sys-1 Mutants?

Why does the SPh not form in *sys-1* mutants? One simple explanation might have been that this defect is secondary to the lack of arm extension. When gonadal arm extension is blocked in wild-type hermaphrodites by laser ablation of the DTCs, the germ-line cells remain aberrantly in the central gonadal region, and the somatic gonadal primordium cannot form. However, removal of germ cells rescues SPh formation in such animals, indicating that the somatic gonadal cells are capable of SPh assembly when not physically impeded (Blelloch *et al.*, 1999). To ask whether SPh formation is similarly rescued in *sys-1* mutants, we removed the two primordial germ cells, Z2 and Z3, in L1 *sys-1(q544)* mutants by laser microsurgery and scored them for SPh formation as early L3s and for generation of mature gonadal structures in adults ($n = 8$). However, removal of the germ line did not rescue SPh formation in *sys-1* mutants. The somatic gonadal precursors did not assume the positions typical of a normal SPh, and mature somatic gonadal structures were still not observed in L4s or adults. In addition to the morphological defects in these animals, we noted that 16 somatic gonadal cells could be counted at early L3, implying that the same cell division defects occurred in these animals as in unablated *sys-1* mutants.

A second hypothesis to explain the *sys-1* defect in SPh formation might have been that the somatic gonadal cells assumed an epithelial character, which caused them to surround the germ line rather than coalescing as normal. To test this idea, we looked in *sys-1* mutants for the expression of the following epithelial cell markers that are not expressed in wild-type L3 hermaphrodite gonads: JAM-1::GFP (Mohler *et al.,* 1998), *hmp-1*::GFP (Raich *et al.,* 1999), and α-LIN-26 antibodies (Labouesse *et al.,* 1996). However, as in wild type, we did not observe expression of these markers in *sys-1* L3 mutant gonads (data not shown). Furthermore, no specialized junctions typical of epithelial cells were observed between the somatic gonadal cells at the EM level, consistent with the lack of JAM-1::GFP and *hmp-1*::GFP.

sys-1 Hermaphrodites Possess Disorganized Somatic Gonadal Tissues

To ask whether the gonadal precursor cells can generate differentiated somatic gonadal tissues in *sys-1* mutant hermaphrodites, we used Nomarski DIC optics as well as tissue-type-specific markers to score differentiation. Sheath differentiation was assessed using the *lim-7*::GFP marker (Hall *et al.,* 1999). Normally, sheath cells incompletely encase the developing germ line (Figs. 11A–11C). In all *sys-1(q544)* mutants examined, some gonadal cells expressed *lim-7*::GFP, suggesting the presence of sheath cells ($n > 15$, Figs. 11D–11F), but the stained cells were found in disorganized patches rather than as a coherent structure. Spermathecal differentiation was assessed using either of two transgenes, *leIs8* or UL26, as well as staining with the monoclonal antibody MH27. *leIs8* is a *lacZ* reporter expressed in spermathecal and some uterine cells, as well as some nongonadal cells (Hope, 1991). In *sys-1(q7)* mutants carrying *leIs8,* X-Gal staining was observed as disorganized patches in all animals ($n = 51$, data not shown). UL26, a spermathecal marker (Hope, 1991), was similarly expressed in all *sys-1(q544)* mutants ($n = 49$, data not shown). *sys-1(q7)* mutants were double-stained with the monoclonal antibody MH27 (Wood, 1988), which recognizes adherens junctions and highlights the spermatheca, and propidium iodide, which stains nuclei (Orsulic and Peifer, 1997). The *sys-1(q7)* mutants showed two or three patches of spermathecal tissue. Furthermore, whereas wild type had an average of 48 spermathecal cells per animal as expected from the lineage (range from 44 to 52, $n = 15$, data not shown), *sys-1(q7)* mutants possessed 57–64 spermathecal cells per animal ($n = 7$). Finally, we used antibodies to LIN-26 (Labouesse *et al.,* 1996) to demonstrate the presence of uterine tissues in *sys-1(q544)* hermaphrodites (not shown)**.** We conclude that *sys-1* mutants produce differentiated somatic gonadal tissues, but that those tissues are not organized into normal coherent structures.

sys-1 Defects in Nongonadal Tissues

Three defects in nongonadal tissues were observed at a low penetrance in *sys-1* mutants. First, vulval development can be abnormal. Approximately 50% of *sys-1(q544)* hermaphrodites bear a protruding vulva. This phenotype roughly corresponds to the partially penetrant generation of two anchor cells in the somatic gonad that we observed (see above). However, the vulva was often misshapen, even when only one anchor cell could be detected, suggesting defects in vulval morphogenesis. Such defects might reflect a problem in the vulval hypodermis itself or with the connection between gonad and hypodermis. Second, some embryonic lethality is observed among progeny of *sys-1(q544)/+* mothers (~6%). The dead embryos arrest rather late in embryogenesis and major tissue types appear to be made. Third, *sys-1* adults, but not *sys-1* larvae, can appear uncoordinated, an effect observed in many gonadogenesis mutants (e.g., Blelloch *et al.,* 1999) and likely to result from the large abnormal mass of gonadal tissues in the animal.

The sys-1 Male Phenotype

The difference in the morphologies of *sys-1* hermaphrodite and male gonads is striking (Fig. 2). To examine this difference in more detail, we examined specific aspects of male gonadogenesis in *sys-1* mutants. The cell lineage of Z1 and Z4 in *sys-1(q544)* males was virtually identical to that of wild-type during L1 and L2. Specifically, the timing and number of cell divisions was essentially normal, and formation of the male somatic gonadal primordium at the proximal end of the gonad by early L2 was indistinguishable from that of wild type ($n = 5$) (Figs. 7C and 7D). We further examined a set of late L2 *sys-1(q544)* males (without prior lineaging) and found all to have a normal SPm ($n = 19$). Furthermore, the regulatory cells typical of the male, linker cell and DTCs, were generated and functional. Therefore, *sys-1* activity is not essential for generation of these regulatory cells in the male.

Although early gonadogenesis was largely wild type in *sys-1* males, three lineage defects were observed in at least some of these animals. First, Z1.p divided in an anterior dorsal/posterior ventral cleavage plane instead of the anterior ventral/posterior dorsal plane typical of wild type. Interestingly, Z1.pp moved dorsally to correct this abnormality. Second, the distally located Z4.p, which never divides in wild type, divided aberrantly in 2 of 4 *sys-1(q544)* males examined through late L2; these divisions produced extra tiny cells toward the distal end (data not shown). An examination of 19 *sys-1* late L2 males without lineaging revealed 8 (42%) with a variable number of extra tiny cells near the distal tip. In *lag-2*::GFP-bearing animals, some but not all of these distally located extra cells expressed GFP. Therefore, Z1.a and Z4.p (the male DTCs) are capable of undergoing extra divisions late in L2 in a fraction of *sys-1* males. Third, in the L4 stage, some *sys-1* males possessed one or more additional cells with intense *lag-2*::GFP (50%, $n = 20$). These extra cells appeared to be additional linker cells that were generated after L3. Only a single linker cell was found at the L3 stage in *sys-1* mutant males, which is

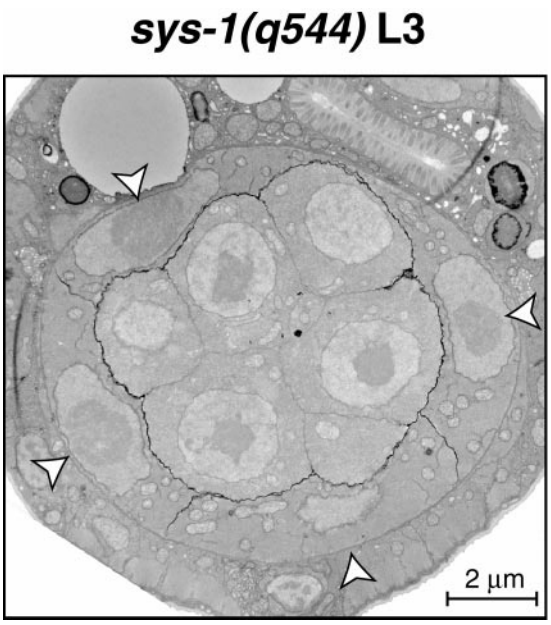

FIG. 10. Somatic gonadal epithelium in a *sys-1* L3 gonad. Transverse section, transmission electron microscopy. Four somatic gonadal cells (open arrowheads) enclose the centrally located germ cells.

the same as wild type (not shown). Finally we tested the ability of *sys-1* homozygous males to mate and produce cross-progeny and found them capable, albeit at a much reduced efficiency (see Materials and Methods). We conclude that *sys-1* plays a role in male gonadogenesis, but that the penetrance of mutant defects is significantly lower and the defects later and less severe in males than in hermaphrodites.

DISCUSSION

The Role of sys-1 in Development of the Hermaphrodite Gonad

In this paper, we demonstrate that the *sys-1* gene is required for early morphogenesis of the hermaphrodite gonad. The cell lineages of the somatic gonadal progenitor cells, Z1 and Z4, are defective in *sys-1* mutant hermaphrodites with extra divisions, extra anchor cells, and a lack of DTCs. One attractive interpretation is that Z1.a and Z4.p have been transformed into their sisters, Z1.p and Z4.a. Consistent with this possibility, Z1.a and Z4.p have lost the ability to generate a DTC and acquired the capacity to generate an anchor cell. An alternate interpretation is that the Z1/Z4 lineage has been transformed into a simpler, perhaps ancestral, pattern of divisions that at one time generated a simple gonadal epithelium.

The *sys-1* mutant somatic gonadal blast cells fail to coalesce into an SPh at the L3 stage. Instead, they encase the germ-line tissue in a single-layered shell. One simple explanation for this defect might have been that the centrally localized germ-line cells serve as an obstacle to SPh formation. This explanation was true for *gon-1* mutants (Blelloch *et al.*, 1999), but is not the case for *sys-1* mutants—when the germ line was removed in early L1 *sys-1* hermaphrodites, the SPh still failed to assemble. Many other explanations are possible. For example, perhaps the DTC controls SPh formation in addition to its other regulatory roles. If true, the failure to make an SPh might be a secondary consequence of the inability to generate a DTC. Alternatively, the cells generated in the mutant Z1/Z4 lineage might impose some new program on the gonad or fail to acquire the necessary adhesive and migratory properties essential for SPh formation.

The Role of sys-1 in Development of the Male Gonad

The *sys-1* mutant males exhibit partially penetrant and relatively late defects in gonadogenesis. In L1 and early L2 *sys-1* males, the Z1/Z4 cell lineage is virtually indistinguishable from wild type, and the male-specific somatic gonadal primordium forms normally. Lineage alterations are observed only later and only in some males. Indeed, some *sys-1* males can produce cross-progeny, whereas *sys-1* hermaphrodites are all sterile.

One male gonadal lineage defect is reminiscent of a hermaphrodite lineage defect: Z1.a and Z4.p can undergo extra divisions in males as in hermaphrodites. However, the details of these extra divisions are distinct in the two sexes. Thus, whereas Z1.a and Z4.p always undergo at least some extra divisions in *sys-1* hermaphrodites, they occur in only about half of the *sys-1* males. Furthermore, whereas the extra divisions in hermaphrodites mimic Z1.p/Z4.a divisions with respect to size and timing, this is not the case of males: the extra divisions occur later in males and generate a variable number of tiny cells. The male Z1/Z4 lineages therefore remain strikingly asymmetric. Nonetheless, the similarity between this hermaphrodite and male defect suggests that *sys-1* is normally required in both hermaphrodites and males to suppress cell divisions in Z1.a and Z4.p.

What do we think about the partial penetrance of the *sys-1* male defects? One explanation might be that *sys-1(q544)* is not a null allele. Until *sys-1* is cloned, this question remains open, although genetic arguments indicate that *sys-1(q544)* is a strong loss-of-function allele and putative null. For other genes, examples abound of mutations that are molecular null, but that nonetheless have variable and/or partially penetrant defects. For example, *lin-12(0)* has a partially penetrant Lag phenotype, which is enhanced by removal of *glp-1* activity (Lambie and Kimble, 1991). We suggest that *sys-1* activity is indeed less critical for male gonadogenesis than for hermaphrodite gonadogen-

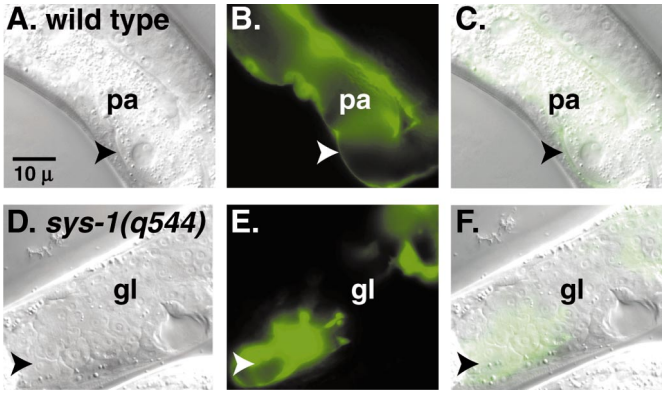

FIG. 11. Differentiated sheath cells in wild-type and *sys-1(q544)* gonads. (A) Wild-type adult hermaphrodite gonad, DIC microscopy. The proximal arm (pa) contains undifferentiated germ cells and oocytes and is encased by a somatic gonadal sheath (arrowhead). Scale marker same for all images. (B) Same animal as in (A) expressing the sheath marker *lim-7*::GFP. (C) Overlay of (B) on (A). (D) *sys-1(q544)* L4 hermaphrodite gonad, DIC microscopy. The germ-line (gl) cells are central, and sheath cells are clustered in patches at the periphery (arrowhead). (E) Same animal in (D) expressing *lim-7*::GFP. (F) Overlay of (E) on (D).

esis, perhaps because its function is covered in males, at least in part, by a different gene.

sys-1 and Sexual Dimorphism

The *sys-1* gene has mutant defects in both sexes, suggesting that it is part of a regulatory program common to both hermaphrodite and male gonadogenesis. The existence of such a common program of gonadogenesis is well established. For example, *gon-1* is essential for gonadal shape in both sexes (Blelloch *et al.*, 1999), and *gon-2* and *gon-4* are essential for gonadal divisions in both sexes (Friedman *et al.*, 2000; Sun and Lambie, 1997). Therefore, development of these two sexual organs relies on at least some of the same genes.

The effects of *sys-1* mutants on gonadogenesis are drastically different in the two sexes: *sys-1* appears to be essential for hermaphrodite gonadogenesis, but to play only a minor role in male gonadogenesis. Based on this clear phenotypic difference, we suggest that the function of *sys-1* has evolved from a role common to both sexes to a role that has become tailored to hermaphrodite gonadogenesis. As such, *sys-1* is likely to provide a genetic entrée into the general problem of how the four-celled gonadal primordium, which is morphologically indistinguishable in hermaphrodites and males, is regulated to generate a symmetrical hermaphrodite gonad in XX animals and an asymmetrical male gonad in XO animals.

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