Genetic and Molecular Analysis of *spe-27*, a Gene Required for Spermiogenesis in *Caenorhabditis elegans* Hermaphrodites

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ABSTRACT

Hermaphrodites with mutations in the *spe-27* gene are self-sterile, laying only unfertilized eggs; mutant males are fertile. Hermaphrodites make spermatids that fail to activate to crawling spermatozoa so passing oocytes sweep them out of the spermatheca. These spermatids do activate and produce self-progeny if young mutant hermaphrodites are mated by fertile (or sterile) males. Spermatids isolated from either mutant males or hermaphrodites initiate activation *in vitro* when treated with proteases, but then arrest with spiky membrane projections that resemble those of a normal intermediate in pseudopod formation. These phenotypes are identical to *spe-8* and *spe-12* mutants. They can be explained if males and hermaphrodites have distinct pathways for spermatid activation, and these three genes are necessary only for the hermaphrodite pathway. Consistent with this model, when *spe-27* mutant male spermatids without seminal fluid are artificially inseminated into hermaphrodites, they fail to activate. The *spe-27* gene has been isolated, sequenced and its regulatory regions identified. The sequence predicts a 131 amino acid polypeptide that has no striking structural motifs and no resemblance to known proteins. Two of the mutations in *spe-27* alter mRNA splicing; a third mutation is a temperature-sensitive missense mutation.

MOST cells require new synthesis of specialized gene products to undergo and sustain their differentiation (*e.g.*, SHAPIRO 1985; SIMONS and FULLER 1985; FINNEY *et al.* 1987; KRAFT *et al.* 1989; SIMONS and WANDINGER-NESS 1990; BLAU 1992; CHANDRASEKHAR *et al.* 1993). However, some cells, such as platelets, undergo rapid differentiation and change their morphology in the absence of macromolecular synthesis (DERENLEAU 1987; NACHMIAS *et al.* 1987). In *Caenorhabditis elegans,* the differentiation of spherical, nonmotile spermatids into asymmetric crawling spermatozoa, the process called spermiogenesis, takes place in the absence of transcription and translation (WARD *et al.* 1983). Thus *C. elegans* spermiogenesis provides an opportunity to use genetics for studying cellular morphogenesis resulting from modification and rearrangement of preexisting cellular components.

The overall process of sperm development, spermatogenesis, has two phases, meiosis and spermiogenesis, which have been described in detail (WOLF *et al.* 1978; WARD *et al.* 1981; ROBERTS *et al.* 1986). At the completion of meiosis in *C. elegans,* haploid spermatids bud from a central residual body. This residual body serves as a repository for cellular components not needed by the sperm, including ribosomes, endoplasmic reticulum, Golgi apparatus, and most of the actin and tubulin. Sperm-specific components are segregated to the spermatid by a specialized transient organelle, the fibrous body-membranous organelle complex. Spermiogenesis is initiated by extracellular signals that activate the nonmotile, spherical spermatids to form bipolar spermatozoa with pseudopods, which crawl by membrane flow. Unlike most crawling cells, the morphology and movement of spermatozoa are not determined by an actin-based cytoskeleton (NELSON *et al.* 1982). *C. elegans* sperm contain little actin and myosin; instead, narrow filaments are polymerized from the major sperm protein (MSP), the most abundant protein in nematode sperm (WARD and KLASS 1982; ROBERTS *et al.* 1989; ROBERTS and STEWART 1995).

Hermaphrodite spermatids activate to become crawling spermatozoa in the spermatheca. Male spermatids accumulate in virgin males and then activate in the uterus of the hermaphrodite where they are deposited together with seminal fluid during mating. Although *in vivo* activators have not been identified, chemicals that trigger spermiogenesis *in vitro* have been found. These activators include the proton ionophore monensin and the weak base triethanolamine (TEA), which act by increasing the intracellular pH of spermatids (NELSON and WARD 1980; WARD *et al.* 1983). Pronase, a mixture of proteases, activates spermatids without altering the intracellular pH (WARD *et al.* 1983). In addition, drugs that inhibit calmodulin in mammals (*e.g.*, trifluoperazine) also initiate spermiogenesis but then inhibit the motility of activated spermatozoa unless washed out; it is unlikely that these drugs act on calmodulin in this system (SHAKES and WARD 1989).

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In this paper, we present a genetic, phenotypic, and molecular analysis of a new gene, *spe-27*, which is necessary for spermiogenesis in *C. elegans* hermaphrodites but not in males.

MATERIALS AND METHODS

Genetics and strains: *C. elegans* var. Bristol, strain N2, was used as the wild type. The strains were maintained at appropriate temperatures on *Escherichia coli* OP50-seeded plates, and genetic manipulations were performed as described (BRENNER 1974).

The *spe-27* gene is defined by three alleles: *it110*, *it132ts* and *hc161*. The first two *spe-27* alleles, *it110* and *it132ts*, were identified among a number of uncharacterized chromosome *IV spe* mutations isolated after EMS mutagenesis (kindly provided to us by DIANE SHAKES). We obtained the third allele, *spe-27(hc161)*, by a screen of 3,480 EMS mutagenized male chromosomes for mutations that failed to complement *spe-27(it110)*. Homozygous *spe-27(it110)* or *spe-27(hc161)* mutant hermaphrodites are sterile at 16, 20, and 25°, laying <0.5 progeny per hermaphrodite; *spe-27(it132ts)* hermaphrodites are completely sterile at 20 or 25° but are weakly fertile at 16°, producing 38 ± 15 (mean ± SD) progeny per worm (a wild-type hermaphrodite produces ~280 progeny). The males of all three mutant alleles have nearly normal fertility at all temperatures. For example, when plates of four males were mated with four hermaphrodites for 12 hr, N2 males yielded a mean of 321 ± 46 (mean ± SE) outcross progeny ($N = 7$); whereas *spe-27(it110)* males yielded a mean of 244 ± 27 outcross progeny ($N = 10$).

We mapped *spe-27(it110)* using chromosome *IV* deficiencies and two factor mapping with the markers *unc-5* and *unc-24*. We analyzed four complete broods from *spe-27(it110) unc-24(e138)/++* worms. Seven out of 345 Unc animals were fertile, and seven out of 986 non-Unc worms were sterile. For *spe-27(it110) unc-5(e53)/++*, we screened all the Unc worms from eight broods. Six out of 539 Unc animals were fertile. This places *spe-27* 1.1 map unit from *unc-24* and 0.5 map unit from *unc-5*, which is a 0.3 map unit region between *skn-1* and *unc-44*, where no deficiencies have been isolated. Consistent with this location, *spe-27(it110)* complements four deficiencies surrounding this region of the genetic map: *eDf18(IV)*, *eDf19(IV)*, *mDf9(IV)* and *mDf4(IV)*. No further genetic mapping was done since *spe-27* was located on the physical map (see RESULTS).

Light microscopy: Sperm development in males and hermaphrodites was analyzed as previously described (NELSON and WARD 1980; SHAKES and WARD 1989) by dissecting the male testis or hermaphrodite spermatheca in sperm medium (SM), pH 7.8 with or without the *in vitro* sperm activators pronase (200 μg/ml) or triethanolamine (60 mM at pH 7.8). For most studies, adult virgin males were used as a source of spermatids, but the phenotypes of hermaphrodite sperm were also examined. For examination of cell nuclei, preparations were fixed and stained with 1 μg/ml of the DNA binding dye 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO).

Electron microscopy: Adult virgin males were dissected in SM, and the testes were transferred to 1% formaldehyde, 1% glutaraldehyde in SM in a depression slide. The testes were fixed in a moist chamber for 60 min at room temperature and then embedded in 1% agar. Small agar blocks containing the tissue were then incubated in the fixative overnight at 4°. The blocks were washed in 10 mg/ml lysine for 20 min, rinsed in SM, then postfixed in 1% OsO_4 in SM. Excess OsO_4 was rinsed out with water, and the blocks were stained in 0.5% aqueous uranyl acetate. The blocks were then dehydrated in a graded series of ethanol and embedded in EPON/Araldite resin for sectioning. Thin sections were examined with a Philips 420 electron microscope operating at 80 kV.

DNA transformation: Young adult *spe-27* mutant hermaphrodites were microinjected with cosmids, plasmids, restriction digests of plasmids, or purified DNA fragments according to standard methods (FIRE 1986; MELLO *et al.* 1991). DNA with the dominant allele, *rol-6(su1006)*, which causes worms to roll, was used as a marker in all transformation experiments. After recovery, injected worms were mated with *spe-27* mutant males to obtain progeny, and virgin F_1 roller hermaphrodites were picked at the L4 stage and scored for fertility. Stable transformed lines were subsequently obtained from the fertile worms.

Molecular analysis: DNA and RNA were isolated from worms following published methods (WOOD 1988; AUSUBEL *et al.* 1994). Differential northern analysis was done using poly (A)+ RNA isolated from strains that either make sperm or do not. Males were purified from *him-5(e1490)*; hermaphrodites that make no sperm were *fem-1(hc17ts)* raised at restrictive temperature (NELSON *et al.* 1978; DONIACH and HODGKIN 1984); hermaphrodites that make only sperm were *fem-3(q23ts)* raised at restrictive temperature where the gain-of-function mutation causes continuous spermatogenesis in adults (BARTON *et al.* 1987). cDNA clones were isolated from Lambda Zap and Unizap libraries prepared from poly (A)+ RNA of male enriched (95% males) adult worms by Stratagene (La Jolla, CA). Genomic and cDNA inserts cloned into a pBluescript (Stratagene) vector were used for dideoxy sequencing. All sequences were read from both strands using mostly standard vector primers, but some internal gene primers were also used. Exonuclease III was used to generate a deletion series for sequencing (AUSUBEL *et al.* 1994) and for transformation analyses to determine the gene boundaries. Specific mutations in *spe-27* alleles were identified by cycle sequencing of amplified DNA generated by polymerase chain reaction (PCR) following the protocol provided in the Cyclist Exo-*Pfu* polymerase DNA sequencing kit (Stratagene). Either isolated genomic DNA or DNA from single worms (WILLIAMS *et al.* 1992) was used as templates for PCR amplification of the *spe-27* gene. Reverse transcription followed by PCR (RT-PCR) was performed to analyze the *spe-27* mRNA products in *spe-27(it110)* and *spe-27(hc161)* mutant worms. The PCR-amplified products were cycle-sequenced as described above.

***In situ* localization of mRNA:** *In situ* localization of the *spe-27* transcript was performed following the protocol of EVANS *et al.* (1994). Briefly, young adult hermaphrodites or young adult males were dissected on a polylysine-coated slide and processed as described. Antisense RNA probes labeled with 11-digoxigenin-dUTP were generated from a *spe-27* cDNA and hybridized to the tissue at 55° for 36 hr. Excess probe was washed away, the tissue incubated with anti-digoxigenin antibody coupled to alkaline phosphatase, excess antibody removed, and the color developed (Genius System, Boehringer Mannheim, Indianapolis, IN).

Artificial insemination: Inseminations were performed as described by LAMUNYON and WARD (1994). Spermatids were drawn into needles from 2-day-old wild-type and *spe-27* males, some were washed in the needles by sedimentation through SM containing 1% BSA, and then injected through the vulva into the uterus of anesthetized young adult *spe-27* hermaphrodites.

RESULTS

Phenotype of *spe-27* mutants: The isolation of three strains with mutations in the *spe-27* gene is described in MATERIALS AND METHODS. All three mutations cause

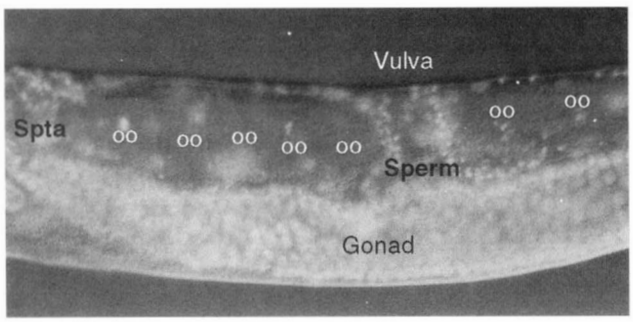

FIGURE 1.—Sperm sweep. The uterus of a young hermaphrodite is shown after fixation. Nuclei are stained with DAPI and visualized by epifluorescence while the cells are visualized by transillumination with Nomarski optics. Sperm can be seen as compact DAPI stained dots among the oocytes in the uterus near the vulva where they have been swept from the spermatheca (Spta) by the passing oocytes (oo). Magnification = ×250.

the same phenotype: mutant hermaphrodites are sterile and lay only unfertilized eggs, whereas mutant males are fertile. Mutant hermaphrodites accumulate spermatids in their spermathecae, but in hundreds of mutant worms examined, these spermatids never activated to form spermatozoa. Without pseudopods, the mutant spermatids cannot adhere to the walls of the spermatheca or return to the spermatheca when displaced. Thus they are swept out of the spermatheca and expelled through the vulva by mature oocytes passing down the gonad (Figure 1). In contrast, *spe-27* mutant male spermatids activate normally upon mating, and the males are fertile (see MATERIALS AND METHODS).

Spermatids of both males and hermaphrodites mutant for *spe-27* appear normal by light microscopy (Figure 2, A, D, G, and J). Similarly, mutant male spermatids appear normal by electron microscopy (data not shown). We tested the ability of *spe-27* mutant hermaphrodite and male spermatids to activate in media containing either pronase or TEA, two *in vitro* activators. In the presence of the weak base TEA, mutant spermatids from either sex, and from any of the three mutant alleles, activated to form normal-looking motile spermatozoa (Figure 2, F, I, and L). In contrast, when exposed to pronase, spermatids from both mutant hermaphrodites and males initiated activation but failed to form pseudopods. Instead, the spermatids formed long, rigid spikes (Figure 2, E, H, and K). These spikes resemble a spiky intermediate seen during TEA activation of wild-type spermatids that is presumed to be a normal intermediate during spermiogenesis since TEA-activated spermatids form viable spermatozoa (SHAKES and WARD 1989; LAMUNYON and WARD 1994). In *spe-27* mutant spermatids, however, these spikes were motionless and did not undergo the rapid collapse and reorganization observed in wild-type spermatids. The observation that both male and hermaphrodite spermatids from *spe-27* mutants activate aberrantly when treated with pronase indicates that mutations in *spe-27* cause a defect in the spermatids themselves and that the male spermatids are not normal, even though they are capable of forming viable spermatozoa.

We used mutants with the temperature-sensitive mutation *spe-27(it132ts)* to see when the phenotype was temperature sensitive. Initial temperature shift experiments showed that the temperature-sensitive period was in the L4 period during spermatogenesis (data not shown), like other sperm defective mutants (ARGON and WARD 1980; L'HERNAULT *et al.* 1988). To see if the spermatids themselves were temperature sensitive after they had formed, mutant hermaphrodites were shifted from 16° (permissive) to 25° (restrictive) and *vice versa* before and after spermatogenesis, which occurs at the time of the L4/adult molt. Hermaphrodites shifted from 25 to 16° just before the L4/adult molt yielded 42 ± 18 ($N = 6$) progeny, whereas the reciprocal shift yielded no progeny ($N = 3$); continuous growth of *spe-27(it132ts)* at 16° results in broods of 38 ± 15 (see MATERIALS AND METHODS). Just after the L4/adult molt, when spermatogenesis has been completed, shifting from 25 to 16° yielded 16 ± 9 ($N = 5$) progeny. The reciprocal shift just after the molt yielded no progeny ($N = 5$), but when shifted 19 hr later, 18 ± 2 progeny ($N = 4$) were obtained. These results suggest that hermaphrodite *spe-27(it132ts)* mutant spermatids are themselves temperature sensitive since their competence to form functional spermatozoa can be altered by temperature shifts after they have been formed.

The phenotypes of *spe-27* mutants are identical to those of mutants in two previously described genes: *spe-8* and *spe-12* (L'HERNAULT *et al.* 1988; SHAKES and WARD 1989). It is likely that the products of all of these genes participate in a pathway that initiates spermiogenesis in the hermaphrodite. If so, mutant gene products might interact, exhibiting extragenic noncomplementation. To look for such interactions, we tested all of the mutations for complementation by constructing pair-wise *trans*-heterozygotes. The brood sizes of these heterozygous hermaphrodites were identical to wild type (data not shown), so there is no indication of lack of complementation between any of these genes. We also looked for interactions between these genes by transforming the cloned *spe-27* gene (see below) into *spe-12* and *spe-8* mutant strains, using the dominant *rol-6* gene DNA as a cotransformation marker. Multiple copies of the cloned *spe-27* gene failed to restore fertility to either *spe-12* or *spe-8* mutants in over 50 roller transformants of each genotype. In controls with *spe-27* hermaphrodites, 16 of 57 roller transformants were fertile.

SHAKES and WARD (1989) presented a model for spermatid activation based on the phenotypes of *spe-8* and *spe-12* mutants. The model proposes that there are two pathways for activation, male and hermaphrodite. Spermatids from males and hermaphrodites have the components of both pathways, but each sex ordinarily uses its own. The *spe-27, spe-8,* and *spe-12* products are postu-

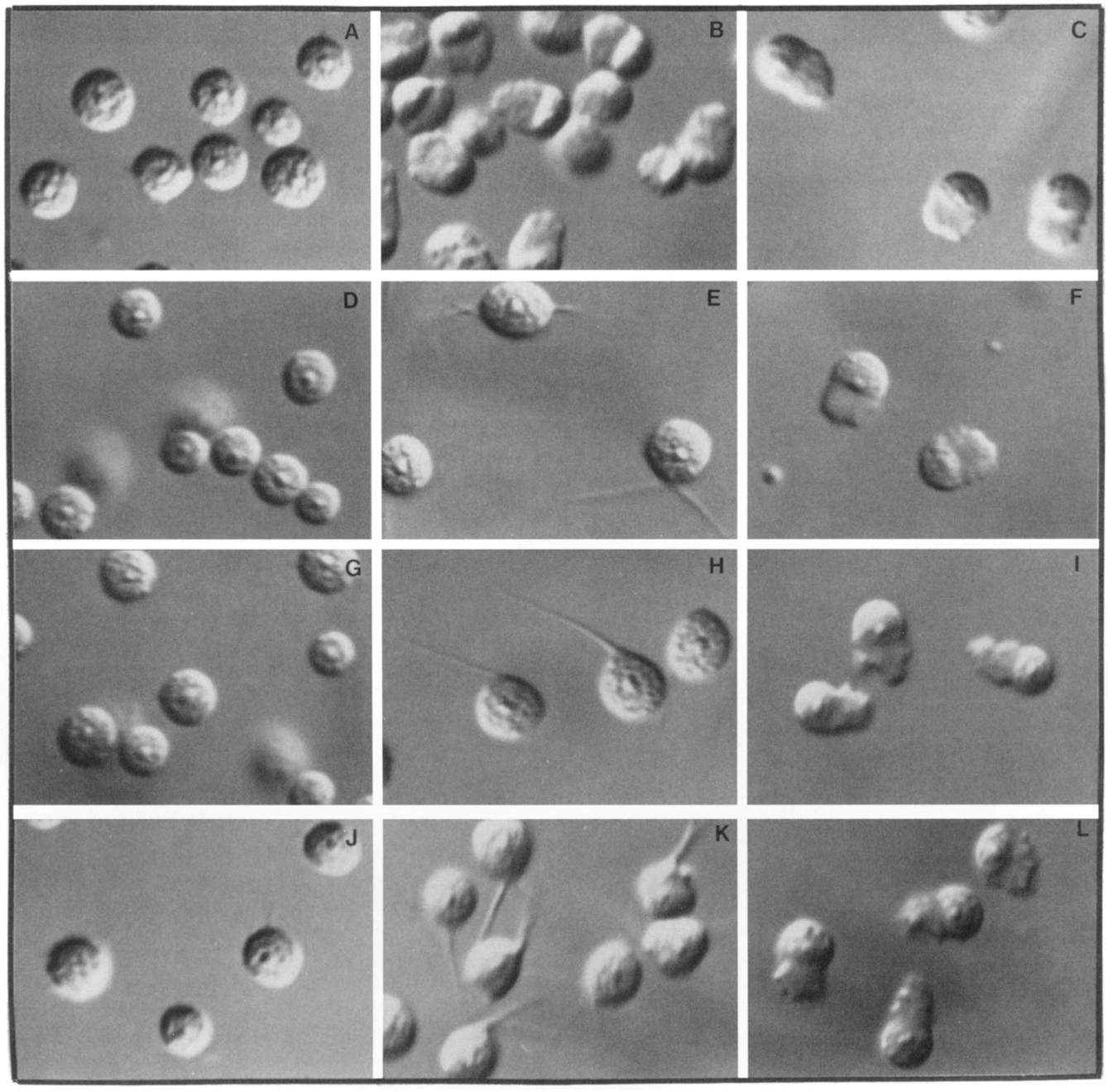

FIGURE 2.—Wild-type and *spe-27* mutant male spermatids activated in pronase and TEA. Wild-type spermatids (A) form pseudopods in the presence of pronase (B) and TEA (C). Spermatids from *spe-27* mutant alleles *it110* (D–F), *hc161* (G–I) and *it132ts* (J–L) arrest as spiky intermediates when exposed to pronase (E, H and K, respectively), but they activate normally in TEA (F, I and L, respectively). Magnification = ×1650.

lated to be components of the pathway used to activate hermaphrodite spermatids. One prediction from this model is that spermatids from *spe-27* mutant hermaphrodites should activate by the male pathway. This was observed in *spe-8* and *spe-12* mutants; mating with fertile or sterile males activated the hermaphrodites' own spermatids to produce self-progeny (SHAKES and WARD 1989, Figure 6). To see if this were true for *spe-27* mutants, *spe-27(it110)unc-5(e53)* mutant hermaphrodites were mated with either *spe-27* or *fer-1* mutant males; *fer-1* males are sterile but transfer seminal fluid and defective sperm during mating. The *unc-5* marker allows self-progeny, which are Unc, to be distinguished from outcross progeny. The *fer-1* and *spe-27* mutant males stimulated the production of 18 ± 11 ($N = 19$) and 23 ± 15 ($N = 15$) self-progeny, respectively. No self-progeny were produced from unmated controls. This shows that *spe-27* mutant hermaphrodite sperm can be activated by the male pathway.

A second prediction of this model is that *spe-27* mutant male spermatids, which become spermatozoa upon mating when they are transferred to hermaphrodites

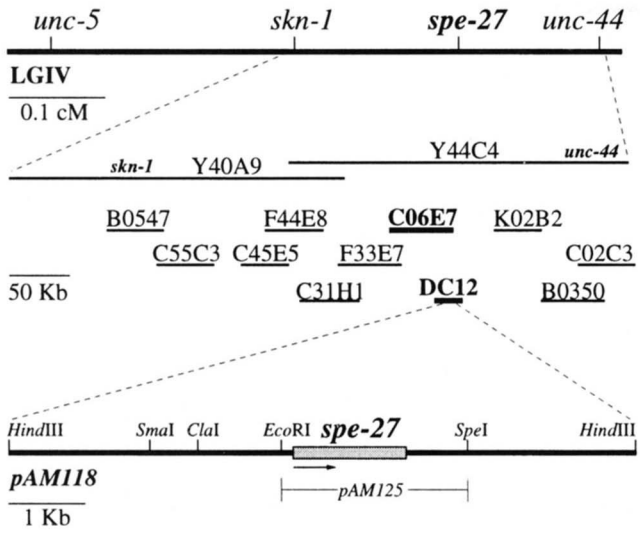

FIGURE 3.—Genetic and physical maps of the *skn-1 unc-44* region of chromosome *IV*. Cosmids *C06E7* and *DC12* rescue the *spe-27* mutant phenotype when microinjected into mutant worms. *pAM118* is an 8.2-kb *Hin*dIII subclone from *DC12* that contains the *spe-27* gene. Further subcloning localized *spe-27* to a 2.5-kb *Eco*RI-*Spe*I fragment, *pAM125*. Restriction enzyme sites are shown above the subclone.

together with male seminal fluid, should not activate when exposed only to the hermaphrodite activator. We tested this prediction using the recently developed method of artificial insemination by microinjection of sperm (LAMUNYON and WARD 1994). Spermatids were drawn into a microneedle from *spe-27(it110)* males or from wild-type males as a control. Although spermatids were taken from above the vas deferens and should not have seminal fluid, most samples were washed in the needles by sedimentation through SM plus 1% BSA to ensure removal of male activator. The washed spermatids were then microinjected into the uterus of *spe-27(it-110)dpy-20(e1282)* young adult hermaphrodites. None of 18 hermaphrodites injected with washed or unwashed *spe-27(it110)* spermatids produced any outcross or self-progeny. In controls done in parallel, three out of four hermaphrodites inseminated with wild-type spermatids produced outcross progeny (1, 19, and 20 progeny, respectively) but no self-progeny; in many other artificially inseminated hermaphrodites, outcross progeny are produced (LAMUNYON and WARD 1994). Thus, *spe-27* male spermatids fail to activate in the hermaphrodite when transferred by microinjection in the absence of male seminal fluid, consistent with a defect in the hermaphrodite activation pathway.

Cloning and sequencing *spe-27*: We isolated the *spe-27* gene to learn more about the function of its product. Genetic mapping located *spe-27* on chromosome *IV* between *skn-1* and *unc-44*. These two genes have been cloned, and they define an ~400-kb region on the physical map (Figure 3). We assayed cosmid clones from the *skn-1 unc-44* region for their ability to rescue the sterile phenotype of *spe-27* mutant hermaphrodites by microinjection transformation rescue, coinjecting the dominant mutant *rol-6* gene DNA as a marker. Only two overlapping cosmids, C06E7 and DC12, restored fertility. We subcloned several restriction fragments from DC12 and tested them for their capacity to rescue the sterility of *spe-27* hermaphrodites. A 2.5-kb *Eco*RI-*Spe*I fragment was the smallest restriction fragment able

TABLE 1

DNA transformations of *spe-27* worms

Plasmid	Description	DNA concentration	No. of *spe-27* worms injected	No. of roller hermaphrodites (transformants)	No. of fertile roller hermaphrodites	No. of F_2 progeny per fertile hermaphrodite	No. of F_2 dead embryos per fertile hermaphrodite
		(μg/ml)					
	wild type					278 ± 15 (14)	0
pAM125	2.5 kbp *spe-27*	200	25	40	9	233 ± 78 (4)	≤1
pAM131	129 bp intron insert	150	15	121	43	207 ± 27 (4)	ND
pAM132	552 bp intron insert	150	10	81	33	260 ± 14 (4)	ND
pAM125-8	3′Δ(+5)	150	22	23	5	190 ± 36 (5)	ND
pAM125-9	3′Δ(−347)	100	22	32	0	—	—
pAM125-10	3′Δ(−404)	100	25	37	0	—	—
pAM135	5′Δ(−112)	20	5	45	11	270 ± 28 (6)	4 ± 3.0
pAM134	5′Δ(−74)	150	5	4	1	ND	ND
pAM134	5′Δ(−74)	50	15	10	1	ND	ND
pAM134	5′Δ(−74)	20	10	81	5	109 ± 37 (6)	35 ± 8.6
pAM133	5′Δ(−27)	150	20	5	0	—	—
pAM133	5′Δ(−27)	100	20	1	0	—	—
pAM133	5′Δ(−27)	50	10	26	0	—	—
pAM133	5′Δ(−27)	20	15	82	0	—	—

Deletions are described by the nucleotides that remain; deletions from the 3′ end are indicated as + from the first nucleotide of the polyA addition; deletions from the 5′ end are indicated as − from the ATG start codon. Progeny and embryo numbers are shown ± SD with number of hemaphrodites in parentheses. ND, not determined.

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1     gaattcctgattcgctgtgaatttctttctgccagaagctctttccgatcagatgaagcc
61    attttcgaagaagccaatcgaattgcaaccgatttgagacaataattaataattttaatt
121   ttaatttttgcataacgtaaacttttctgtttcacgtgggacatgaattaacaacatttaa
181   agtttgttattactactttttatttCAATTATCGCAATGAATAAATCACTGATCTTTTTAT
                              cDNA START M  N  K  S  L  I  F  L  L

241   TATCATTTGCATATTCATGTTACTCGACAAAAACTGAAAAgtaagttgtattgtcttaca
        S  F  A  Y  S  C  Y  S  T  K  T  E  N

301   ttttggttgagaatttatcaaaacatatataatttttttcagCAATTTTGACATAAACGAC
                                                 N  F  D  I  N  D

361   GTGGAGAATAAAGCGTGCCAATATGGATGTGGGTTTGAAAATGTCAGAAGAACTTCGTTT
      V  E  N  K  A  C  Q  Y  G  C  G  F  E  N  V  R  R  T  S  F
         A(E→K)
         it132ts

421   AGAAAgtaagtagatttttttttttgaagaatttgaaagaaaattagttgtactcaagaat
      R  K

481   aatggttcgaattgattttaaatacgtatgtaggtaacatttttttttaatttttaaaaaaa
541   tgtactgtttcactattataatttaaaactaaggttttcaaatatacatttatgagtcct
601   atttaatcgaatgttagctgatttttttgtttactatttaattttcacataatttttcaaac
661   agaatcagaatgaatcggtaaacgtttataaatcttccagGACATACGACATACTGTACA
                                               T  Y  D  I  L  Y  T

721   CCTGCTCTGAATGTTCCACACTTTTCGATTTGTGCATCAAGgtatagtttgaaaatgttt
        C  S  E  C  S  T  L  F  D  L  C  I  k
                                                  A
                                                  hc161

781   gtttcattaatcagctcaatattctttcagTACCGTACTTGTCAAGATGGTTGTGTTCCA
                                     Y  R  T  C  Q  D  G  C  V  P
                                  A
                                  it110

841   GAAATGCCAGTTCTTCCCTATGATCAAGCAAAGGATGTGTCGGCGAGAATTgtaagattt
      E  M  P  V  L  P  Y  D  Q  A  K  D  V  S  A  R  I

901   taatttataaaaaaaccgttgttgaaacggaatgtggatgtcttgagtgaagtaattatca
961   aactatttatctatagttcccagttgtggttataagttatgtgaaacagcagaaaattat
1021  gaatcaaagaaatccctaaaatttaaccagacttattaaaatgttgtgaaatttttcaaaa
1081  cgaaaaattcccgtttcacatgttactgaaaatgattcaagaacacaagttttctgattc
1141  ataaatgatcttttatttttcagaaaatttgttttgaaaaatccaatgtttcgatttctcc
1201  acataaagtctgaatacaaacaatttatttggatcaaaaaacaaaaactgaatttatcaa
1261  aaaaaaatataaaataaaattttaatttttatacggccaacccgcaaaagaacgaaatgat
1321  ttttgttttcttggattatgttttgctggttttttgttttgtcgcaaaaaaacaaaaaaaa
1381  aatttttcccgaaacaatccaaaaaacaacttccctggttccattgaattaatttttcag
1441  CGTCCCCCAATGAATCCCTGCTTGGATTTGGTCACTGCGTGCAATTACGAGTCAATGGAT
      R  P  P  M  N  P  C  L  D  L  V  T  A  C  N  Y  E  S  M  D

1501  AGCTATGAAATTCTTCGATCTGAAAATCCATTCCTTTACATTTGAATAAATTTTATTCCT
      S  Y  E  I  L  R  S  E  N  P  F  L  Y  I  END

1561  Attcggtggcttcattacaaatttctcacttttcaacacatttcaacattccgattcaaa
       POLY A

1621  gaaattacgtaagaggtgacgaaagataacatcttcagcttggaacattgtcgttttacc
1681  tcttcttcttctcattaagagataccaatctgaaaggcttttccctcatttatgtctgtc
1741  tgtcacaacaacaacttttcaacattatccttcatttttcctgaaaattcattgaaaggt
1801  gaaaagagtgg
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FIGURE 4.—The *spe-27* gene sequence. Lower case letters indicate introns and the 5′ and 3′ untranslated sequences. Upper case letters indicate exons present in cDNA clone *pAM132*. The predicted amino acid sequence of the *spe-27* protein is shown below the DNA sequences in the single letter code. Translation start and stop sites and the polyadenylation site are labeled. A 32-bp near perfect inverted repeat sequence in the 5′ untranslated region is underlined. The nucleotide change in each mutant allele is indicated, as is the amino acid change of *it132ts*.

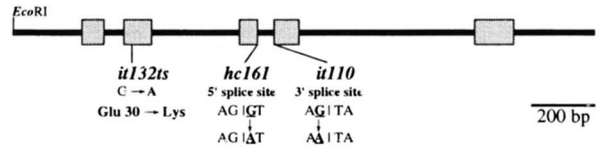

FIGURE 5.—The *spe-27* gene structure. Shaded boxes indicate exons. Black bars indicate introns and the 5′ and 3′ untranslated sequences. Two of the mutations, *spe-27(hc161)* and *spe-27(it110)* are G to A transitions in the 5′ and 3′ splice sites of intron III, respectively. *spe-27(it132ts)* is also a G to A transition causing a missense mutation that changes Glu30 to Lys in exon II.

to restore fertility to *spe-27* mutants (Table 1). Subsequent deletion analysis showed the 3′ end of this clone was unnecessary and so delimited the gene to an ~1.8-kb region (see below). All three mutants in the *spe-27* complementation group were rescued with *pAM125*, confirming that they are allelic (data not shown).

To analyze the *spe-27* transcript, we used the 2.5-kb fragment from *pAM125* to probe two cDNA libraries prepared from male RNA. We isolated five cDNA clones after screening 10^6 plaques. We sequenced the five cDNA clones isolated; all five were identical in their region of overlap, and their sequences were contained in the 1.8-kb *spe-27* genomic region that restores fertility. By aligning genomic and cDNA sequences, we determined that the gene is composed of five small exons and four introns (Figures 4 and 5). Like most *C. elegans* introns (WOOD 1988), introns I and III are very small, 49 and 61 bp, respectively. Introns II and IV are much larger, 275 and 551 bp, respectively.

The *spe-27* gene encodes a putative 131 amino acid polypeptide with a molecular weight of 15,182 D. The protein is mostly hydrophilic, with an estimated isoelectric point of 4.6, and it has no extended hydrophobic regions. The predicted amino acid sequence has no significant similarity to any gene products in the databases when searched by BLASTP or TBLASTN (ALTSCHUL *et al.* 1990). In addition, searches for conserved motifs and domains using the programs MOTIFS and PROFILESCAN from the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI) revealed no convincing matches.

The sequence of the *spe-27* gene from each of the mutants confirmed that we have, indeed, cloned the *spe-27* gene and that the three members of the *spe-27* complementation group are allelic. We sequenced 1.8 kb of DNA corresponding to the *spe-27* gene from each mutant using cycle sequencing of PCR-amplified DNA. Each mutant has a single mutation in this region, a G to A transition characteristic of EMS mutagenesis. The *spe-27(it132ts)* allele is a missense mutation in the second exon, changing Glu30 to Lys. The mutation in *spe-27(hc161)* converts the conserved G at the 5′ splice site of intron III to an A; the mutation in *spe-27(it110)* is in the same intron, changing the conserved G at the 3′ splice site to an A (Figures 4 and 5). The latter two mutations are predicted to generate aberrantly spliced transcripts.

Finding two out of three mutations in the same intron raised the possibility that their mutant phenotypes might arise from disrupting the intron rather than the coding region. Although rare, some introns have functions on their own (FRAGAPANE *et al.* 1993; PRISLEI *et al.* 1993; CAFFARELLI *et al.* 1994). If this were the case, disrupting the intron might alter the ability of an otherwise wild-type *spe-27* gene to rescue *spe-27* mutants. Because we could not delete only this intron readily, we constructed two plasmids containing insertions of plasmid vector DNA into intron III: *pAM131* contains a 129-

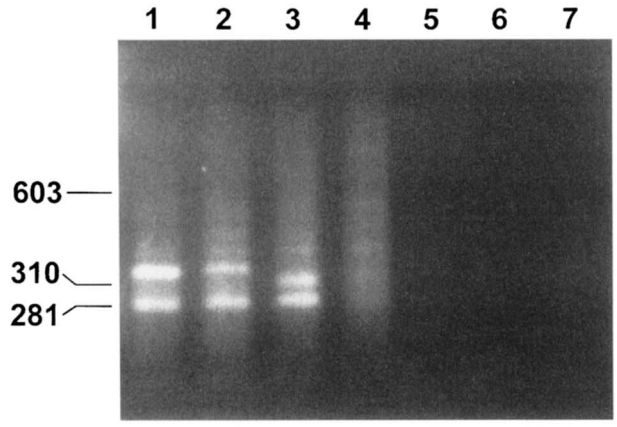

FIGURE 6.—Misspliced mRNA caused by the two splice site mutations. Ethidium bromide stained gel of RT-PCR products using *spe-27*-specific primers; sizes of standards in base pairs. Lanes: (1) Normal sperm RNA from *fem-3(q23)* mutant RNA; (2) *spe-27(hc161)* mutant male RNA showing a slightly larger RT-PCR product; (3) *spe-27(it110)* mutant male RNA showing a slightly smaller RT-PCR product; (4) *fem-1(hc17)* mutant RNA (this mutant makes no sperm); (5) Control, no RNA; (6) Control, *fem-3(q23)* mutant RNA, PCR reaction with no reverse transcriptions; (7) Control, *spe-27(hc161)*RNA, PCR reaction with no reverse transcription. The band at 281 bp is an unidentified PCR product whose sequence does not resemble *spe-27*. The integrity of all RNAs used in this experiment was confirmed by Northern blot analysis using an actin gene as a probe (data not shown).

bp *Ssp*I fragment from pBlusecriptSK+ inserted in a unique *Ssp*I site of intron III; *pAM132* has a 552-bp *Ssp*I fragment from pBluescriptSK− inserted into the same site. Both plasmids restored fertility of *spe-27* mutants nearly as well as the wild-type plasmid (Table 1), so insertions into intron III do not alter the function of the *spe-27* gene.

To see what messenger RNAs, if any, were produced in the two putative splicing mutants, we examined *spe-27* mRNA from mutant worms using reverse transcription and PCR amplification of the product (RT-PCR) with *spe-27*-specific primers. RT-PCR products were obtained only with RNA from worms making sperm (Figure 6). The most abundant *spe-27* mRNA species made in *spe-27(hc161)*, the 5′ splice defect mutant, is slightly larger than normal (Figure 6, lane 2); that made in *spe-27(it110)*, the 3′ splice defect mutant, is slightly smaller (Figure 6, lane 3). Sequencing of the PCR products from the mutants revealed that the 5′ splice mutation (*hc161*) causes the next best 5′ splice consensus within intron III to be used to splice to the normal 3′ junction of intron III (Figure 7). This yields a larger mRNA with part of intron III incorporated into the mature message, which causes a shift in reading frame creating a premature stop codon. The predicted polypeptide encoded by this aberrantly spliced message would contain amino acids 1–70 plus a carboxy terminus of 32 different amino acids that arise from the shifted reading frame. The 3′ splice mutation (*it110*) causes the normal 5′

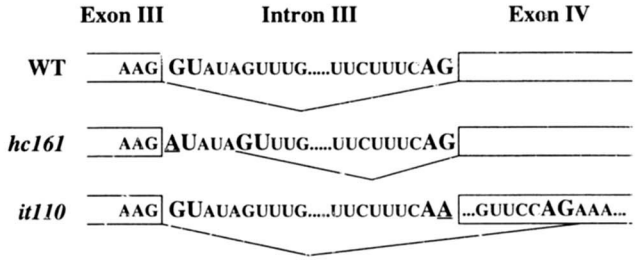

FIGURE 7.—mRNA splicing of wild-type and splice-site mutants. The mRNA splicing pattern of wild type and mutants is diagrammed showing the spliced sequences based on sequencing the RT-PCR products shown in Figure 6.

splice junction of intron III to splice to a cryptic 3′ site within exon IV (Figure 7). This produces a shortened message, which encodes a truncated protein that includes 20 of the different amino acids that were encoded by the *hc161* mutant message. An unidentified smaller PCR product is also obtained in these experiments that is unrelated in sequence to *spe-27* but is apparently sperm-specific.

Determination of the 5′ and 3′ boundaries of *spe-27*: The 3′ untranslated region of *spe-27* is short. The last base of the translation stop codon (UG**A**) is the first A of the polyadenylation signal (**A**AUAAA), and the polyadenylation site identified from cDNA clones is located only 16 bases downstream from the stop codon (Figure 4). At the 5′ end of *spe-27*, the largest cDNA extends 11 nucleotides 5′ to the putative AUG start site. The nucleotides immediately adjacent to this AUG closely match the *C. elegans* consensus sequence for translation initiation (W. WOOD, personal communication). There is a 32-bp inverted repeat in the genomic sequence ending just 21 nucleotides 5′ of the start codon which might play a role regulating *spe-27* expression.

To estimate which sequences were required for functional expression of *spe-27*, we made deletions of the 5′ and 3′ ends of the 2.5-kb fragment *(pAM125)* and assayed their ability to restore fertility in *spe-27* mutant worms by DNA transformation (Figure 8 and Table 1). A construct that deletes the 3′ end up to 5 nucleotides 3′ from the polyadenylation site, 3′Δ(+5), restored nearly wild-type fertility in *spe-27* mutants; therefore, little of the 3′ region is necessary for *spe-27* function. As expected, constructs that delete the last exon, 3′Δ(−347) and 3′Δ(−404), did not rescue *spe-27* mutants.

The 5′ untranslated region of *spe-27* in *pAM125* is 215 bp long. A deletion up to −112 from the ATG start site, 5′Δ(−112), still restored nearly wild-type fertility in *spe-27* mutants (Figure 8 and Table 1). A deletion to −74 from the ATG start site, 5′Δ(−74), produced fertile worms that laid about half the number of eggs of wild-type worms, suggesting that sequences required for proper *spe-27* expression are missing. Finally, 5′Δ(−27), which deletes to −27 from the ATG start site, including most of the inverted repeat, did not yield any fertile worms when transformed into *spe-27* mu-

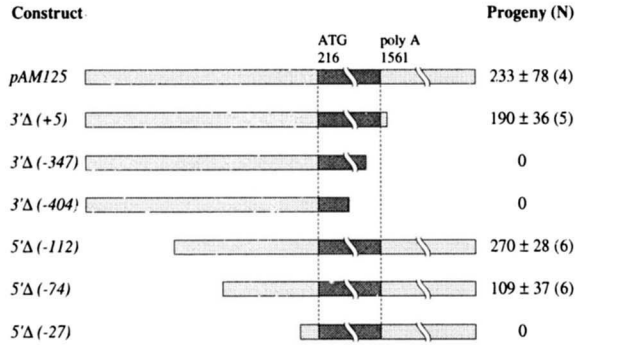

FIGURE 8.—Deletion analysis of the ends of *spe-27*. The deletion constructs diagrammed were microinjected along with a marker plasmid carrying the dominant *rol-6* allele into *spe-27* hermaphrodites. F_1 hermaphrodites that rolled were assayed for fertility by counting their F_2 progeny. Deletions are labeled by the nucleotides that remain: from the 3′ end labeled as + from the first nucleotide of the polyA addition; from the 5′ end labeled as − from the ATG start codon.

tants. To assay these 5′ end deletion constructs by microinjection transformation, it was necessary to decrease the DNA concentration about 10-fold compared with the concentration normally used for transformation with the intact construct (Table 1). When injected at the same concentration used for *pAM125* (200 ng/μl) along with the *rol-6* gene DNA marker, few transformants (roller worms) were obtained and many dead embryos appeared, perhaps due to ectopic expression of *spe-27*.

***spe-27* expression:** Mutations in *spe-27* appear to affect only the sperm; this predicts that *spe-27* should be expressed in tissues undergoing spermatogenesis. We tested this prediction using differential Northern blot analysis and *in situ* RNA hybridization with an antisense RNA probe, in addition to the RT-PCR described previously.

We probed a Northern blot containing mRNA isolated from worms that are somatically female but have germ lines that produce only sperm [*fem-3(q23)*] or oocytes [*fem-1(hc17)*]. We also probed mRNA from males purified from a *him-5(e1490)* strain. An RNA of ~0.5 kb was present in the RNA from the *fem-3* strain and from males; these are the two strains that produce sperm (Figure 9). The size of this transcript, 0.5 kb, corresponds roughly to that of the longest cDNA *(cAM132)*, which indicates that this cDNA clone is close to full length. No hybridization to *fem-1* mRNA was detected. Therefore, at this level of sensitivity, *spe-27* appears to be expressed only in worms that produce sperm. The more sensitive RT-PCR experiment (Figure 6) also indicated that *spe-27* RNA is present in *fem-3(q23)* but not in *fem-1(hc17)* worms, in agreement with the Northern analysis.

By *in situ* hybridization experiments, we detected *spe-27* message in the testis (Figure 10). The transcript seems to be absent in spermatogonial cells undergoing mitosis but is present in the spermatocytes. The message

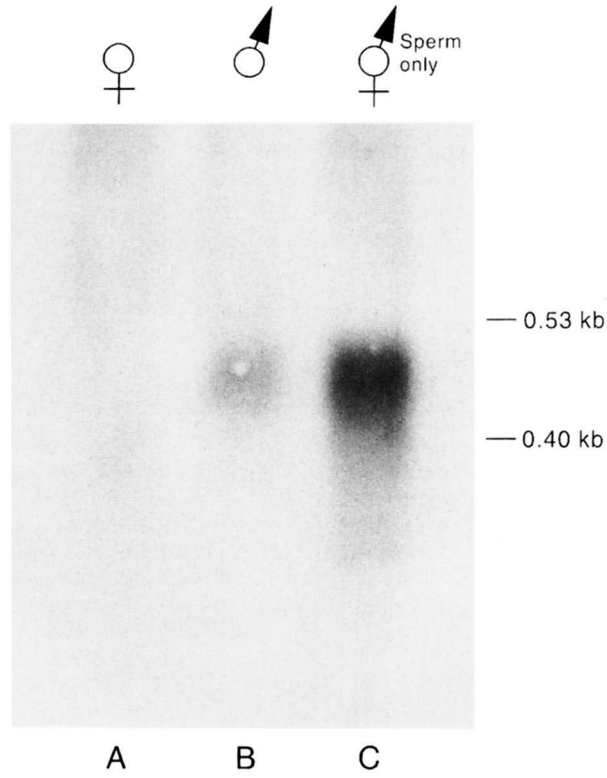

FIGURE 9.—Differential northern analysis of *spe-27* expression. Poly A+ RNA probed with *spe-27* cDNA, *pAM132*. (A) RNA from *fem-1(hc17)* worms that are females that produce oocytes and no sperm. (B) RNA from purified male worms (this lane was underloaded relative to A and C). (C) RNA from *fem-3 (q23)* worms that are somatically females but produce only sperm. An ~0.5-kb transcript is present in the RNA from worms that produce sperm and absent from those that do not.

is also present in the residual bodies but absent in the spermatids; this result is expected, since ribosomes are segregated into the residual body when the spermatids form. No hybridization to somatic tissues was observed, although there is some background staining of the intestine, probably caused by endogenous phosphatase activity.

DISCUSSION

The *spe-27* mutant phenotype: Spermiogenesis in *spe-27* mutants is summarized in Figure 11, which diagrams mutant and wild-type spermatid activation both *in vivo* and *in vitro*. Mutant hermaphrodite spermatids fail to activate to form spermatozoa so the hermaphrodites are sterile. Their spermatids are swept out of the spermatheca by passing oocytes and eventually expelled from the uterus when oocytes are laid. In contrast, mutant males are fertile and their spermatids activate normally upon mating. Mutant hermaphrodite spermatids look indistinguishable from wild type. They can be activated to form spermatozoa by mating young hermaphrodites with males, either sterile or fertile, which induces the production of self-progeny.

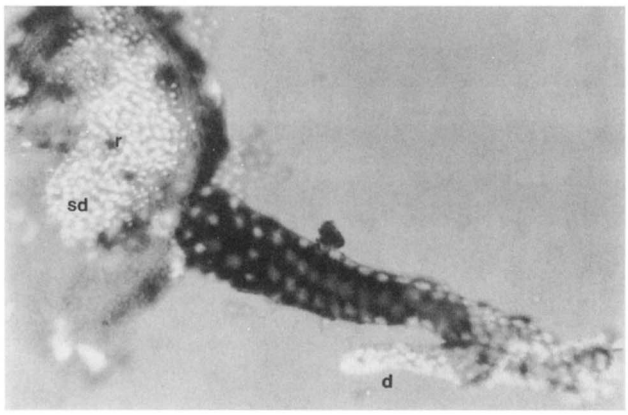

FIGURE 10.—Localization of *spe-27* expression by *in situ* hybridization. Males were dissected and hybridized with a digoxygenin antisense RNA probe to a *spe-27* cDNA followed by antidigoxygenin antibodies coupled to alkaline phosphatase and a substrate that shows up as dark brown staining. Nomarski optics is combined with fluorescence of DAPI stained nuclei. Dark staining is found in the spermatocytes, which are in the lower middle of the figure, and in the scattered residual bodies (r) outside the testis. Spermatids (Sd) and the distal tip of the testis (d), where cells are still in mitosis, lack staining. Controls with no antisense RNA were essentially blank except for staining to the gut, which presumably represents endogenous alkaline phosphatase activity (data not shown). The gut is the dark staining region wrapping around the spermatids.

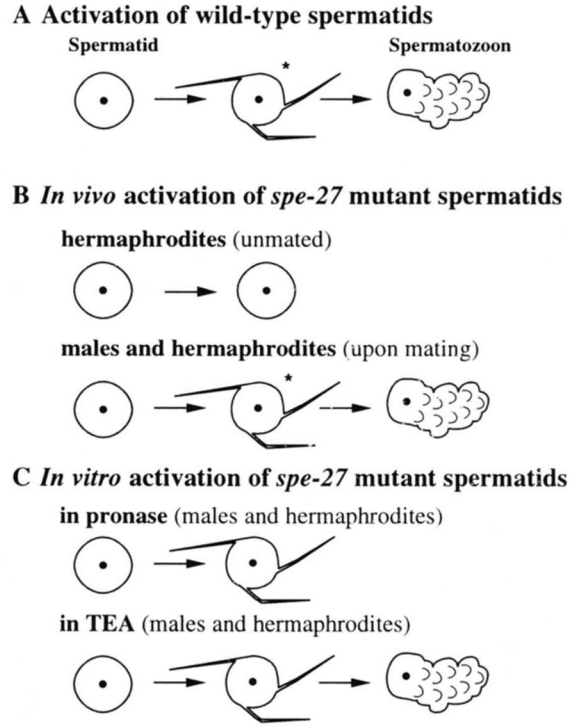

FIGURE 11.—Summary of spermiogenesis in wild-type and *spe-27* mutant spermatids. (A) Normal *in vivo* and *in vitro* activation of wild-type hermaphrodite and male spermatids. (B) *In vivo* activation in *spe-27* mutant spermatids. Hermaphrodite spermatids do not activate. Male spermatids activate normally upon mating and hermaphrodite spermatids are also activated by mating. (The * on the spiky intermediate in A and B indicates this intermediate has not been seen *in vivo* since spermiogenesis cannot be visualized inside the worm with sufficient resolution to detect the spikes.) (C) *In vitro* activation of *spe-27* mutant spermatids. In pronase, both male and hermaphrodite spermatids initiate spermiogenesis but never form pseudopods. In TEA, mutant spermatids from both sexes form normal looking pseudopods.

A simple explanation for these phenotypes might be that *spe-27* hermaphrodites are missing the signal that activates spermatids to become spermatozoa. Three observations suggest that this is unlikely. First, both hermaphrodite and male mutant spermatids activate with pronase to form spiky membrane projections but not pseudopods. This defect shows that there is some alteration in the mutant spermatids themselves and not just in an external signal. Second, when wild-type male spermatids are washed and artificially inseminated into mutant hermaphrodites, they activate to form viable spermatozoa and produce outcross progeny, showing that an activation signal is present in *spe-27* hermaphrodites. In contrast, *spe-27* mutant male sperm inseminated into *spe-27* hermaphrodites fail to activate, showing again that there is a defect in these sperm. Third, *in situ* hybridization indicates that the *spe-27* gene appears to be expressed in spermatocytes but not in somatic tissue, the likely source of an activating signal.

The phenotypes of *spe-27* mutants are identical to those of mutants defective in two other genes, *spe-8* and *spe-12* (L'HERNAULT *et al.* 1988; SHAKES and WARD 1989), and can be explained by the model suggested by SHAKES and WARD (1989). This model proposes that spermatids of both sexes have separate receptors for male and for hermaphrodite activators. These receptors connect to distinct pathways that transduce the signals to form spermatozoa. Mutations in *spe-8, spe-12* and *spe-27* disrupt an early step in the hermaphrodite signal pathway. Pronase triggers the hermaphrodite activation pathway by proteolysis of surface proteins, and TEA either triggers the male pathway or bypasses the signal transduction pathways by increasing the intracellular pH and directly activating spike formation and pseudopod extension. Because the defective hermaphrodite spermatids still retain a functional male activation pathway, mating by males transfers the male activator and activates hermaphrodite spermatids to spermatozoa. The artificial insemination experiments described here provide direct support for this model because they show that *spe-27* male sperm do not activate when inseminated in the absence of seminal fluid. This is consistent with the model's prediction that mutant male sperm, like the hermaphrodite sperm, should be unable to respond to the hermaphrodite activator. Moreover, the observation that wild-type male sperm can respond to the hermaphrodite activator when inseminated without seminal fluid shows that male sperm can respond to either activator and confirms that *spe-27* hermaphrodites do make the hermaphrodite activator.

***spe-27* sequence and expression:** The *spe-27* muta-

tions are rescued by a 1.8-kb DNA fragment from the region identified by genetic mapping. This fragment contains a gene that is expressed only in spermatocytes and encodes a 131 amino acid polypeptide. The predicted *spe-27* polypeptide sequence does not resemble any known proteins nor does it contain recognizable functional motifs or likely hydrophobic regions; therefore, the protein is likely to be cytoplasmic, but the sequence provides no clues to SPE-27 function.

Temperature shift experiments with the temperature-sensitive mutation *spe-27(it132ts)* shows that in this mutant hermaphrodite spermatids are themselves temperature sensitive. Mutant hermaphrodites shifted from restrictive to permissive temperature after spermatids have been made become fertile, whereas shifting from permissive to restrictive temperature reduces fertility. These results suggest that mutant SPE-27 function is reversibly temperature sensitive since no new gene products are made in spermatids. They also imply that SPE-27 must be present in the spermatids and must function there, as opposed to acting earlier in spermatogenesis, otherwise the spermatids themselves would not be temperature sensitive. This result is consistent with the proposed model that SPE-27 functions in the hermaphrodite spermatid activation pathway.

The wild-type *spe-27* gene appears to require little flanking DNA outside its coding region to rescue *spe-27* mutants. This conclusion is tempered by the uncertainty of how well the multigene arrays that form after transformation mimic the quantitative regulation of the normal gene. Nonetheless, deletion analysis of the 3′ regions of *spe-27* shows that only five nucleotides 3′ beyond the polyadenylation site are required for the restoration of fertility in *spe-27* mutants. At the 5′ end, *spe-27* appears to have a defined promoter, but it extends at most 112 nucleotides 5′ of the translation start site. Constructs that delete ≤74 nucleotides 5′ to the start site only restore fertility to about half that of the longer constructs. Constructs deleting up to 27 nucleotides 5′ of the start site fail to restore any fertility to transformed *spe-27* mutant hermaphrodites. This last deletion removes the 5′ inverted repeat suggesting that this sequence may play a *cis*-regulatory role.

Initial microinjections with two of the 5′ end deletion constructs, 5′Δ(−112) and 5′Δ(−74), did not result in any transformed progeny with the roller phenotype (*rol-6* was the dominant marker used for transformation). Instead, injected worms laid many dead embryos. This may indicate that the DNA is toxic at the concentration used (MELLO *et al.* 1991). By decreasing the DNA concentration 10-fold, we were able to obtain transformed worms. As more of the 5′ sequence was deleted, the fertility of transgenic worms decreased and the embryonic mortality increased. This suggests that the loss of sequences from the 5′ region not only affects *spe-27* expression in the testis, but might also cause toxic ectopic expression in the embryo.

Mutations in *spe-27*: Two of the three mutations in the *spe-27* gene (*hc161* and *it110*) are G to A transitions in the conserved Gs at the 5′ and 3′ splice sites, respectively, of intron III. Mutations in splice sites can promote the use of cryptic splice sites. Sequencing of the cDNAs from the mutant *spe-27* mRNA indicates that both use cryptic splice sites causing frameshifts in the encoded polypeptides. The resulting disruptions of the polypeptide sequence are the most likely cause of the mutant phenotype. The mutation that causes temperature-sensitive sterility, *spe-27(it132ts)*, is also a G to A transition. It causes a missense mutation changing Glu30 to Lys in SPE-27. At the higher temperature, this mutation could alter the conformation of the *spe-27* protein or disrupt its interactions with other proteins, since an acidic amino acid is changed to a basic one.

Activation pathways: It is not apparent why there are two distinct pathways for activating spermiogenesis, nor why both male and hermaphrodite sperm have both pathways. Hermaphrodites do not appear to need the male pathway, nor do males need the hermaphrodite pathway since their sperm are transferred with seminal fluid. It is generally accepted that hermaphroditism in nematodes is derived from dioecious species (GHISELIN 1969; POINAR 1983). If so, a possible way for hermaphrodites to arise from females would be by changes in the regulation of the germ-line sex determination pathway so that spermatids are briefly produced (HODGKIN 1992). In this case, changes must also occur to activate these spermatids to spermatozoa. If the source of the activating signal is somatic, then either the male activating signal needs to be induced in these hermaphrodites or a separate pathway of activation must be invoked. A separate pathway might be advantageous because, in addition to activating hermaphrodite spermatids, it would also provide a "backup" activation system for male sperm. There are many examples of such redundancy of genetic systems in development that are thought to have evolved to safeguard against developmental errors (*e.g.*, FERGUSON and HORVITZ 1989, BRENNER *et al.* 1990; TAUTZ 1992). If hermaphrodites did arise as surmised here, it would predict that dioecious species should only have one system of spermatid activation and would not need to express the *spe-27* gene or the other members of the hermaphrodite pathway. Now that the *spe-27* gene has been isolated, this can be tested by analyzing the expression of its homologues, if they exist, in related dioecious species such as *C. remanei* and *Rhabditis* species, and compared with expression in other hermaphroditic species such as *C. briggsae*.

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Arizona Undergraduate Biology Research Program. The *spe-27* sequence is available in GenBank, accession number U37713.

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