Mutation of a Putative Sperm Membrane Protein in *Caenorhabditis elegans* Prevents Sperm Differentiation but Not Its Associated Meiotic Divisions

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Abstract. Spermatogenesis in the nematode *Caenorhabditis elegans* uses unusual organelles, called the fibrous body-membranous organelle (FB-MO) complexes, to prepackage and deliver macromolecules to spermatids during cytokinesis that accompanies the second meiotic division. Mutations in the spe-4 (spermatogenesis-defective) gene disrupt these organelles and prevent cytokinesis during spermatogenesis, but do not prevent completion of the meiotic nuclear divisions that normally accompany spermatid formation. We report an ultrastructural analysis of spe-4 mutant sperm where the normally close association of the FB's with the MO's and the double layered membrane surrounding the FB's are both defective. The internal membrane structure of the MO's is also disrupted in spe-4 mutant sperm. Although sperm morphogenesis in spe-4 mutants arrests prior to the formation of spermatids, meiosis can apparently be completed so that haploid nuclei reside in an arrested spermatocyte. We have cloned the spe-4 gene in order to understand its role during spermatogenesis and the molecular basis of how mutation of this gene disrupts this process. The spe-4 gene encodes an ~1.5-kb mRNA that is expressed during spermatogenesis, and the sequence of this gene suggests that it encodes an integral membrane protein. These data suggest that mutation of an integral membrane protein within FB-MO complexes disrupts morphogenesis and prevents formation of spermatids but does not affect completion of the meiotic nuclear divisions in *C. elegans* sperm.

Development of sperm in the nematode *Caenorhabditis elegans* offers a number of advantages as a model system for studies of cellular morphogenesis. Spermatogenesis occurs via a series of steps that are cytologically distinct, and these stages occur sequentially along the length of the gonad. Additionally, many aspects of spermatogenesis will occur in vitro and many genes in which mutations specifically arrest spermatogenesis have been recovered. The mature spermatozoon that results from this differentiation pathway has a single pseudopod and moves by crawling on the substrate; nematode sperm lack both a flagellum and acrosome (for review see Kimble and Ward, 1988).

Unequal partitioning of cellular constituents during cell division can play an important role during development (for review see Davidson, 1986). In *C. elegans*, unequal cytoplasmic partitioning occurs at several stages during spermatogenesis. During meiosis II, for instance, part of the system that ensures the orderly segregation of components to the spermatid are unusual organelles called the fibrous-body membranous organelle (FB-MO) complexes. Several of the genes in which mutations disrupt aspects of spermatogenesis (spermatogenesis-defective) do so by affecting cytoplasmic partitioning (Hirsh and Vanderslice, 1976; Ward, 1986; Ward and Miwa, 1978; Argon and Ward, 1980; Edgar, 1982; Burke, 1983; Sigurdson et al., 1984; L'Hernault et al., 1988; Shakes, 1988, 1989a,b). Several spe mutants have defects in formation and/or function of the FB-MO complexes and a subset of these mutants also prevent formation of haploid spermatids (Ward et al., 1981; Shakes and Ward, 1989b; Varkey, J., and S. Ward, unpublished observations).

Mutations in the spe-4 gene disrupt the coordination of cytokinesis with meiotic nuclear divisions during spermatogenesis so that a spermatocyte-like cell that contains four haploid nuclei forms instead of four spermatids (L'Hernault et al., 1988). In this paper, we present electron microscopic analysis showing that developing spe-4 spermatocytes contain abnormal FB-MO complexes. An allele specific restriction fragment length polymorphism (RFLP) and transgenic rescue have been used to localize and clone the spe-4 gene. Molecular analysis suggests that spe-4 encodes a 465 amino acid integral membrane protein that is expressed during spermatogenesis. We suggest that, as a likely FB-MO complex protein, the spe-4 product might play a role in spermatid formation during meiosis II.
Table I. Single Stranded DNA Primers Used for Primer Extension and PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Sequence</th>
<th>Primer #3 Hybridizes to the 5' End of Exon 2 and the 3' End of Exon 1; It has No Homology to Introns</th>
<th>Position in spe-4 Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATTACCTGTCTAAAAATGGACAC</td>
<td>1-23; Sense primer</td>
<td>1-23; Sense primer</td>
</tr>
<tr>
<td>2</td>
<td>CCAAATAAGACATGTCAGTACATTAGGCC</td>
<td>91-120; Antisense primer</td>
<td>91-120; Antisense primer</td>
</tr>
<tr>
<td>3</td>
<td>CCCACCTCCATTTGAAAATGCTCCCATCC</td>
<td>213-222; Antisense primer</td>
<td>213-222; Antisense primer</td>
</tr>
<tr>
<td>4</td>
<td>TTGGCCGTTTTAGCACGCAGTGGG</td>
<td>619-641; Sense primer</td>
<td>619-641; Sense primer</td>
</tr>
<tr>
<td>5</td>
<td>TCGAGATGGCAACATGCTGCT</td>
<td>1036-1058; Antisense primer</td>
<td>1036-1058; Antisense primer</td>
</tr>
<tr>
<td>6</td>
<td>CGACTAATGATTTCTGTCGC</td>
<td>last 21 nt.'s; Antisense primer</td>
<td>last 21 nt.'s; Antisense primer</td>
</tr>
<tr>
<td>7</td>
<td>GAAACACATGTCAGTACGTAGTAC</td>
<td>n.a.; Bluescript vector primer</td>
<td>n.a.; Bluescript vector primer</td>
</tr>
</tbody>
</table>

* See Fig. 7 for the numbered positions of primers #1-5. Primer #3 hybridizes to the 5' end of exon 2 and the 3' end of exon 1; it has no homology to introns. Primer #6 is designed to the last 21 nucleotides of genomic sequence printed in Fig. 7. Primer #7 includes the start codon of the B-galactosidase gene in Bluescript and can be used together with an appropriate second primer to PCR amplify inserts cloned into the polylinker of this vector.
PCRs on DNA samples either to localize the deletion in the spe-4(q347) allele or to verify the presence of cloned DNA in transgenic worms. Samples containing either 100 ng of genomic or 1 ng of cosmid DNA were amplified by PCR with primers #4 and 5 (see Table I) in order to characterize the spe-4(q347) deletion. 100 µl reactions were set up under standard conditions (Innis and Gelfand, 1990). 25 cycles were performed in a Perkin-Elmer DNA Thermal Cycler under the following conditions: denaturation at 94°C for one min, annealing at 63°C for two min, and extension at 72°C for one min. PCR products were fractionated by agarose gel electrophoresis and gel bands containing the desired PCR product were excised and pooled, and the contained DNA was purified with GeneClean (Biotool, La Jolla, CA) for direct sequencing.

PCR was also performed on small numbers of worms to characterize transgenic worms and various fertile and sterile spe-4 homozgyous controls. Worms were first picked to individual plates to verify if they were fertile or laid unfertilized oocytes. Worms of the appropriate genotype were then pooled and processed for PCR by the techniques of Barstead and Waterston (1991). Aliquots containing the DNA from eight worms were subjected to 30 cycles of PCR with primer #6 and either primer #1 or #7 (see Table I for primer positions) under the following conditions: denaturation at 94°C for one min, annealing at 53°C for two min, and extension at 72°C for one min. The resulting PCR products were fractionated by agarose gel electrophoresis.

DNA was sequenced by the chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Sequenase II, U.S. Biochemicals, Cleveland, OH), and both double stranded templates in BlueScript and single stranded M13 templates were used. Sequencing was initiated from oligonucleotides complementary to previously determined sequences. One cDNA and the entire genomic region from which it was derived plus flanking regions at both ends were sequenced, the former on the sense strand and the latter on both strands. Sequences were compiled and analyzed using GENEPRO (Riverside Scientific, Bainbridge Island, WA) and FASTA (Pearson and Lipman, 1988).

Germline Transformation of C. elegans

Transformation of C. elegans by microinjection of recombinant plasmid DNA was carried out essentially as described by Mello et al. (1991). This technique involves a single, high-volume injection into the central cytoplasmic syncytium of each of the two distal gonadal arms. Worms were microinjected with either 100 µg/ml pOA1, which is a BlueScript recombinant plasmid that contains the spe-4 gene (see Results) or 10 µg/ml pOA1 mixed with 100 µg/ml pRF4. The pRF4 plasmid contains rol-6(su0068), which confers a dominant rolling phenotype that aids in identifying worms that have been genetically transformed by the microinjection procedure (Mello et al., 1991). DNA for microinjection was suspended in 10 mM Tris-HCl, 1 mM EDTA (TE), pH 7.5.

The recessive nonconditional sterility exhibited by all spe-4 mutants required that we microinject heterozygous non-Spe hermaphrodites, and then test for the effect of the transforming DNA on their Spe progeny. The recipient strain for all microinjections was gld-1(q268)/unc-13(e1091spe-4(q347))lin-10(c439). Mutation of the lin-10 gene prevents formation of a functional vulva so that embryos hatch within a "bag" of worms because they form a functional vulva, and these animals permit outcrossing to test a spe-4 transgene in other genetic backgrounds. Outcrossing was performed with DPy spe-4(q347) heterozygous males in order to establish transgenic strains in which the unc-13 lin-10 chromosomes were replaced by spe-4 chromosomes. Pedigree analysis and PCR were performed in order to ensure that these strains contained the transgene.

Results

Review of C. elegans Spermatogenesis

Development of sperm in C. elegans males has been described in detail (Wolf et al., 1978; Ward et al., 1981; Ward, 1986; Kimble and Ward, 1988) and these cellular events are reviewed in Fig. 1. The primary spermatocyte initially forms in syncytium with a cytoplasmic core called the rachis. Before the meiotic divisions, the primary spermatocyte buds off the rachis and completes development without any requirement for intimate association with other cells. As in most cells, nuclear divisions are closely coordinated with cytokinesis and other aspects of cytodifferentiation. The primary spermatocyte undergoes the two nuclear divisions of meiosis to give rise to four haploid spermatids. These spermatids form by budding off of a residual body, and spermatids only contain a subset of cellular constituents present in the secondary spermatocyte. Material within the residual body, which includes all the ribosomes and most of the actin and tubulin, is apparently resorbed (Wolf et al., 1978; Ward et al., 1981; Nelson et al., 1982; Ward, 1986). Sessile spermatids, since they lack ribosomes, complete differentiation into motile spermatozoa in the absence of cellular protein synthesis (Ward et al., 1983).

The asymmetric partitioning of cellular constituents to the spermatids as they bud off of the residual body partly occurs via specialized organelles called the FB-MO complexes, and the morphogenesis of these structures is reviewed in Fig. 2 (Wolf et al., 1978; Ward et al., 1981; Ward, 1986; Roberts et al., 1986). The MO's begin to form from the Golgi apparatus in pachytene spermatocytes, and the FB's form a short time later in close association with the MO's (Fig. 2 a). Growth of the fibrous body in the primary spermatocyte occurs within the confines of a MO-derived membrane enve-
The fibrous body (FB) develops in close association with, and is surrounded by the membranous organelle (MO) within the primary spermatocyte. The MO is separated by a collar (C) region into a head (speckled region at left) and body (region to the right of the collar). (b) The FB-MO complex reaches its largest size within mature spermatocytes. The double layered MO-derived membrane surrounds the striped fibers within the FB, and the fibers of the FB contain MSP; (c) the membranes surrounding the FB retract and fold up as the FB begins to disappear and disperse its contents during budding of spermatids from the residual body; (d) the head of each MO (arrow) moves to a position just below the plasma membrane (PM) of the spermatid, and the FB's disappear; (e) the head of the MO fuses at the collar to the plasma membrane and exocytoses its contents (speckles at the arrow) onto the cell surface. A permanent fusion pore remains at the point of each MO fusion (each cell has many MO's).

Figure 2. Summary of morphogenesis of the FB-MO complex. (a) The fibrous body (FB) develops in close association with, and is surrounded by the membranous organelle (MO) within the primary spermatocyte. The MO is separated by a collar (C) region into a head (speckled region at left) and body (region to the right of the collar); (b) the FB-MO complex reaches its largest size within mature spermatocytes. The double layered MO-derived membrane surrounds the striped fibers within the FB, and the fibers of the FB contain MSP; (c) the membranes surrounding the FB retract and fold up as the FB begins to disappear and disperse its contents during budding of spermatids from the residual body; (d) the head of each MO (arrow) moves to a position just below the plasma membrane (PM) of the spermatid, and the FB's disappear; (e) the head of the MO fuses at the collar to the plasma membrane and exocytoses its contents (speckles at the arrow) onto the cell surface. A permanent fusion pore remains at the point of each MO fusion (each cell has many MO's).

Figure 3. Aberrant morphogenesis of the FB-MO complexes in spe-4 mutants. (A and B) Developing FB-MO complexes in dpy-5 (control; A) and spe-4(q347) dpy-5 (B) primary spermatocytes. The association of FB's with MO's and the presence of a double layered membrane around the FB observed in controls is not seen in spe-4. (D and H) Division of spe-4 spermatocytes. Both micrographs depict cells with at least two nuclei (there could be two more nuclei in each of these cells in another section plane). Sometimes more than one nucleus resides in a cell that is not attempting to divide (D; q347) and other times the cell is obviously constricted (H; hc81). Note distended MO's that are not associated with FB's; the latter are not membrane bound. Also note the granular "necklace" (arrowheads) surrounding the nuclei, and vacuoles (V) that are not observed in wild type and are presumed to be MO-derived in D. (G) Higher magnification view of an example of abnormal MO's in spe-4(q347) terminal spermatocytes. (C) Control spermatids budding from the residual body (not evident in this section plane) in which the FB's are still at least partly membrane bound and (F) after completion of budding and completion of FB disassembly. Note the prominent MO's near the cell surface. (E) Region of a spermatozoon showing a MO that has fused with the cell surface. A permanent fusion pore (arrow) is evident. Bars: (A-D, F, and H) 1 μm; (E and G) 0.5 μm.
The MOs move to the cell surfaces (Fig. 3 f). The wild type during meiosis H (Fig. 3 c) and the FBs disassemble while FBs remain surrounded by a double membrane until the spermatid buds off the residual body (Wolf et al., 1978; Ward et al., 1981; Roberts et al., 1986). The MOs then fuse with the cell surface and undergo exocytosis during formation of the mature spermatoozoon (Fig. 3 e). We have not observed spe-4 terminal spermatocytes (Fig. 3, d, g, and h) that contained mature spermatozoon (Fig. 3 e). We have not observed any MOs like those found in wild-type spermatids (Fig. 3, c and f) or spermatozoa (Fig. 3 e).

Genetics

The spe-4 gene has been mapped to chromosome I and shown to complement sDf5 but failed to complement sDf6 (L'Hernault et al., 1988). This placed spe-4 near the unc-15 unc-13 interval, which is <0.25 map unit, and to the right of unc-15 (Rose and Bailtie, 1980) (Fig. 4 a). We recovered Unc recombinants from spe-4(hc78)/unc-15(e73) unc-13(e51) animals and 2/2 unc-15 animals were Spe while 0/3 unc-13 animals were Spe, which also suggests that spe-4 is to the right of unc-15. Two factor mapping in cis was then performed on unc-13(el091) spe-4(q347)/+ +. 10 complete broods comprised of 2,951 animals were analyzed; only one of 730 unc-13 animals was fertile and all 2,221 wild types were fertile. This suggests that spe-4 is ~0.7 map unit from unc-13, based on the previously described two factor mapping equation (Fig. 4 a; Brenner, 1974).

The correspondence of the genetic map with the physical map can vary in different regions of the C. elegans genome (e.g., Greenwald et al., 1987) but in the unc-15 unc-13 interval, 1 map unit is ~3,200 kb of DNA (Maruyama and Brenner, 1991). This meant that spe-4 was possibly within 225 kb of unc-13.

Molecular Localization of spe-4

The physical map of chromosome I is nearly complete in the region where spe-4 is located (Coulson et al., 1986, 1988), and we obtained a number of cosmid clones that are located in this region. Genomic DNA was prepared from C. elegans strains heterozygous for one of the three spe-4 alleles, transferred to Southern blots, and probed with several recombinant cosmid cosmids containing a portion of chromosome I in order to search for a RFLP. Two of these cosmids, C44E1 (Fig. 4 a) and ZK524 (data not shown), identified a RFLP as- 

Figure 4. Genetic and physical maps of the spe-4 region. (A) The position of spe-4 relative to other nearby genes is shown at the top of the figure. The position of spe-4 was determined by combined genetic and molecular techniques. The corresponding C. elegans physical map is shown at the bottom of the figure, and each horizontal line represents a cosmid (the construction and analysis of the physical map is explained in Coulson et al., 1986). The molecular localization of the left breakpoint of sDf6 was discovered by Maruyama and Brenner (1991) and confirmed during this study (data not shown). The spe-4 gene lies near the right end of the C44E1 insert (see Results). (B) Restriction map of the spe-4 region deduced from genomic DNA. The lines under the map are cDNA (pMA7) or cloned, genomic restriction fragments that were sequenced (pMA2 and pMA6) or used for transgenic rescue (pOA1).
Figure 5. (A) DNA polymorphisms in spe-4. DNA was prepared from wild type N2 and the heterozygous balanced spe-4 strains sDf5/spe-4(hc78), sDf5/spe-4(hc81), and sDf5/spe-4(q347). 5 μg of DNA was digested with EcoRV, subjected to electrophoresis in a 0.7% agarose gel, transferred onto Hybond N, and hybridized to 32P-labeled cosmid C44E1. The arrowhead indicates the polymorphic restriction fragment associated with spe-4(q347). The arrow indicates the corresponding wild type restriction fragment. (B) Northern hybridization with a spe-4-specific probe. Total RNA was prepared from fem-1(hcl7) or fem-3(q23) worms reared at 25°C. Under these growth conditions, both of these mutant strains have female somatic tissues but the germline contains oo- and sperm, while the germline in fem-1(hcl7) contains oo- but no sperm, while the germline of fem-3(q23) contains sperm but no oo-ocytes. 20 μg of total RNA was subjected to electrophoresis in a 1.4% agarose gel containing formaldehyde, transferred to nylon and hybridized to the spe-4 genomic clone pMA2 (see Fig. 4 for the position of pMA2). The probe detects one ~1.5-kb spermatogenesis specific RNA. The sex nonspecific 5.9-kb mRNA encoded by unc-13 (Maruyama and Brenner, 1991) is present in these fem-1 and fem-3 RNA samples and has been detected with unc-13 specific probes (data not shown). For both A and B, size standards are indicated along the left margin of each figure.

These three recombinant plasmids were used as probes, DNA sequencing templates, and/or constructs for creation of transgenic worms, as described below. The right breakpoint of cosmid C44E1 lies between the rightmost EcoRI and EcoRV restriction sites depicted in Fig. 4 b (data not shown). This places the spe-4 gene at the right end of the C44E1 recombinant insert and to the right of unc-13, which begins closer to the other end of this cosmid (Maruyama and Brenner, 1991). Both unc-13 and spe-4 are transcribed left to right, and a near full length unc-13 cDNA does not hybridize to either pMA2 or pMA6 (data not shown).

The ultrastructural phenotype of spe-4 mutants suggests that a polypeptide within sperm might be affected by mutation of this gene. These data suggest that spe-4 transcription might occur only within animals engaged in spermatogenesis. This was examined by performing Northern hybridizations to blots containing RNA from worms that either contained sperm (genotype: fem-3(q23)gf raised at 25°C) or lacked sperm (genotype: fem-1(hcl7)gf raised at 25°C). An ~1.5-kb RNA was found only in fem-3 RNA when this blot was probed with a wild type 1.8-kb restriction fragment that included the region affected by the q347 deletion (pMA2, see Fig. 4 b for its position). Both fem-1(hcl7) and fem-3(q23) have female somatic tissues and, since they differ only in their germline, this suggests that pMA2 contains part of a gene that is spermatogenesis or sperm specific (Fig. 5 b).

Complementary DNA clones corresponding to the ~1.5-kb RNA were isolated by screening C. elegans libraries with a pMA2 insert probe. Insert size and Southern hybridization experiments (data not shown) indicated that the largest cDNA, pMA7, was near full length (see below), and within a region encompassed by subclones pMA2 and pMA6 (Fig. 4 b).

The above-mentioned genetic and molecular data all are consistent with a spe-4 location near and to the right of unc-13 on chromosome I. We sought to confirm the location of spe-4 by microinjecting worms with a recombinant plasmid that contained DNA from the presumed spe-4 region. A total of 34 gld-1/fem-3 spe-4 lin-10 heterozygous hermaphrodites were microinjected with the recombinant plasmid pOA1 (for the position of pOA1, see Fig. 4 b); 18 of these 34 hermaphrodites were co-injected with the rol-6 plasmid pRF4 as a dominant behavioral marker. Fertility was restored to spe-4 worms microinjected with pOA1, and the results of these experiments are summarized in Table II. The pOA1 plasmid contains a 3.8-kb EcoRV restriction fragment that includes the entire putative spe-4 transcription unit, ~450 bp 5' to the transcription start and ~1,200 bp 3' to the proposed polyadenylation signal (see below). Three independently derived stable transformed lines were created and, in one case, a transgenic line (ebEx3) was outcrossed to replace both unc-13 spe-4 lin-10 chromosomes with spe-4 chromosomes. This outcross was performed in order to eliminate the lin-10 gene because its vulvalless phenotype results in broods that are much smaller than non-
Table II. Characterization of Three spe-4 Transgenes

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Chromosome I genotype</th>
<th>Germline inheritance of transgene</th>
<th>Brood size (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>unc-13 spe-4 lin-10</td>
<td>-</td>
<td>0 (n = 45)</td>
</tr>
<tr>
<td>ebEx1</td>
<td>unc-13 spe-4 lin-10</td>
<td>19 of 70 were fertile</td>
<td>13 ± 7 (n = 21)</td>
</tr>
<tr>
<td>ebEx2</td>
<td>unc-13 spe-4 lin-10</td>
<td>33 of 58 were fertile</td>
<td>20 ± 8 (n = 20)</td>
</tr>
<tr>
<td>ebEx3</td>
<td>unc-13 spe-4 lin-10</td>
<td>ND</td>
<td>28 ± 9 (n = 12)</td>
</tr>
<tr>
<td>-</td>
<td>unc-15 + lin-10</td>
<td>-</td>
<td>27 ± 13 (n = 10)</td>
</tr>
<tr>
<td>ebEx3</td>
<td>+ spe-4 +</td>
<td>103 of 128 were fertile*</td>
<td>169 ± 50 (n = 12)</td>
</tr>
<tr>
<td>ebEx3</td>
<td>+ + +</td>
<td>ND</td>
<td>212 ± 48 (n = 11)</td>
</tr>
</tbody>
</table>

Stable transgenic strains bearing the extrachromosomal arrays ebEx1, ebEx2, or ebEx3 were recovered from unc-13(e1091) spe-4(q347) lin-10(e1439)I hermaphrodites that were microinjected with plasmid DNA. Inheritance of these transgenes and the brood sizes of transgenic hermaphrodites were compared to non-transgenic control strains. These transgenes all contain the spe-4 plasmid pOA1, and ebEx2 and ebEx3 also contain the rol-6(sul004)II plasmid pRF4 dominant selectable marker (see Materials and Methods). Brood sizes were determined for the bracketed number of hermaphrodites (n) and are presented as the mean number of progeny with the standard deviation (SD). ebEx3 was crossed into a spe-4(q347) or wild type chromosome I background in order to examine the brood size of non-Lin non-Unc hermaphrodites carrying this transgene.

* ~6% of the worms that inherit ebEx3 are mosaics that are either sterile rollers, fertile non-rollers, or express the rolling phenotype in only a portion of their cuticle. ND, not determined.

Lin hermaphrodites (Table II). Construction of this strain relied on following the dominant rolling phenotype associated with transgenes that include a rol-6(sul004)II mutant plasmid, such as ebEx3 (Mello et al., 1991). The continued presence of spe-4 wild type sequence within the ebEx3 transgene following this cross was directly demonstrated by PCR in which an amplified product formed only when spe-4 sequences were continuous with at least a portion of the Bluescript cloning vector (Fig. 6). The resulting ebEx3/spe-4 hermaphrodites had an average brood size that was ~81% of ebEx3/+ non-spe-4 controls (Table II). Detailed examination of the progeny of ebEx3/spe-4 hermaphrodites indicated that this transgene behaves like an extrachromosomal array that is mitotically unstable (Table II), which could explain why ebEx3/spe-4 did not have a wild type brood size. Consequently, it appears that pOA1 contains most, or perhaps all, sequence information required to restore fertility to spe-4 hermaphrodites.

**Sequence of the spe-4 Gene**

We determined the sequence of cDNA pMA7 on the sense strand and the corresponding, as well as flanking, genomic region on both strands (Fig. 7). Comparison of the genomic and cDNA sequences reveals that the spe-4 gene is composed of eight exons, and that pMA7 has a 10 nucleotide polyA tail at its 3' end. The polyA tail is preceded by an imperfect match to the AATAAA consensus polyadenylation signal (Proudfoot and Brownlee, 1976). Primer extension indicates that the 5' end of the spe-4 transcript is 23 nucleotides longer than cDNA pMA7 (Fig. 8a). This experiment predicts that the 5' end of the spe-4 transcript is at position 1 (Fig. 7), assuming no additional intron splicing. A sense primer (primer #1, Table I) at this presumed 5' end was then used to show that it could prime PCR sense strand products following reverse transcription PCR with an antisense primer (primer #3, see Table I). The resulting product of this PCR closely corresponds in size to the predicted 236-bp fragment (Fig. 8b), indicating that nucleotides 1-23 of the genomic sequence are found in the spe-4 mRNA and are not interrupted by an intron. Furthermore, both primer extension and reverse transcription PCR confirm that the transcription of spe-4 occurs only in animals engaged in spermatogenesis.

The data discussed above establish the transcription initiation point and indicate that the spe-4 transcript is 1,484 nucleotides without the polyA 3' tail (Fig. 7). The first ATG responsive to translation occurs at position 1 (Fig. 7), assuming that this band is also present in lane 8). The size of molecular weight standards appear along the left margin of the figure.
Figure 7. Sequence of the spe-4 gene and flanking regions. A total of 2,539 nucleotides of genomic sequence are shown with the deduced amino acid sequence appearing below the line in one-letter code. The transcription start, based on primer extension, is indicated by +1. The proposed initiator methionine begins at position +16 and the termination codon begins at position 2,511.

cDNA sequence appears in upper case, non-italicized letters, introns and flanking regions in lower case. The positive numbers at the right are nucleotide positions from the sequence of cDNA, the 5'23 nucleotides are upper case and italicized to reflect the sequence data are available from EMBL/GcnBank/DDBJ.

Downloaded from jcb.rupress.org on July 9, 2017
ORF and a table of codon usage provided by C. Fields (Institute for Genomic Research, Gaithersburg, MD). The vertical position of dots

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Figure 9. Analysis of ORFs within the pMA7 cDNA sequence and the ORF that extends from the 5' end of pMA7 to position -108 in the
genomic sequence (see Fig. 7). All six potential reading frames are displayed by the GENEPRO program (Riverside Scientific), which
can evaluate the codon usage within ORFs. Any potential ORFs that start with a methionine are indicated by a thick dashed horizontal
bar. Superimposed on the horizontal bars are a series of dots. Each dot represents a comparison of the codon used at that position in the
ORF and a table of codon usage provided by C. Fields (Institute for Genomic Research, Gaithersburg, MD). The vertical position of dots
relative to the horizontal dashed bar indicates similarity to the C. elegans codon usage table. Dots above the bar are more similar to the
codon usage table than dots below the bar. The ORF in reading frame one (indicated on the ordinate) is open to the left border of the plot and
the first ATG methionine codon (the start of the thick, dashed bar) is the proposed translational start. This ATG (arrow; also see position
16 in Fig. 7) is followed by a single long ORF (1395 nucleotides, 465 amino acid residues) that shows excellent agreement with C. elegans
codon usage (note the large number of dots above the thick, dashed line). This ORF terminates in a TGA stop codon (at arrowhead; also
see position 1,411 in Fig. 7).

Figure 8. Primer extension and reverse transcription PCR of spe-4 transcripts. For both techniques, the template total RNA was either
fem-3 (which has a female soma and makes sperm but not oocytes),
him-5 males (male soma and germline) or, as a negative control,
fem-1 (female soma and germline). (a) Identification of the 5' end
of the spe-4 transcript by primer extension. The three rightmost
lanes contain the products of a primer extension reaction using a 32P end-labeled oligonucleotide (primer #2, see Table I) hybrid-
ized to 20 µg of total RNA. The size of the DNA products of reverse
transcription was determined by comparison to DNA sequencing
reactions primed with primer #2 and shown in the four leftmost
lanes. These primer extension results indicate that the 5' end is an
A (the sense strand complement of the T at the arrow), which is po-
sition +1 in Fig. 7. Note that both fem-3 and him-5 (which have sperm) form products (arrowheads) while no product is formed
from fem-1 females (which lack sperm). (b) Confirmation of the 5'
end of the spe-4 transcript by reverse transcription PCR. Reverse
transcription was performed on 250 ng of total RNA hybridized to
primer #3. Primer #1, designed to be at the 5' transcription start
determined by primer extension (see Fig. 8 a), was then added,
PCR was performed, and the three rightmost lanes of this ethidium
bromide stained 1.5% agarose gel contain 5% of the products of
these reactions. Note that both fem-3 and him-5 (which have sperm)
form the expected 236-bp product (arrowhead) while no product
is formed from fem-1 females (which lack sperm). The size stan-
dards for b are a 100-bp DNA ladder, and the positions of the 100-
200-, and 300-bp fragments appear along the left margin of the
figure.
probe is homologous to two EcoRl/KpnI restriction fragments that
eosmid and all samples of (7.
products of 1 ng C44E1 recombinant cosmid
the wild type product
L'Hernault and Arduengo C.
sDf3/spe-4(hc78), sDjff /spe-4 (hc81),
Figure 10
golabeled large EeoRl insert fragment of the eDNA pMA7. The
on a 1% agarose gel, transferred to nylon, and hybridized to an oli-
omic DNA, or a "no added template" DNA control is shown. The
PCR was performed as described in Materials and Methods. The
stained 1.5 % agarose gel that had been loaded with 10% of the PCR
PCR primers were #4 and #5 (see Table I). An ethidium bromide
used two different heterozygous
4(q347), in
addition, produced a smaller fragment (arrow). We
were deleted by the q347 mutation are shown in bold in Fig. 7.
The position of the spe-4(q347) deletion indicates that the structure of the spe-4 protein will be affected by this mutation. The q347 deletion removes part of both exon 5 and intron 5, including the splice donor sequence between this exon and intron (Fig. 4 b). Unless a cryptic splice donor is present, the q347 deletion will shift the reading frame and add one amino acid now capable of being encoded by intron 5 (and not found in wild type spe-4 protein) followed by a TAA stop codon in intron 5. Assuming that the q347 mRNA is translated, a polypeptide of 244 amino acids rather than the wild type 465 amino acids would result.
A hydrophathy plot (Kyte and Doolittle, 1982) of the spe-4 protein indicates that it is likely to be an integral membrane protein (Fig. 11). There are at least seven regions that could span the membrane using the conservative window of 19 residues and the first, third, and sixth regions are followed by a sequence of charged residues. As compared with other membrane proteins, the deduced amino acid sequence near the NH2 terminus and the position of the first proposed membrane spanning region suggests that the spe-4 polypeptide lacks a cleavable NH2 terminal signal sequence (von Heijne, 1985). The region from residues 211–394 is hydrophilic, as it contains many charged and polar residues, and presumably faces either the extracellular space or the lumen of a vesicle. There are a total of five potential N-linked glycosylation sites in the spe-4 protein at positions 27, 159, 172, 313, and 334; we do not know if these sites are used but the last two are located within the above-mentioned hydrophilic region (Fig. 11). The spe-4 protein has no significant homology to other proteins in either the GENPEPT (update 71) or SWISS-PROT (update 21) databases. The most significant matches were to membrane spanning domains within several other integral membrane proteins (data not shown).

Discussion
We have presented phenotypic, genetic, and molecular analyses of spe-4, a gene involved in sperm morphogenesis. We have discovered that the genetic lesion within a spontaneous allele is a small deletion and, after localizing this deletion on the physical map of chromosome I, have cloned the spe-4 gene. This cloned DNA can rescue spe-4 associated sterility in germline transformation experiments. Transcription of the spe-4 gene results in an ~1.5-kb polyA + mRNA that is produced only in animals engaged in spermatogenesis. This spe-4 mRNA encodes a protein that is not homologous to

Figure 10. Further characterization of the genomic region affected by spe-4(q347). (A) 5 µg of genomic DNA from N2(wild-type), sDf5/spe-4(hc78), sDf5/spe-4(hc81), or sDf5/spe-4(q347) was dou-
ble-digested with EcoRI and KpnI and subjected to electrophoresis on a 1% agarose gel, transferred to nylon, and hybridized to an oligo-
golabeled large EcoRI insert fragment of the cDNA pMA7. The probe is homologous to two EcoRI/KpnI restriction fragments that are both ~1.3 kb (arrow). Deletion of the single KpnI site between these two EcoRI sites by the spe-4(q347) mutation results in a fusion fragment of ~2.4 kb (arrowhead). (B) PCR strategy to obtain the sequence of the region deleted by the spe-4(q347) mutation. PCR was performed as described in Materials and Methods. The PCR primers were #4 and #5 (see Table I). An ethidium bromide stained 1.5% agarose gel that had been loaded with 10% of the PCR products of 1 ng C44E1 recombinant cosmid DNA, 100 ng genomic DNA, or a "no added template" DNA control is shown. The cosmids and all samples of C. elegans genomic DNA produced the wild type product (arrowhead) while strains containing spe-4(q347), in addition, produced a smaller fragment (arrow). We used two different heterozygous spe-4(q347) strains in order to ensure that the balancer chromosome (sDf5 or nDp4) was not the tem-
plate for this smaller fragment (arrow). For both A and B, size stan-
ards are indicated along the left margin of each figure.
previously characterized proteins, but hydropathy analysis indicates that it is probably an integral transmembrane protein that passes through the lipid bilayer multiple times.

The sterile phenotype of spe-4 mutants is caused by their failure to form normal spermatids and, consequently, functional spermatozoa. All three spe-4 alleles are completely penetrant for this sterile phenotype and form terminal spermatocytes in which meiotic nuclear divisions have occurred without the usually concomitant cell divisions. The major subcellular defect revealed by ultrastructural analyses of spe-4 mutant spermatocytes is disruption of the FB-MO complexes. These structures, which are first observed in spermatocytes prior to the first meiotic division, apparently play a role that is unique to nematode sperm. Unlike flagellated sperm, where spermatids contain ribosomes engaged in translation and a lengthy time interval separates the second meiotic division from loss of ribosomes to the residual body (for review see Olds-Clarke, 1988), nematode spermatids do not contain ribosomes because they are discarded into the residual body as these cells form (Wolf et al., 1978; Ward et al., 1981). Consequently, all protein components that are necessary for subsequent differentiation into spermatozoa must be included in the residual body as these cells form (Wolf et al., 1978; Ward and Klass, 1982). These studies reveal that fiber integrity in spe-4 mutants requires neither continuous association of an FB with a MO nor the presence of a surrounding membrane. Detailed study of the early morphogenesis of FB's in spe-4 mutants should reveal how FB's form and if there is ever association of MO's with FB's.

FB-MO complexes contain several proteins that are required by the C. elegans spermatozoon, and they are segregated to the spermatid during meiosis II when this cell separates from the residual body. The membrane around each FB retracts towards its associated MO, the fibers of the FB disassemble, releasing their MSP contents to the cytoplasm (Ward and Klass, 1982; Roberts et al., 1986) and the MO moves to the cell surface. The MO then fuses with the cell surface and forms a permanent fusion pore as it releases its contents onto the cell surface by exocytosis during conversion of spermatids to spermatozoa (Wolf et al., 1978; Nelson and Ward, 1980; Ward et al., 1981). MSP localizes within the pseudopod where it appears to play a cytoskeletal role (Roberts, 1983; Roberts et al., 1989; Seppenwol et al., 1989). Unfortunately, it is presently not possible to determine whether the spe-4 protein functions in gametes because mutations in this gene arrest cellular morphogenesis prior to spermatid formation. Recovery of appropriate temperature sensitive spe-4 mutations might allow spermatids to form.
under permissive conditions. These spermatids could then be shifted to restrictive conditions in order to mutationally disrupt the spe-4 protein and examine its role within spermatids and during the conversion of spermatids into spermatozoa.

Several genes that affect FB-MO ultrastructure have been identified (e.g., Ward et al., 1981; J. Varkey and S. Ward, personal communication), and one of these genes (spe-10) causes a mutant phenotype that shares similarities with some of the spe-4-associated defects (Shakes and Ward, 1989b). In spe-10 mutants, each MO prematurely retracts the double membrane that surrounds its associated FB just before or as spermatids are forming; normally, an FB does not lose its association with an MO until after spermatid formation. These FBs do not disassemble their fibrous contents and they end up in the residual body, rather than the spermatid. The MO's are segregated to the spermatid where, instead of developing the compact mushroom-like morphology that is characteristic of wild type MO's, they become large and vacuolated. The MO's within spe-10 spermatids and the stable FB's that lack surrounding membranes are both similar in appearance to the MO's and FB's observed within spe-4 terminal spermatocytes. The mutant phenotypes of spe-4 and spe-10 both suggest that fibers within an FB that lacks a surrounding membrane only disassemble when they are within spermatids. Additionally, in both spe-10 and spe-4, the MO's are separate from the FB's at the point when they become swollen and abnormal in appearance.

Previous results had indicated that the phenotypic effects of spe-4 were probably limited to the disruption of spermatogenesis (L'Hernault et al., 1988). This study has revealed that the spe-4 gene encodes an ~1.5-kb mRNA that is expressed during spermatogenesis. Furthermore, the cloning and sequencing of spe-4 and a deletion mutation within this gene (q347) indicate that the probable spe-4 null phenotype is limited to disruption of spermatogenesis. Analysis of the spe-4 sequence suggests that it encodes an integral membrane protein of 465 amino acids. This inference is based on hydropathy plots (Kyte and Doolittle, 1982) that indicate the spe-4 protein spans the membrane several times. We have used this algorithm at a window of 19 residues for our sequence analyses. A window of this size empirically has been found to permit one to distinguish between hydrophobic regions that span the membrane and those that are located within interior regions of globular proteins. If the average hydrophathy of a region is greater than 1.6, this region probably spans a membrane (Kyte and Doolittle, 1982); the spe-4 protein has at least seven regions that meet this criterion. Integral membrane proteins are synthesized on rough endoplasmic reticulum and usually have a signal sequence that permits their passage into the ER membrane (reviewed by Singer, 1990). The proposed signal sequence in the spe-4 protein does not appear to be cleavable because it is too far from the NH2 terminus and lacks the appropriate distribution of charged residues near the putative membrane spanning domain (von Heijne, 1985). Consequently, if this model is correct, the spe-4 encoded protein should be co-translationally inserted into the endoplasmic reticulum via an internal signal sequence, and the protein should retain its NH2 terminus after completion of polypeptide synthesis.

Molecular data together with the ultrastructural analyses discussed above suggest spe-4 encodes an integral membrane protein that resides within membranes that are part of the FB-MO complex. It is also possible that the spe-4 encoded protein resides within other membranes in sperm. To address this question, we are raising antisera to the spe-4 encoded protein that has been synthesized in bacteria. Electron microscopic localization of the spe-4 antigen with specific antibodies should allow unambiguous determination of its subcellular localization in wild type spermatocytes and spermatozoa.

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