**ABSTRACT**

*Caenorhabditis elegans* spermatids complete a dramatic morphogenesis to crawling spermatozoa in the absence of an actin- or tubulin-based cytoskeleton and without synthesizing new gene products. Mutations in three genes (*spe-8, spe-12*, and *spe-27*) prevent the initiation of this morphogenesis, termed activation. Males with mutations in any of these genes are fertile. By contrast, mutant hermaphrodites are self-sterile when unmated due to a failure in spermatid activation. Intriguingly, mutant hermaphrodites form functional spermatozoa and become self-fertile upon mating, suggesting that spermatids can be activated by male seminal fluid. Here we describe a mutation in a fourth gene, *spe-29*, which mimics the phenotype of *spe-8, spe-12*, and *spe-27* mutants. *spe-29* sperm are defective in the initiation of hermaphrodite sperm activation, yet they maintain the ability to complete the morphogenetic rearrangements that follow. Mutant alleles of *spe-12, spe-27*, and *spe-29* exhibit genetic interactions that suggest that the wild-type products of these genes function in a common signaling pathway to initiate sperm activation. We have identified the *spe-29* gene, which is expressed specifically in the sperm-producing germ line and is predicted to encode a small, novel transmembrane protein.

**CELLS** acquiring specialized functions often undergo morphogenetic rearrangements to form structures crucial for their performance. Though our understanding of how these structural changes are regulated is incomplete, mechanisms controlling cellular morphology have been described in a few well-studied cell types and in many cases have been conserved. For example, the polarized morphology that precedes mating in the budding yeast *Saccharomyces cerevisiae* initiates when haploid cells respond to a mating pheromone secreted by neighboring cells of the opposite mating type. Mating pheromone activates a mitogen-activated protein (MAP) kinase signaling cascade and the small GTPase Cdc42p, which ultimately results in modification of the actin cytoskeleton and cell wall (reviewed in Leberer *et al.* 1997; Madden and Snyder 1998). In fibroblasts, members of the MAP kinase signaling cascade and the small GTPases Cdc42 and Rac function to regulate morphological responses to growth factors (reviewed in Kyriakis and Avruch 1996). Cdc42 and Rac are also thought to mediate cytoskeletal changes as anucleate platelets activate during blood clotting (Azim *et al.* 2000). In these well-studied examples, morphogenetic change is largely accomplished through alteration of the actin cytoskeleton.

Spermatids from the nematode *Caenorhabditis elegans* undergo a dramatic and sudden morphogenesis during their activation to form crawling spermatozoa and thus provide an ideal model for examining cellular morphogenesis in a genetically tractable metazoan. Activation of self-sperm begins within the hermaphrodite when spermatids are first pushed into the spermatheca by oocytes and perceive an unidentified endogenous activator (Ward and Carrel 1979). Spermatids from males activate soon after they are ejaculated into the hermaphrodite uterus. Within minutes, membranous organelles fuse with the spermatid plasma membrane, a polar pseudodop extends, and the major sperm protein polymerizes and organizes into a pseudopodial cytoskeleton (Nelson and Ward 1980). All of these changes are needed for spermatozoa to crawl and fertilize oocytes. Remarkably, these rearrangements occur without regulation of a classical actin- or tubulin-based cytoskeleton (spermatids have neither) and in the absence of new gene product synthesis (spermatids have no ribosomes; Ward *et al.* 1981; Ward 1986; Pavalko and Roberts 1989). Because of their unusual cell biology, nematode sperm may utilize unconventional mechanisms to regulate their morphogenesis to spermatozoa. Several mutations, identified through screens for genes required for sperm development, specifically impair the initiation of spermatid activation and cause nearly identical phenotypes: spermatids from *spe-8, spe-12*, or *spe-27* mutants fail to activate in virgin hermaphrodites, yet both self-spermatids and male-derived spermatids activate normally in mated hermaphrodites. The phenotype of these...
members of this pathway interact genetically. We have ually picked at the L4 stage to seeded plates and allowed to self-

describe a fourth gene, Worm synchronization and progeny counts:

t127

Hermaphrodites Outcross males (non-Unc) were mated to various restriction fragments into the pBluescript II SK

hermaphrodites. Males were mated to

spe-27

dpy-20

spe-29

dpy-20; him-5 hermaphrodites. The spe-27 spe-29 double mutant was isolated from a non-Unc non-Dpy Spe child of this cross.

In all cases, the genotype of double mutants was verified by complementation analysis using multiple hermaphrodites from each strain. Since double-mutant males were fertile, strains could be maintained unbalanced. The him-5(elt490) mutation was included in each double mutant strain to aid in the initial isolation of males.

Worm synchronization and progeny counts: Synchronized virgin hermaphrodites were obtained by allowing gravid hermaphrodites to lay eggs on a seeded plate for 5 hr. When males were present in the brood, hermaphrodites were separated from males during the final larval stage (L4) to preserve their virginity.

To determine self-brood sizes, hermaphrodites were individually picked at the L4 stage to seeded plates and allowed to self-fertilize at the indicated temperature. Gravid hermaphrodites were transferred to new plates daily until no new eggs were laid in a 24-hr interval. Progeny were recorded as they were removed by aspiration. If hermaphrodites were nearly sterile (Spe mutants), F, were recorded and removed from the plate before reaching sexual maturity. When him-5 hermaphrodites were utilized, the progeny number reflected the sum of dead eggs (a result of the him-5 mutation) and live offspring. Hermaphrodites that died prematurely (while still holding fertilized eggs) were excluded from the analysis. All errors presented are the standard error of the mean (SEM).

Transactivation assays: Sperm from Spe hermaphrodites were transactivated by mating synchronized virgin hermaphrodites with three sterile fer-1 males for a 24-hr interval. Animals were placed together when hermaphrodites were at the L4 stage; males were at the L4 or young adult stage. fer-1(hcl13s); him-5 males were utilized in assays that were conducted at 25°, while fer-1(b223s); him-5 males (which are sterile at 20°) were utilized in assays that were conducted at 20°.

Sperm counts: Synchronized virgin hermaphrodites grown at 25° were picked at the L4 stage to individual plates. After maturing to adulthood and laying eggs or oocytes for 3–5 hr, each was fixed and stained with DAPI (4’,6-diamidino-2-phenylindole) and the sperm nuclei present in the spermatheca and uterus were recorded as described by NANCE et al. (1999). For him-5 controls, the number of fertilized eggs laid before fixation was added to the sperm count. him-5 hermaphrodites laid 45 ± 8.8 (SEM) eggs before fixation, spe-29 hermaphrodites laid 23 ± 1.3 oocytes before fixation, and spe-12 hermaphrodites laid 24 ± 2.3 oocytes before fixation.

Transformation of spe-29 hermaphrodites: Adult hermaphrodites were transformed with cosmid or plasmid DNA (at 20 ng/µl) as described by MELLO and FIRE (1995). PRF4, which contains the dominant rol-6 (su1006) allele, was injected at (100 ng/µl) to facilitate recognition of transformed animals (which roll; KRAMER et al. 1990). After recovery, each injectee was mated overnight with four spe-29 males. Transformed hermaphrodite F1 progeny of injected worms were picked to individual plates as virgins and scored for self-fertility. Fertility was always assessed in the F1 generation to minimize germ-line silencing effects on the transformed DNA (KELLY et al. 1997; KELLY and FIRE 1998).

Construction of wild-type and mutated genomic subclones of cosmid F25H8: Standard techniques were used to isolate and manipulate DNA (AUSUBEL et al. 1995). When PCR-amplified DNA was cloned, all relevant coding regions were sequenced in their entirety to ensure that no mutations were introduced. All oligonucleotide sequences are presented in the 5′ to 3′ orientation.

Unmodified subclones of F25H8 were obtained by cloning various restriction fragments into the pBluescript II SK+ vector.
RNA from each mutant was analyzed by Northern analysis as described in materials and methods.

Plasmid p[N136 was constructed from p[N115 by an inverse PCR-based site-directed mutagenesis procedure. Briefly, oligonucleotides were designed from opposite strands such that their 5' bases were abutting, save an additional base that was added to the 5' end of one oligonucleotide (CGGTCGACGAGAAGAAGAAGA; boldfaced G is the added base). Using these oligonucleotides and p[N115 as template, a linear version of p[N115 with an additional nucleotide at one terminus was amplified. Purified PCR product was phosphorylated, circularized, and transformed into bacteria for recovery. The inserted base pair is predicted to result in a frameshift early in the first exon of P518H, which would terminate translation after the 12th codon.

Plasmid p[N142 was constructed from p[N137, a version of p[N115 mutated by the above procedure to introduce a SpeI site early in the spe-29-translated region (first four codons of spe-29 in p[N137: ATGCGACGACTAGT; SpeI site is underlined). To create p[N142, p[N137 was cleaved with SpeI, sticky ends were filled with Klenow, and the linear plasmid was circularized. The Klenow fill creates a 4-bp insertion that disrupts the reading frame and is predicted to terminate translation of spe-29 mRNA after the fourth codon.

Isolation of spe-29 cDNAs and identification of the it127 lesion: spe-29 cDNAs were isolated by PCR from a fem-3(gf)
cDNA library directionally cloned into a pBluescript-derived plasmid vector (library generously provided by H. Smith). To amplify the 5' end of spe-29, PCR reactions were performed with a vector-specific oligonucleotide (ACGTTGAAAACGCAGCCACCATTA) and a spe-29 oligonucleotide from exon II (GACCTCGGAGAAGAAGAAGA). The 3' end of the spe-29 transcript was amplified with an oligonucleotide from exon I (CGGATTTTGGTTCATCTGAGGT) and a vector-specific oligonucleotide (GAAAACGCTATGACCATGATTAG). PCR products were cloned into the pCR2.1 vector (Invitrogen, San Diego). Three clones from the 5' end of the gene and three clones from the 3' end of the gene (which partially overlapped the 5' end clones) were sequenced.

The spe-29(it127) lesion was identified by comparing the sequences of genomic DNA amplified by PCR from individual wild-type and spe-29 worms (Williams 1995). Oligonucleotides CCGAATTTCAGGTCACTGGACG and GATTCGCCCAATACCGAAGG were used in the amplification and sequencing reactions. A single G-to-A base change was detected in this interval. To ensure that this mutation was present in the genomic template and was not established during amplification, new genomic DNA was amplified from spe-29 worms and sequenced. All sequencing reactions were performed by the University of Arizona Laboratory of Molecular Systematics and Evolution sequencing facility using an ABI Prism sequencer (Applied Biosystems, Foster City, CA) and with defects in spermatogenesis nearly identical to those found in spe-8, spe-12, and spe-27 mutants. We positioned it127 on LGIV in a region that defines it as a new spe gene, which we designated spe-29 (see materials and methods). It127 is the only mutant allele of spe-29. We abandoned efforts to identify additional alleles of spe-29 after realizing that the gene was unusually small and was closely abutted by flanking genes, so it provided an exceptionally small target for random mutagenesis (see molecular characterization below).

In vitro, when treated with proteases that prompt wild-type spermatids to form pseudopods (Figure 1, A and matogenesis-defective mutants, we identified one, it127, with defects in spermatogenesis nearly identical to those found in spe-8, spe-12, and spe-27 mutants. We positioned it127 on LGIV in a region that defines it as a new spe gene, which we designated spe-29 (see materials and methods). It127 is the only mutant allele of spe-29. We abandoned efforts to identify additional alleles of spe-29 after realizing that the gene was unusually small and was closely abutted by flanking genes, so it provided an exceptionally small target for random mutagenesis (see molecular characterization below).

**RESULTS**

**spe-29 spermatids fail to activate to spermatozoa:** Among a collection of previously uncharacterized spe-29 mutants, we identified one, it127, with defects in spermatogenesis nearly identical to those found in spe-8, spe-12, and spe-27 mutants. We positioned it127 on LGIV in a region that defines it as a new spe gene, which we designated spe-29 (see materials and methods). It127 is the only mutant allele of spe-29. We abandoned efforts to identify additional alleles of spe-29 after realizing that the gene was unusually small and was closely abutted by flanking genes, so it provided an exceptionally small target for random mutagenesis (see molecular characterization below).

*In vitro*, when treated with proteases that prompt wild-type spermatids to form pseudopods (Figure 1, A and
spe-29 spermatids arrested their morphogenesis after extending spiky projections, although a few formed apparently normal pseudopods (Figure 1, C and D). Sperm from spe-12 (as well as spe-8 and spe-27) mutants extend identical spiky projections (Figure 1, E and F; Shakes and Ward 1989). Also, like the previous mutants, spe-29 spermatids treated with triethanol-amine, which increases intracellular pH, activated normally and formed motile pseudopods (data not shown).

Spermatids within spe-29 virgin hermaphrodites are unable to activate, mimicking the phenotype of spe-8, spe-12, and spe-27 sperm. Although mutant hermaphrodites produce many spermatids (see below), these spermatids rarely activate to form normal crawling spermatozoa (Figure 1, G and H). Consequently, hermaphrodites are unable to self-fertilize their oocytes. Many of these immotile spermatids are dislodged from the spermatheca and swept away as oocytes parade through the reproductive tract (Figure 2). Some spermatids (~50 per spermatheca; see below) manage to avoid being dislodged and are retained in the spermatheca.

Self-sterility in spe-29 mutants is not absolute and is somewhat temperature sensitive; virgin hermaphrodites lay only occasional self-fertilized eggs at 15° and 25°, but produce more at 20° (6.9 ± 1.2 progeny, n = 15; wild type produced 257 ± 13.6 progeny at 20°, n = 10). In wild type and many mutants with a leaky Spe phenotype, oocytes are self-fertilized nearly continuously until the supply of spermatozoa is exhausted (Ward and Carrel 1979; L’Hernault et al. 1988). By contrast, spe-29 hermaphrodites lay infrequent self-fertilized eggs and many unfertilized oocytes for up to 5 days after the onset of ovulation, suggesting that a few spe-29 spermatids activate sporadically well after ovulation begins (data not shown). These eggs develop into Spe hermaphrodites with no other obvious developmental defects.

**spe-29 self-spermatids can be efficiently rescued by mating:** spe-29 spermatids could fail to initiate activation because of defects in signaling. Alternatively, spe-29 spermatids may initiate but fail to complete activation because of defects in the cellular machinery required for morphogenesis. spe-8, spe-12, or spe-27 sperm are defective specifically in the initiation of activation because these mutant spermatids are able to form functional spermatozoa when exposed to male seminal fluid after mating, a phenomenon we refer to as “transactivation.” [spe-8, spe-12, and spe-27 males are also fertile because their sperm are bathed in seminal fluid during ejaculation (Shakes and Ward 1989; Minniti et al. 1996; Nance et al. 1999).] To test whether spe-29 spermatids could be transactivated, we mated spe-29 dpy-20 virgin hermaphrodites with sterile fer-1; him-5 males, which function as donors of seminal fluid. Mated spe-29 dpy-20 hermaphrodites produced self-brood sizes (recognized by their Dpy phenotype) that in some cases approached levels of wild-type (dpy-20) controls and were substantially higher than those obtained from virgin spe-29 dpy-20 hermaphrodites (Table 1). The ability of spe-29 spermatids to activate when exposed to a signal (which we refer to as male activator) supplied in the ejaculate indicates that they have all the components necessary to form functional spermatozoa but are defective in their response to an activation signal (which we refer to as hermaphrodite activator) present within virgin hermaphrodites.

**spe-29(it127) and spe-12(hc76) are dominant enhancers of spe-27(it132):** The similarity in phenotype of spe-8,
sple-12, sple-27, and sple-29 mutants suggested that the products of these genes are needed specifically for the function of a signaling pathway required for spermatid activation. If so, this pathway might be compromised by manipulating the levels of these gene products in combinations by varying gene dosage. We looked for such genetic interactions among mutant alleles of these four genes (Table 2). Few sple-27(it132s) self-spermatids activate and fertilize oocytes at the permissive temperature (Table 2; Minniti et al. 1996). We added mutant alleles of the other activation genes to sple-27 mutants and examined the self-fertility of the resulting worms. The phenotypes of sple-27 hermaphrodites also homozygous for the nonconditional alleles sple-29(it127) or sple-12(hc76) were not informative, since each double mutant adopted the epistatic phenotype of the nonconditional allele. However, when sple-27 hermaphrodites heterozygous for either sple-29 or the null allele sple-12(hc76) were selfed, brood sizes were reduced significantly (MANOVA; P < 0.001). Both sple-29(it127) and sple-12(hc76) were completely recessive in an otherwise wild-type background. Thus sple-29(it127) and sple-12(hc76), while normally recessive, are each dominant enhancers of sple-27(it132s) when sperm are activated in virgin hermaphrodites, consistent with the products of these genes functioning in a common signaling pathway.

sple-29 spermatids are normal in their response to male activator: In null alleles of sple-12, such as hc76, transactivation of self-spermatids by mating is never efficient, rescuing only a small fraction of spermatids present within hermaphrodites at the time of mating (Nance et al. 1999). When we transactivated sple-29 spermatids, we noticed that hermaphrodites produced many more self-progeny than typically observed when sple-12 spermatids are transactivated. To confirm that the efficiency of

![Figure 3.—sple-29 male fertility. Single young adult males of the indicated genotype were placed with a single young adult unc-24 dpy-20 hermaphrodite and allowed to mate for 24 hr. Outcross progeny (non-Unc non-Dpy) were counted. Wild type, n = 21; sple-29, n = 23; sple-12, n = 12. Error bars represent the SEM.](image)

**TABLE 2**

Enhancement of sple-27 self-sterility by sple-29 and sple-12

<table>
<thead>
<tr>
<th>Hermaphrodite genotype</th>
<th>Self-progeny</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>sple-27</td>
<td>8 ± 1.2</td>
<td>22</td>
</tr>
<tr>
<td>sple-29</td>
<td>0.5 ± 0.1</td>
<td>20</td>
</tr>
<tr>
<td>sple-12</td>
<td>0.07 ± 0.07</td>
<td>15</td>
</tr>
<tr>
<td>sple-27 sple-29</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>sple-27; sple-12</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>sple-27 sple-29/sple-29 sple-29(+)</td>
<td>3.4 ± 0.6</td>
<td>25</td>
</tr>
<tr>
<td>sple-27; sple-12(+)</td>
<td>1.4 ± 0.3</td>
<td>24</td>
</tr>
<tr>
<td>Wild type</td>
<td>274 ± 10.5</td>
<td>11</td>
</tr>
<tr>
<td>sple-29/sple-29(+)</td>
<td>282 ± 7.3</td>
<td>14</td>
</tr>
<tr>
<td>sple-12/sple-12(+)</td>
<td>292 ± 5.7</td>
<td>14</td>
</tr>
</tbody>
</table>

* All animals were also homozygous for the him-5(e1490) mutation. Wild type refers to him-5(e1490).

* Self-progeny includes the sum of live progeny and dead eggs (a consequence of the him-5 mutation). All animals were selfed at 25°C. Values are expressed as the average ± the SEM.

**TABLE 3**

Transactivation efficiency and retention of sple-29 and sple-12 sperm

<table>
<thead>
<tr>
<th>Hermaphrodite genotype</th>
<th>Self-progeny</th>
<th>Range</th>
<th>n</th>
<th>Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>sple-29</td>
<td>0.7 ± 0.4</td>
<td>1–11</td>
<td>10</td>
<td>88 ± 5.4</td>
</tr>
<tr>
<td>sple-12</td>
<td>0.2 ± 0.1</td>
<td>0–1</td>
<td>10</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>sple-29 (/ x fer-1 male)</td>
<td>82 ± 13</td>
<td>14–166</td>
<td>14</td>
<td>NA</td>
</tr>
<tr>
<td>sple-12 (/ x fer-1 male)</td>
<td>3.4 ± 1.2</td>
<td>0–16</td>
<td>15</td>
<td>NA</td>
</tr>
<tr>
<td>Wild type</td>
<td>110 ± 11</td>
<td>43–162</td>
<td>10</td>
<td>137 ± 14</td>
</tr>
</tbody>
</table>

* All animals were also homozygous for the him-5(e1490) mutation. Wild type refers to him-5(e1490).

* Self-progeny includes the sum of live progeny and dead eggs (a consequence of the him-5 mutation). All animals were selfed at 25°C. Values are expressed as the average ± the SEM.

* n, sample sizes for worms used to determine brood sizes.

* Separate virgin hermaphrodites were used to determine the average number of sperm in each strain (him-5, n = 5; sple-29; him-5, n = 15; sple-12; him-5, n = 11; see materials and methods).
transactivation of spe-12 and spe-29 spermatids differed significantly, we compared the self-brood sizes of mated spe-12 and spe-29 hermaphrodites to the number of spermatids present in control unmated mutants of equivalent age. As shown in Table 3, mating restores the self-fertility of spe-29 hermaphrodites to a much greater extent than it does for spe-12. Two observations suggest that most or all spermatids present within a mated spe-29 hermaphrodite activate to spermatozoa. First, broods from some spe-29 hermaphrodites mated to fer-1 males were as large as brood sizes from unmated wild-type controls (Table 3). Second, the average brood size of transactivated spe-29 hermaphrodites (82) nearly matched the average number of sperm present within similarly aged virgin spe-29 hermaphrodites (88). Only a few self-progeny were produced by mated spe-12 hermaphrodites even though spe-12 hermaphrodites had as many sperm as spe-29 hermaphrodites, indicating that spe-29 sperm are transactivated more efficiently than spe-12 sperm.

We also tested the ability of spe-29 male-derived sperm to activate by assessing the fertility of individual spe-29 males. spe-29 males were as fertile as wild-type males; in comparison, fertility of spe-12 males was significantly impaired (Figure 3) even though both spe-12 and spe-29 males produce seemingly normal numbers of sperm (data not shown) and spe-12 males copulate normally (NANCE et al. 1999). Thus, while spe-29 spermatids fail to activate in response to hermaphrodite activator alone, unlike spe-12 mutants they are wild type in response to male activator.

spe-29(it127) impairs response to male activator in a sensitized genetic background: spe-12 sperm are defective in their response to both hermaphrodite and male activator, while spe-29 sperm are defective only in their response to hermaphrodite activator. We reasoned that if spe-29(+) is not needed for response to male activator, then the efficiency of transactivation of another activation mutant should not be influenced by the spe-29 genotype. To test this hypothesis, we compared the self-fertility of spe-27(it132ts) hermaphrodites to spe-27(it132ts) spe-29 hermaphrodites after each had been mated to fer-1 males. At 20°, mated spe-27 hermaphrodites had a Spe-29-like phenotype, producing many more self-progeny than mated spe-12 null mutants (Table 4). However, when spe-27 spe-29 double mutants were mated, self-brood sizes were substantially smaller than those of mated spe-27 single mutants. By contrast, mated spe-12 and spe-12, spe-29 hermaphrodites had equivalent brood sizes (Table 4). At a higher temperature (25°), mated spe-27 hermaphrodites yielded only a few self-progeny (Table 4), similar to mated spe-12 mutants (see Table 3). Mutating spe-29 had little effect on the already poor activation of spe-27 sperm at 25°, since mated spe-27 spe-29 hermaphrodites produced broods similar in size to those of mated spe-27 hermaphrodites. Thus spe-29 can impair the ability of self-sperm to respond to male activator, but only when the activation signaling pathway is compromised [at 20° in a hypomorphic spe-27(it132ts) background] but not blocked [in a null spe-12 background or a spe-27(it132ts) background at 25°]. These data suggest that spe-29(it127) limits, but does not abolish, the effectiveness of the sperm activation signaling pathway.

spe-29 is predicted to encode a small, novel membrane protein: Through a series of genetic mapping experiments, we positioned spe-29 on LGIV within the overlap of deficiencies dpy-19 and mDf7 and between egl-20 and pKP614 (see MATERIALS AND METHODS). We assayed genomic cosmid clones from this ~100-kb region for their ability to restore self-fertility to spe-29 hermaphrodites when introduced into the germ line. One cosmid, F25H8, significantly increased the self-fertility of mutants (data not shown). The sequence from this cosmid and its predicted genes was available from the C. elegans SEQUENCING CONSORTIUM (1998). We used this information to design subclones that contained individual

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**TABLE 4**

<table>
<thead>
<tr>
<th>Hermaphrodite genotype*</th>
<th>Average self-progeny</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>spe-29 (virgin)</td>
<td>3.9 ± 0.7</td>
<td>18</td>
</tr>
<tr>
<td>spe-29 (× fer-1 male)</td>
<td>214 ± 8.5</td>
<td>15</td>
</tr>
<tr>
<td>spe-12 (virgin)</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>spe-12; spe-29 (virgin)</td>
<td>60 ± 5.7</td>
<td>20</td>
</tr>
<tr>
<td>spe-12; spe-29 (× fer-1 male)</td>
<td>0.1 ± 0.1</td>
<td>10</td>
</tr>
<tr>
<td>spe-27 (virgin)</td>
<td>55 ± 7.8</td>
<td>16</td>
</tr>
<tr>
<td>spe-27; spe-29 (virgin)</td>
<td>0.1 ± 0.1</td>
<td>15</td>
</tr>
<tr>
<td>spe-27 (× fer-1 male)</td>
<td>196 ± 16</td>
<td>20</td>
</tr>
<tr>
<td>spe-27; spe-29 (virgin)</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>spe-27; spe-29 (× fer-1 male)</td>
<td>50 ± 5.0</td>
<td>29</td>
</tr>
<tr>
<td>spe-27 (× fer-1 male; 25°)</td>
<td>2.5 ± 0.4</td>
<td>49</td>
</tr>
<tr>
<td>spe-27; spe-29 (× fer-1 male; 25°)</td>
<td>1.5 ± 0.3</td>
<td>49</td>
</tr>
</tbody>
</table>

*All animals were also homozygous for the him-5(e1490) mutation. Unless indicated, animals were incubated at 20°.

1 Self-progeny includes the sum of live progeny and dead eggs (a consequence of the him-5 mutation). Error presented is the SEM.

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Figure 4.—Genes present on the pJN115-rescuing plasmid. pJN115 contains genes F25H8.1 (5’ portion only) and F25H8.2 (predicted by GENEFINDER), the 3’ end of gene-1 (BLELLOCH and KIMBLE 1999), and spe-29. The 5’ end of each gene is marked with an arrow showing the direction of transcription. The location of the it127 lesion within the spe-29 gene is indicated with a line (see Figure 6).
TABLE 5

<table>
<thead>
<tr>
<th>Construct</th>
<th>F25H8.2*</th>
<th>spe-29*</th>
<th>Rescued lines</th>
<th>Self-progeny</th>
<th>Maximum progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>—</td>
<td>40</td>
<td>NA</td>
<td>4</td>
</tr>
<tr>
<td>pJN115</td>
<td>+</td>
<td>+</td>
<td>64</td>
<td>7</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>pJN136</td>
<td>—</td>
<td>+</td>
<td>72</td>
<td>13</td>
<td>81 ± 15</td>
</tr>
<tr>
<td>pJN142</td>
<td>+</td>
<td>—</td>
<td>123</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

* + or — refers to the presumed functionality of the indicated gene in each construct. See MATERIALS AND METHODS or details on gene disruption and construct synthesis.

**n** sample size of F1 transformants (Rol phenotype).

F1 transformants were judged to be rescued if their F2 brood sizes were ≥24 (2 standard deviations greater than the maximum number of progeny observed in untransformed animals) and contained some Rol individuals.

Average self-progeny numbers were calculated only from rescued individuals, except for control injections, where broods from all F1 transformants were averaged to determine the baseline fertility.

We searched for transcribed sperm-specific genes in this genomic interval by identifying oligonucleotides that could amplify a small cDNA from a *fem-3* (sperm) cDNA library but not a *fem-1* (oocyte) cDNA library. By utilizing this strategy, we pieced together a full-length cDNA that incorporated the *it127* lesion present in *spe-29* mutants (see MATERIALS AND METHODS). The sequence of this cDNA corresponded to a single small gene located entirely in the region between F25H8.2 and *gon-1* (Figure 4).

Using probes from the full-length cDNA, we detected a single sperm-specific transcript equal in size to the originally observed transcript (~400 bp) and full-length cDNA (~307 bp, see below; Figure 5).

To verify that *spe-29* was this small, previously unpredicted gene rather than the upstream gene F25H8, we created derivatives of the rescuing plasmid pJN115 that contained frameshift mutations in either F25H8.2 (pJN136) or *spe-29* (pJN142; see MATERIALS AND METHODS) and assayed their ability to restore self-fertility to *spe-29* hermaphrodites (Table 5). pJN136, which contained mutated F25H8.2 but wild-type *spe-29*, restored self-fertility to *spe-29* hermaphrodites as effectively as pJN115. However, pJN142, which contained wild-type F25H8.2 but mutated *spe-29*, failed to restore self-fertility to *spe-29* hermaphrodites. On the basis of the identification of a missense mutation in this gene, its sperm-specific expression, and its ability to restore self-fertility to *spe-29* hermaphrodites, we conclude that it is indeed *spe-29*.

*spe-29* is divided by three introns and potentially encodes a small, quite basic (pI = 9.6) peptide of 66 amino acids (Figure 6). While the predicted protein is novel, SPE-29 contains a strongly predicted transmembrane domain that occupies a full third of the protein; only...
Spermatids initiate activation upon receiving either of two activation signals: one that originates from within the hermaphrodite or one that is introduced by the male during mating. The signal, through the action of SPE-8, SPE-12, SPE-27, and SPE-29, likely induces an increase in pHi. Morphogenetic rearrangements that lead to spermatozoon formation follow. SPE-12 (which is localized to the cell surface) and SPE-29 (which contains a transmembrane domain) are tentatively placed upstream of SPE-27 (a predicted cytoplasmic protein). In this model, absence of the SPE proteins would only impair the effectiveness of the pathway, such that weak hermaphrodite activator could not induce activation but more potent male activator could induce activation (albeit less effectively than in wild-type spermatids).

**DISCUSSION**

**SPE-29 is needed for sperm activation by both male and hermaphrodite activators:** In severe alleles of spe-8, spe-12, and spe-27, spermatids do not activate at all in virgin hermaphrodites, while spermatids in mated animals (both self-sperm and male-derived sperm) activate with a lower efficiency than wild-type spermatids (NANCE et al. 1999). The unusual phenotype of these mutants suggests that the corresponding wild-type gene products function in an activation signaling pathway that responds to either a hermaphrodite signal or a male signal introduced by mating, which is presumably carried in the seminal fluid (SHAKES and WARD 1989; MINNITI et al. 1996; Figure 7). In this model, sperm could activate when supplied with either activator; the difference in phenotype of sterile hermaphrodites and fertile males could occur if hermaphrodite activator were less effective than male activator. Mutations in these genes would impair the signaling pathway such that weak hermaphrodite activator could not trigger activation, but stronger male activator could induce at least some sperm to activate.

The Spe-29 phenotype suggests that SPE-29 function may be needed only when sperm utilize the hermaphrodite activator since self-sperm in virgin hermaphrodites do not activate, yet both male-derived and self-sperm in mated hermaphrodites activate normally. However, this phenotype could also arise if the it127 mutation resulted in a partial loss of SPE-29 function sufficient to prevent activation via the hermaphrodite activator but retained enough activity to allow normal activation via the male activator. Several observations suggest that this is the case. First, unlike spe-12 null mutants, spe-29 hermaphrodites are slightly fertile, so some mutant sperm successfully activate even in virgin hermaphrodites. Second, some spe-29 spermatids activate to spermatozoa when treated in vitro with proteases (this study) or the chloride channel blocker DIDS (MACHACA et al. 1996); this is in sharp contrast to spe-12 spermatids, which never activate spermatids when treated in vitro with proteases (this study) or the chloride channel blocker DIDS (MACHACA et al. 1996). This third, while the Spe-29 phenotype is shared by some spe-8 mutants (sperm activate inefficiently when utilizing male activator), at least one spe-8 allele results in a more severe Spe-12-like phenotype (sperm activate inefficiently when utilizing male activator; NANCE et al. 1999). These observations demonstrate that presumably hypomorphic alleles of a gene known to function in male activation cause less severe phenotypes than other, presumably null, alleles of this gene. Finally, the it127 lesion is predicted to result in a single-amino-acid substitution, an alteration that could cause a partial loss of function of the mutant protein. Together, these observations suggest that spe-29(it127) is a hypomorphic allele and that spe-29 null mutants would have the same pheno-
type as spe-8, spe-12, or spe-27 null mutants. However, it remains possible that spe-29(it127) is a null allele and that spe-29 plays a less critical role in sperm activation than do spe-8, spe-12, and spe-27.

Isolation of spe-29 null mutants would be the best way to determine if spe-29 is needed for sperm activation utilizing male activator, but this was not practical since the gene is exceptionally small and closely flanked by neighboring genes so that it provides an extremely small target for random mutagenesis. In addition, RNA-mediated interference with spe-29 double-stranded RNA fails to produce any phenotype (as is true with nearly all spe genes assayed; data not shown; E. B. Davis, unpublished observations). Instead, we addressed this question by examining the effect of spe-29(it127) on sperm activation utilizing male activator in a sensitized genetic background. We found that spe-27 spe-29 double-mutant spermatids were transactivated less efficiently than in either single mutant alone, indicating that spe-29 is required for spermatids to activate efficiently in response to male activator.

**SPE-12, SPE-27, and SPE-29 may function in a single signaling pathway:** Despite the remarkable similarity in phenotype between spe-8, spe-12, spe-27, and spe-29 mutants, we had no evidence that their encoded proteins were required for the function of a single signaling pathway. However, several genetic arguments described here support this hypothesis.

We have demonstrated that mutations in either spe-12 or in spe-29, while completely recessive alone, function as dominant enhancers of the phenotype of a hypomorphic spe-27 mutant in sperm that activates using hermaphrodite activator. Likely hypomorphic spe-29 and spe-27 mutations also act synergistically to reduce the efficiency of activation when sperm utilize male activator (discussed above). There are several interpretations of such genetic interactions. First, spe-29 (and spe-12) could enhance the Spe-27 phenotype indirectly. This phenomenon has been observed in the fly eye, where genes such as Notch, Star, and hedgehog, which affect growth within the morphogenetic furrow of the developing eye disc, dominantly enhance the phenotype of glass mutants (MA et al. 1996). The Glass transcription factor clearly functions well downstream of these genes in a distinct pathway that controls the terminal differentiation of eye photoreceptor cells. To avoid detecting such indirect genetic interactions, it is important to look for interactions among alleles of genes that are hypothesized to function toward the same endpoint. Since spe-12, spe-27, and spe-29 all have remarkably similar phenotypes that implicate their involvement in transducing an activation signal, it is likely that enhancement of the Spe-27 phenotype by these mutants is direct.

Direct genetic interactions among mutants implies that the wild-type gene products function in the same biochemical pathway (Gu et al. 1996; Qu et al. 1999) or in partially redundant pathways (Thomas 1993). If two genes are required for the function of a single signaling pathway, then the phenotype resulting from a null mutation in one of these genes should not be worsened by loss-of-function mutations in the other. We observed that while spe-29(it127) dramatically lowers the self-fertility of mated hypomorphic spe-27 mutants, spe-29(it127) does not reduce the self-fertility of mated spe-12 null mutants or spe-27 mutants grown at 25°C (a temperature at which they behave as null mutants). These results are consistent with SPE-12, SPE-27, and SPE-29 functioning in a common sperm activation signaling pathway, though it remains possible that they act in separate pathways that lead to sperm activation.

Known null mutations in spe-12 (as well as likely null mutations in spe-8 and spe-27) completely block the activation of sperm in virgin hermaphrodites. However, mutant sperm that have been exposed to male seminal fluid are capable of activating, since spe-12 males are somewhat fertile and since some self-sperm in a spe-12 hermaphrodite can be transactivated. These observations suggest that the products of the activation genes may only have a supplementary role in the initiation of sperm activation, perhaps functioning to localize or stabilize effectors of the activation signal. Alternatively, male seminal fluid may contain an additional sperm activator that can initiate activation at a low level through a distinct pathway that does not require spe-12 and the other activation genes.

**Possible functional roles of SPE-29:** Defective activation of spe-29 spermatids in vitro (in protease) indicates that SPE-29 is not functioning as an exogenous activation signal (or a protein required for activator synthesis or delivery) and suggests that SPE-29 functions either to transduce the activation signal or properly localize or modulate proteins that are transducing this signal. The spe-29 sequence reveals little about the likely molecular function of its product. SPE-29 is exceptionally small (a predicted 66 amino acids) and quite basic (pI = 9.6); its only feature of note is a strongly predicted internal membrane domain.

One interpretation of the observed genetic interactions among spe-12, spe-29, and spe-27 alleles is that the wild-type gene products interact directly. Since SPE-12 is localized to the spermatid plasma membrane (NANCE et al. 1999) and SPE-29 is predicted to be an integral membrane protein, one model for their function is that SPE-29 joins SPE-12 at the cell surface in a signaling complex that responds to the hermaphrodite and male activators (Figure 7). SPE-27, a predicted cytoplasmic protein, may function downstream of the membrane proteins (MINNITI et al. 1996; Figure 7).

It is not known how these proteins mediate their signaling effects. One possibility, given that protease treatment of normal spermatids is sufficient to induce activation (WARD et al. 1983), is that SPE-12 and/or SPE-29 is proteolytically cleaved by an endogenous acti-
vator to commence the signaling cascade. These proteins are required at least indirectly for in vitro protease activation since spe-8, spe-12, spe-27, and spe-29 spermatids activate defectively in proteases (Shakes and Ward 1989; Minniti et al. 1996; Figure 1).

The signaling pathway induces multiple changes in the spermatid leading to pseudopod formation. These include membrane rearrangements, membrane fusion, and assembly of the major sperm protein cytoskeleton to form the pseudopod. All of these processes can be induced simply by increasing the intracellular pH of spermatids with protonophores or weak bases (Ward and Carrel 1979; Nelson and Ward 1980). Spermatozoa induced by pH increase appear normal and are capable of fertilization (Ward et al. 1983; Lamunyon and Ward 1994). pH is also known to be a vital element in the control of sperm activation in the nematode Ascaris; upon activation with purified male vas deferens extract, the pH of Ascaris spermatids rises and a stable pH gradient is established in the pseudopod where it is thought to directly regulate the assembly of major sperm protein fibers. SPE-8, SPE-12, SPE-27, and SPE-29 may act to increase intracellular pH since spe-8, spe-12, spe-27, and spe-29 spermatids activate to form normal spermatozoa when their pH is increased in vitro with the weak base triethanolamine (Shakes and Ward 1989; Minniti et al. 1996; this study; Figure 7). Since a pH increase can bypass the entire activation signaling pathway it is likely that the endpoint of the pathway is regulation of intracellular pH, which would serve as a second messenger controlling the morphogenesis of spermatids.

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Literature Cited


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