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A Novel Member of the Tob Family of Proteins Controls Sexual Fate in *Caenorhabditis elegans* Germ Cells

Pei-Jiun Chen,* Amit Singal,* Judith Kimble,† and Ronald E. Ellis*,[1]

**Department of Biology, University of Michigan, Ann Arbor, Michigan 48109; and*
†Howard Hughes Medical Institute and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Although many cell fates differ between males and females, probably the most ancient type of sexual dimorphism is the decision of germ cells to develop as sperm or as oocytes. Genetic analyses of *Caenorhabditis elegans* suggest that *fog-3* might directly control this decision. We used transformation rescue to clone the *fog-3* gene and show that it produces a single major transcript of approximately 1150 nucleotides. This transcript is predicted to encode a protein of 263 amino acids. One mutation causes a frame shift at the sixth codon and is thus likely to define the null phenotype of *fog-3*. Although the carboxyl-terminus of FOG-3 is novel, the amino-terminal domain is similar to that of the Tob, BTG1, and BTG2 proteins from vertebrates, which might suppress proliferation or promote differentiation. This domain is essential for FOG-3 activity, since six of eight missense mutations map to this region. Furthermore, this domain of BTG1 and BTG2 interacts with a transcriptional regulatory complex that has been conserved in all eukaryotes. Thus, one possibility is that FOG-3 controls transcription of genes required for germ cells to initiate spermatogenesis rather than oogenesis. This model implies that FOG-3 is required throughout an animal's life for germ cells to initiate spermatogenesis. We used RNA-mediated interference to demonstrate that *fog-3* is indeed required continuously, which is consistent with this model.

Key Words: **germ-line development; sex determination; FOG-3; Tob; BTG1; BTG2.**

INTRODUCTION

With few exceptions, animals reproduce sexually. This process usually requires that males and females develop distinctive bodies, both for mating and for specialized roles in producing and raising offspring. Many examples of sexual dimorphism, such as the formation of mammary glands by female mammals, are restricted to particular groups of animals. However, a few aspects of sexual dimorphism are common to all species. Perhaps the most ancient example is the production of oocytes by females and sperm by males. Three factors make the control of this decision particularly interesting. First, these two cell fates are strikingly different—sperm are compact, motile cells, whereas oocytes are large cells that contain many of the products needed to regulate and fuel embryonic development. Second, despite these differences, primordial germ cells appear to develop identically in both sexes. For example, in embryonic mice, the primordial germ cells of males and females appear identical until day 13, when female germ cells first enter meiosis and begin to differentiate as oocytes (McLaren, 1995). Third, although we understand how a variety of somatic fates are specified, we know much less about this process in the germ line. Thus, these studies might reveal new mechanisms for the control of cell fate. To understand how the decision of germ cells to form sperm or oocytes is regulated, we are studying the nematode *Caenorhabditis elegans.*

There are two sexes of *C. elegans.* The *XX* individuals develop as hermaphrodites; these are essentially females that produce sperm during larval development, which they store for use in self-fertilization. Upon maturation, hermaphrodites switch to producing oocytes, which they continue throughout adulthood (Hirsh *et al.*, 1976). In contrast,

[1] To whom correspondence should be addressed at the Department of Biology, University of Michigan, 3065D Natural Science Building, 830 North University Avenue, Ann Arbor, MI 48109. Fax: (734) 647-0882. E-mail: ronellis@umich.edu.

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the *XO* individuals develop as males and produce only sperm. The initial signal that regulates these sexual fates is the ratio of *X* chromosomes to autosomes (Madl and Herman, 1979; Nigon, 1951). This ratio controls the activity of *xol-1*, which acts through a cascade of regulatory genes to specify whether an animal becomes male or hermaphrodite (reviewed by Ellis, 1998; Meyer, 1997). The final genes in the cascade are *tra-1*, which promotes female development, and *fem-1*, *fem-2*, and *fem-3*, which promote male development by inhibiting the activity of *tra-1*.

How the *fem* genes and *tra-1* control each sexually dimorphic fate is not clear. However, two groups of target genes have been identified. First, *mab-3* acts downstream of *tra-1* to prevent yolk production in the male intestine (Raymond *et al.*, 1998; Shen and Hodgkin, 1988). Surprisingly, *mab-3* encodes a transcription factor that is similar to Doublesex of *Drosophila* and thus provides the first example of a sex-determination gene that has been conserved during nematode and insect evolution (Raymond *et al.*, 1998). Second, two genes that might directly regulate the decision of germ cells to become sperm or oocytes have been identified—*fog-1* and *fog-3* (Barton and Kimble, 1990; Ellis and Kimble, 1995). Both genes are required for germ cells to initiate spermatogenesis rather than oogenesis, but neither controls other sexual fates in the animal. Furthermore, because mutations in either *fog-1* or *fog-3* cause germ cells to become oocytes rather than sperm, these genes must be controlling which fate germ cells adopt, rather than being required for spermatogenesis per se. For example, *fog-1* or *fog-3 XO* animals appear male but produce oocytes. Finally, analyses of double mutants show that these genes act downstream of *tra-1* and are consistent with the hypothesis that both genes also act downstream of *fem-3*. This result suggests that *fog-3*, unlike *fog-2*, *gld-1*, or the *mog* genes, does not influence germ cell fates by modulating the activity of upstream sex-determination genes (reviewed by Ellis, 1998), but might instead control these fates directly.

To learn how *fog-3* regulates germ cell fate, we have cloned the gene. Our results show that *fog-3* produces a single major transcript, which encodes a protein of 263 amino acids. The amino-terminal portion of FOG-3 resembles that of the Tob and BTG1 proteins in vertebrates (Matsuda *et al.*, 1996). Analysis of the known *fog-3* mutations confirms the importance of this domain and demonstrates that at least one of these mutations completely inactivates the FOG-3 protein. Finally, FOG-3 is required continuously for germ cells to initiate spermatogenesis rather than oogenesis.

MATERIALS AND METHODS

Genetic Nomenclature

The genetic nomenclature for *C. elegans* was described by Horvitz *et al.* (1979), with two exceptions. First, we use "female" to designate a hermaphrodite that makes oocytes but no sperm; by definition, female worms are unable to self-fertilize. Second, we use capital letters and plain font to indicate the protein encoded by a gene. Thus, the protein produced by the *fog-3* gene is FOG-3.

Genetic Techniques

We employed techniques for culturing *C. elegans* described by Brenner (1974) and raised strains at 20°C unless indicated otherwise. All *C. elegans* strains were derived from the Bristol strain N2 (Brenner, 1974), with the exception of the following wild isolates: DH424 (Liao *et al.*, 1983), TR403 (Collins *et al.*, 1989), and AB1, CB4857, and RC301 (Randy Cassada, personal communication; Hodgkin and Doniach, 1997). In addition, we used these mutations: *unc-13(e1091)* (Waterston and Brenner, 1978), *unc-29(e1072)* (Lewis *et al.*, 1980), *spf-1(q7)* (J. Miskowski and J. Kimble, personal communication), 10 *fog-3* alleles (Ellis and Kimble, 1995), *lin-11(n566)* (Ferguson and Horvitz, 1985), *ncl-1(e1865)* (Hedgecock and Herman, 1995), *unc-36(e251)* (Brenner, 1974), *glp-1(q224*ts*)* (Austin and Kimble, 1987), *fem-3(q96*ts*)* (Barton *et al.*, 1987), *dpy-20(e1282)* (Hodgkin, 1983), and *him-5(e1490)* (Hodgkin *et al.*, 1979). We also used the deficiencies *nDf23*, *nDf24*, *nDf25* (Ferguson and Horvitz, 1985); *mnDf111* (Lundquist and Herman, 1994); and *qDf5*, *qDf8*, *qDf9*, *qDf10*, *qDf11*, *qDf12*, *qDf13*, *qDf14*, and *qDf15* (Ellis and Kimble, 1995).

Restriction Fragment Length Polymorphism (RFLP) Mapping

To identify RFLPs between different strains of *C. elegans*, we used cosmids from the *unc-29* to *lin-11* region to probe Southern blots of N2, AB1, CB4857, DH424, RC301, and TR403 DNA (Southern, 1975). The probes were prepared by labeling whole cosmids with [^{32}P]dCTP by random priming. Using the overlapping cosmids B0379 and T07H2 as probes, we detected several *Hha*I polymorphisms between N2 and CB4857 DNA. In particular, one fragment measures approximately 2.4 kb in N2 animals, but only 2.35 kb in CB4857 animals; furthermore, this polymorphism is detected by an 8.2-kb *Hin*dIII fragment we subcloned from B0379. We call the CB4857 form of this polymorphism *qP5*.

To determine the location of *fog-3* with respect to *qP5*, we isolated recombinants from *unc-13 fog-3(q470) + lin-11 [N2]/+ + qP5 + [CB4857]* heterozygotes. After establishing homozygous lines for each recombinant, we prepared DNA and analyzed it by Southern blot. Of the Unc non-Fog recombinant chromosomes, 10/10 contained *qP5*, suggesting that *fog-3* might be located to the left of *qP5*. Of the Lin non-Fog recombinant chromosomes, 5/8 contained *qP5*. These results show that *qP5* lies midway between *fog-3* and *lin-11*.

Polymerase Chain Reaction (PCR) Analysis of Deficiencies

To determine the extent of deficiencies that map to the *fog-3* region, we prepared homozygous deletion embryos, as described by Ellis and Kimble (1995), and used PCR (Mullis *et al.*, 1986; Saiki *et al.*, 1988) to test each for the presence of DNA corresponding to primers from the region. The primer pairs were designed using sequence data from cosmids we had characterized or from the published sequences of cDNAs that map in this region. After the *C. elegans* Genome Sequencing Consortium (1998) finished characterizing these cosmids, we determined the precise location of each primer by BLAST search.

First, we tested DNA from four deficiencies that complement

TABLE 1
Primers Used to Map Deficiencies in the *fog-3* Region

DNA	Primer	Sequence	Cosmid	Target site
A	tT23H11+	ATGACCGACGAGAGTACAATT	**T05F1**	27094 to 27012
	tT23H11−	AATCGCATCGCCTAGAGTCT	(−154 to −133)	
B	cm5c5+	AACATGCGTGAGGTCATCTC	**F26E4**	32520 to 32457
	cm5c5−	AGCAAGCATTACCGATTTGG	(−79 to −60)	
C	cm11d71	T(g)AGGTATTAGGAATAGGTGA	**T24D1**	12532 to 12367
	cm11d7r	TCCTTTCCTGCTCTTCGCGT	(37 to 49)	
D	cm16f9+	AAGAACAnTTTGGAnGGAACA	**T23D8**	13334 to 13252
	cm16f9−	ATTAACAGCTCGCATGTGCT	(48 to 61)	
E	RE3	TCAACATGATATGGCATCGGA	**T23D8**	21861 to 21972
	RE4	AGGCAGACTGTCATCTGGAA	(48 to 61)	
F	RE21	GAACAATACAATCTTCAACTCG	**T23D8**	29095 to 29176
	RE22	GCTCTCCTTTTCGTTTCAATTC	(48 to 61)	
G	RE13	(tc)AAGCTTCTAAATGGAAGAA	**T23D8**	33434 to 33345
	RE14	AGGCCCAACTTAT(a)GTAATGA	(48 to 61)	
H	RE7	AACATAGGAAGTCGACGGCA	**B0379**	10990 to 10830
	RE8	ACGAGGATCGCCGTTATCAG	(84 to 109)	
I	B16T3+	ACAATAATTTTCCGCTTCGCT	**B0379**	38529 to 38419
	B16T3−	TGGTCGAAGGGAGGAGAGT	(84 to 109)	
J	cm13b1+	ATTCCACAATTTCCACCCGAA	**Y106G6**	20000 bp to the
	cm13b1−	TCCTTGAATGTGATTTTCCCT	(right of 109)	right of B0379

Note. Target sequences are listed according to their positions on chromosome I, starting from the left. Nucleotides in primers that do not match the genomic sequence are shown in parentheses. The cosmids that contain each target sequence are shown in boldface, and the location of each cosmid on the physical map is shown in parentheses, using the arbitrary units assigned by the *C. elegans* physical mapping project. (These values might change as the map is refined, but the relative positions of the cosmids should remain constant; for details about each cosmid, see ACeDB at http://probe.nalusda.gov:8300/cgi-bin/browse/acedb). The location of each target sequence within its respective cosmid is shown, using cosmid sequences from the EMBL database.

fog-3. Our results show that the right breakpoints of *nDf23*, *nDf24*, and *nDf25* lie to the left of test DNA A, but that the right breakpoint of *mnDf111* is located farther to the right, probably between test DNAs A and B (Table 2A, Fig. 1). Each test DNA is defined in Table 1.

Next, we studied deletions that fail to complement *fog-3*, testing only DNA located to the right of C on the physical map. Our results indicate that six deficiencies span the entire region from D to J, and three deficiencies have breakpoints that lie within this region. First, the left breakpoint of *qDf14* appears to lie between test DNAs D and E. Second, the left breakpoint of *qDf5* lies between F and G. Third, the right breakpoint of *qDf13* appears to lie between test DNAs I and J. These data indicate that *fog-3* is located in the region between test DNAs F and J (Table 2B, Fig 1).

Transgenic Nematodes

We used transformation rescue to determine the precise location of the *fog-3* gene. To produce stable lines of transgenic animals, we injected *unc-29 fog-3(q504)/spf-1* animals with both the plasmid pRF4 [*rol-6(su1006*dm)] at 100 ng/μl (Mello *et al.*, 1991) and a test cosmid or subclone. After identifying stable, transformed lines of worms that showed the Rol phenotype of our marker gene, we tested homozygous *unc-29* progeny to determine if the extrachromosomal array allowed *fog-3(q504) XX* animals to produce sperm (Table 3). We analyzed the progeny of all self-fertile *unc-29* animals to see if the ability to make sperm was linked to the *rol-6(sd)* mutation, and had thus been caused by *fog-3(+)* on the extrachromosomal array, rather than by a recombination event between *unc-29* and *fog-3* on chromosome I. For the location of our subclones, see Fig. 2.

Analysis of fog-3 cDNAs

The central portion of the *fog-3* cDNA was isolated by reverse transcriptase PCR using primers RE101 and RE102. The 5′ end was isolated by rapid amplification of cDNA ends (RACE; Frohman *et al.*, 1988), using primers Q_0 and RE102 for the primary amplification and Q_1 and RE104 for the secondary amplification; the 3′ end was also isolated by RACE, using primers Q_0 and RE105 first and then Q_1 and RE12. These products were each sequenced on one strand using the dideoxy nucleotide method (Sanger *et al.*, 1977) with fluorescently labeled terminators (Halloran *et al.*, 1993).

Northern Analysis

We used acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski, 1993; Chomczynski and Sacchi, 1987) to isolate total RNA from nematodes grown in liquid culture (Sulston and Brenner, 1974). From these total RNA samples, we prepared poly(A) RNA by selecting for transcripts that bind oligo(dT) cellulose. To analyze these RNAs by Northern analysis (Alwine *et al.*, 1977), we separated them on an agarose gel containing formaldehyde, transferred them to a positively charged nylon membrane,

TABLE 2
Mapping the Extent of Deficiencies in the *fog-3* Region

(A)

DNA	*nDf23*	*nDf24*	*nDf25*	*mnDf11*
A	2/2	2/2	1/1	0/1
	(2/2)	(2/2)	(2/2)	(0/1)
B	nd	nd	nd	(2/2)
C				1/1
	(2/2)	(2/2)	(3/3)	(7/7)

(B)

DNA	*gDf5*	*gDf8*	*gDf9*	*gDf10*	*gDf11*	*gDf12*	*gDf13*	*gDf14*	*gDf15*
D	2/2	0/4	0/1	0/2	0/2	0/3	0/4	(4/4)	0/2
E	(2/2)	nd	nd	nd	nd	nd	nd	0/2	0/2
F	2/2	nd	nd	nd	nd	nd	nd	nd	nd
G	0/3	nd	nd	nd	nd	nd	nd	nd	nd
H	0/1	nd	nd	nd	nd	nd	0/2	0/2	0/1
I	nd	nd	nd	nd	nd	nd	0/2	nd	nd
J	0/2	0/4	0/4	0/3	0/2	0/2	(4/4)	0/2	0/2

Note. (A) Deficiencies that complement *fog-3*. (B) Deficiencies that fail to complement *fog-3*. The test DNAs are listed in Table 1. Values are (eggs containing the test DNA)/(total eggs). The numbers without parentheses represent embryos shown by control reactions to be homozygous for the tested deficiency. The numbers in parentheses represent eggs likely to be homozygous for a deficiency, but not tested with control primers. nd, not determined.

and used an antisense RNA probe from the region between primers RE101 and RE107. As a control for RNA purity and loading, we probed the same blots with a DNA probe to the *actin-3* message (Krause *et al.*, 1989).

Identification of fog-3 Mutations

The entire *fog-3* gene was amplified from the genomic DNA of single mutant animals using the PCR (Mullis *et al.*, 1986; Saiki *et al.*, 1988) and cloned into the pGEM-T vector (Promega). For each mutant, we sequenced two independent clones on one strand, using the dideoxy nucleotide method (Sanger *et al.*, 1977) with fluorescently labeled terminators (Halloran *et al.*, 1993). The primers used for these sequencing reactions were RE108, RE109, RE105, and RE12.

RNA-Mediated Interference

To prepare double-stranded RNA, we used the PCR to generate a template containing nucleotides 4 through 455 of the *fog-3* cDNA, flanked by T7 promoters at each end. We prepared RNAs by *in vitro* transcription, precipitated them, resuspended them in 1× injection buffer (Fire, 1986), and allowed them to anneal at 37°C after brief denaturation. The final concentration was estimated to be 1 to 2 mg/ml by ethidium bromide staining. Injections were carried out as described by Guo and Kemphues (1995) and Fire *et al.* (1998). For control injections, we used a 653-nucleotide dsRNA from the 5′ end of the *ced-9* gene (Hengartner and Horvitz, 1994) and a 750-nucleotide dsRNA from a novel gene on cosmid K08E3.

Genetic Mosaic Analysis

The extrachromosomal array *vEx20* [*fog-3(+) ncl-1(+) rol-6(su1006*sd*) unc-54::gfp*] was created by co-injection of 0.14 ng/μl pRE11 [*fog-3(+)*], 10 ng/μl C33C3 [*ncl-1(+)*], 100 ng/μl pRF4 [*rol-6(sd)*], and 10 ng/μl pPD94.81 [*unc-54::gfp*] into N2 hermaphrodites. Rol progeny carrying this array were crossed with *unc-29 fog-3(q504); unc-36 ncl-1* females; we recovered homozygous *unc-29 fog-3; unc-36 ncl-1; vEx20* animals by screening their descendents for Unc-29 Unc-36 Rol individuals. The presence of each gene on the array was confirmed by its ability to rescue the mutant copy on the chromosome. We screened Rol larvae for animals in which all pharyngeal cells that were descended from MS showed the Ncl phenotype, indicating loss of the array during formation of MS, and then scored these individuals for self-fertility and presence of the array in the germ line.

RESULTS

The fog-3 Gene Is Located on a 3.9-kb Fragment from Cosmid C03C11

Genetic mapping placed *fog-3* in the interval between *unc-29* and *lin-11* on chromosome I, a region of approximately 1.2 Mb. To determine the physical location of *fog-3*, we used three methods. First, we identified and characterized the RFLP *qP5* and found by genetic mapping that it lies to the right of *fog-3* (Materials and Methods). Second, we determined the physical extent of two deficiencies that delete *fog-3* and whose breakpoints define the leftmost

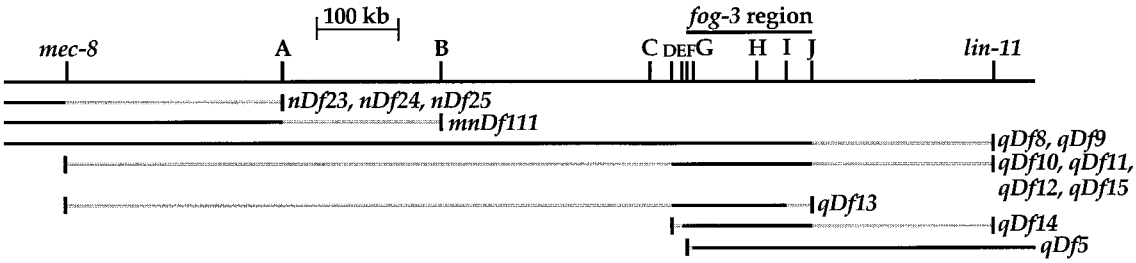

FIG. 1. Location of deficiencies in the *fog-3* region. The location of each target DNA sequence is shown on the *C. elegans* physical map. For each deficiency, DNA known to be deleted is shown in black and DNA that might be deleted in gray.

boundary of the gene (Fig. 1, Materials and Methods). Third, we made transgenic animals using clones from this region and tested the ability of each to rescue *fog-3(q504)* mutants (Fig. 2, Table 3). From these studies we identified a 3.9-kb subclone, pRE11, that contains *fog-3*. This transgene restores self-fertility to most animals (Table 3), but these worms produce fewer self-progeny than the wild type (data not shown). Two cosmids that partially overlap this subclone, E02G12 and F10G8, fail to rescue *fog-3* mutants, suggesting that much of this region is required to provide *fog-3* activity.

Many genes that act in the germ line of *C. elegans* function poorly in transgenic animals (Kelly *et al.*, 1997), with the possible exception of those required for spermatogenesis. For example, mutations in *spe-4* (L'Hernault and Arduengo, 1992), *spe-17* (L'Hernault *et al.*, 1993), *spe-26* (Varkey *et al.*, 1995), *spe-11* (Browning and Strome, 1996), *spe-27* (Minniti *et al.*, 1996), *fer-1* (Achanzar and Ward, 1997), and *spe-9* (Singson *et al.*, 1998) can each be rescued easily by extrachromosomal copies of the wild-type genes. Like these genes, *fog-3* functions well in transformation rescue experiments (Table 3). Furthermore, the extrachro-

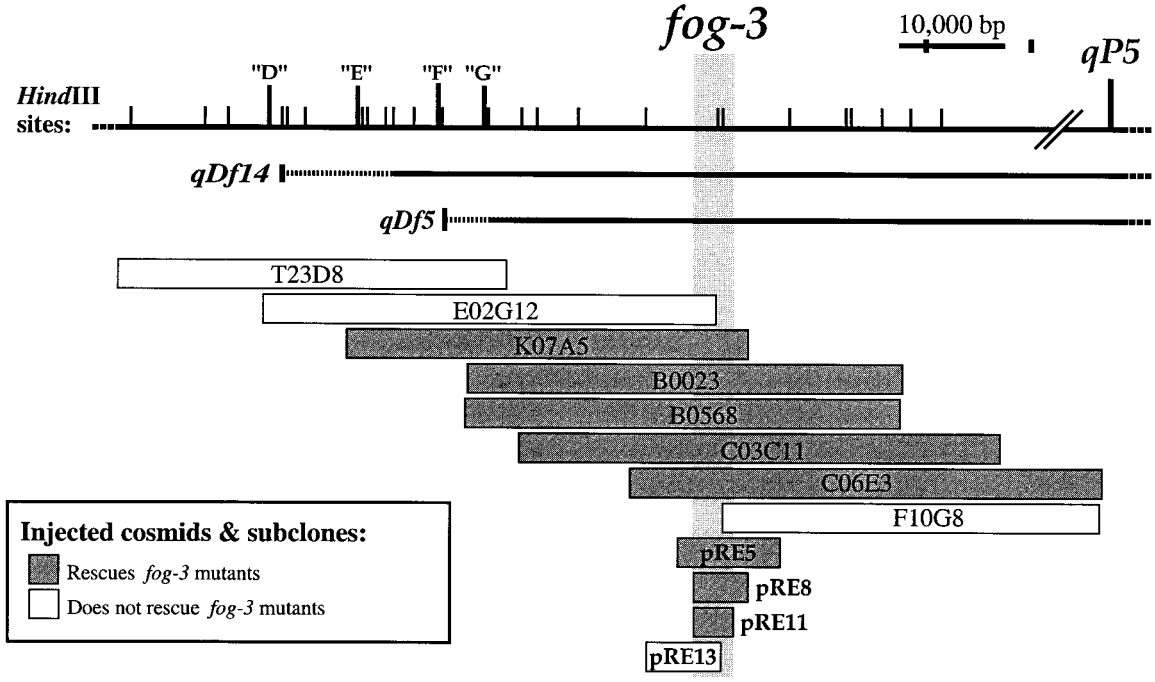

FIG. 2. Location of *fog-3* on the *C. elegans* physical map. The upper line shows a map of this region of the *C. elegans* genome, with the location of *qP5*. The locations of each test DNA in this region are indicated as "D" through "G". Below are two deficiencies that uncover *fog-3*, with deleted portions drawn as solid lines and potentially deleted portions as dashed lines. The minimal rescuing region is defined by pRE11 and is shown as a light gray box.

TABLE 3
Rescue of *fog-3(q504)* in Transgenic Animals

Injected DNA	[DNA] ng/μl	Stable lines	Rescued lines	% Rescue (best line)	% Rescue (all lines)
F14G10, K07A5	1.0, 1.0	1	1	85% (13)	85% (13)
F14G10	5.0	3	0		
T24D1	1.0	8	0		
E02G12	1.0	4	0		
K07A5	1.0	9	9	93% (15)	65% (149)
B0568	1.0	8	5	100% (12)	82% (92)
B0023	1.0	8	4	100% (5)	64% (39)
C03C11	1.0	7	6	73% (15)	43% (69)
C06E3	1.0	4	4	70% (10)	62% (24)
F10G8	1.0	9	0		
pRE5	0.25	6	3	90% (10)	59% (29)
pRE8	0.15	3	3	100% (10)	92% (28)
pRE11	0.14	6	3	80% (10)	71% (24)
pRE13	0.67	4	0		

Note. In these assays, % Rescue indicates the percentage of *unc-29 fog-3 XX* animals that developed as self-fertile hermaphrodites, rather than as females. The number of sperm produced by these animals was usually less than in the wild type.

mosomal arrays that express *fog-3* are remarkably stable and can be propagated indefinitely (data not shown).

The fog-3 Gene Encodes a Transcript of 1150 Nucleotides

The sequence of the 3.9-kb fragment that rescues *fog-3* mutants has been determined by the *C. elegans* Genome Sequencing Consortium (1998). Computer analysis suggests that it encodes a single transcript of eight exons, with an open reading frame of 792 nucleotides (Fig. 3A). To test this prediction, we prepared a probe to the 5′ half of the gene for use in Northern analysis. In poly(A)-purified RNA from a population that contained worms of all ages and both sexes, we detected one transcript of 1150 nucleotides (Fig. 3B, lane 1).

We amplified the central portion of the transcript using reverse transcriptase PCR, and the ends using RACE, and sequenced the products. Our cDNA sequence matched the predicted transcript completely (Fig. 4). In addition, the sequence of the RACE products revealed that the 5′ end of the message is *trans*-spliced to the SL1 leader sequence (Bektesh *et al.*, 1988) and that the 3′ untranslated region is 189 nucleotides long, with a polyadenylation site 13 nucleotides downstream of an AAUAAA signal sequence. This cDNA is 1008 bp long; if the poly(A) tail contained ~140 nucleotides, the total size of the transcript would exactly match that seen by Northern analysis.

Three tests confirm that this transcript is indeed the product of the *fog-3* gene. First, the known *fog-3* mutations map to this transcript (Fig. 4). Second, injection of double-stranded RNA corresponding to this transcript replicates the phenotype of *fog-3* mutants—80/80 such *fog-3(dsRNAi) XX* individuals produced oocytes rather than sperm, but were otherwise normal. Third, a subclone that lacks the last 17 codons of this gene is unable to rescue *fog-3* mutants (Fig. 2, Table 3).

FOG-3 Functions in the Germ Line, Rather Than the Somatic Gonad

The *fog-3* transcript is not found in *XX* adults that lack a germ line (Fig. 3B, lane 2), but is present in *XX* adults that have a germ line and produce sperm (lane 3). Thus, *fog-3* either is expressed in germ cells or is induced in other cells by the germ line. To determine if *fog-3* actually functions in

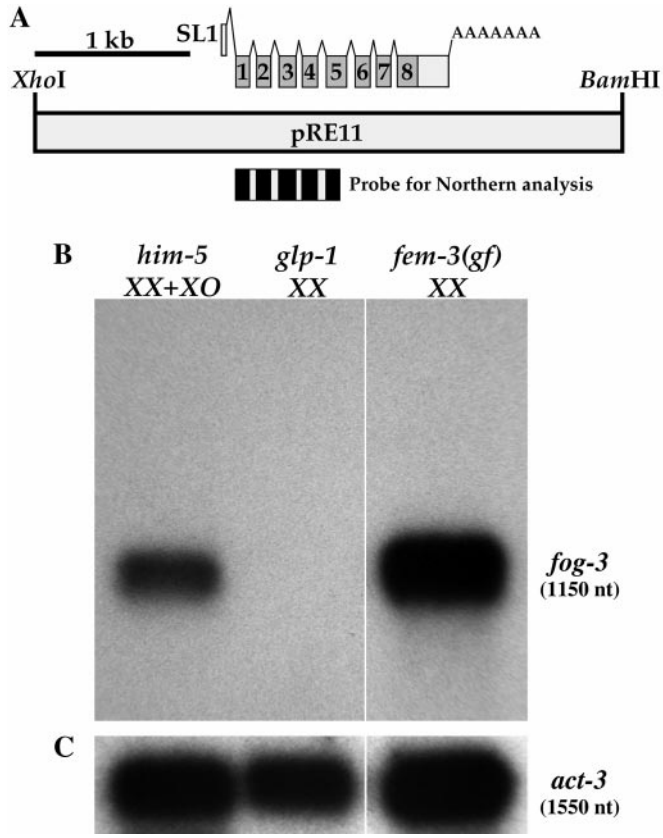

FIG. 3. The *fog-3* transcript. (A) The location and structure of the *fog-3* transcript is shown with respect to the subclone pRE11. The coding regions are in dark gray, and the untranslated regions in light gray. The portion of the transcript used as a probe for Northern analysis is shown in black. (B) Northern blot of *fog-3*. (C) The same blot, after hybridization with a probe for *actin-3*, to determine if the samples were of equal size. The *him-5* animals were of mixed age and mixed sex. The *glp-1* and *fem-3* animals were adults raised at the restrictive temperature of 25°C. At this temperature, the Glp animals lack almost all germ cells, and the *fem-3(gf)* animals produce only sperm.

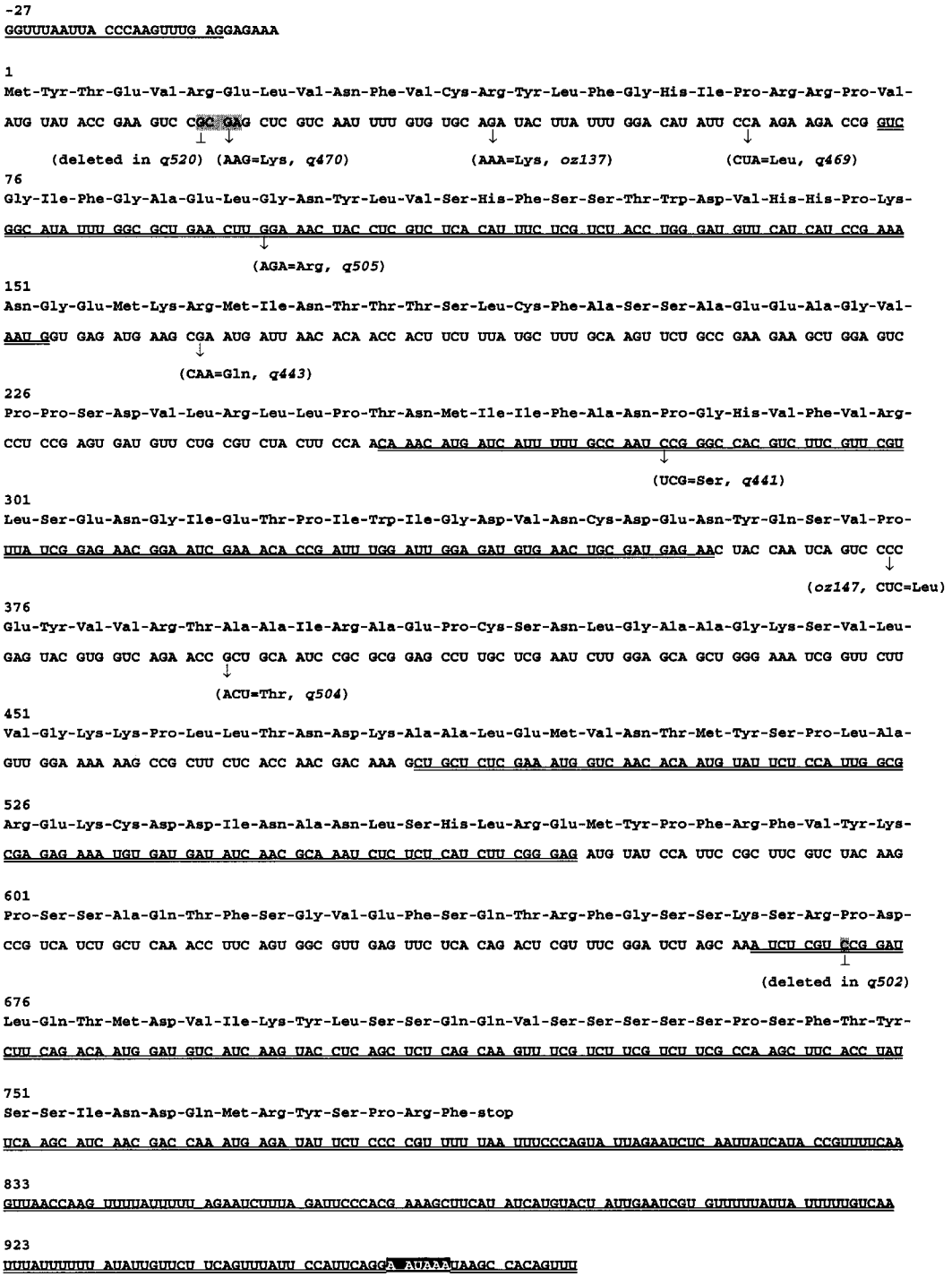

FIG. 4. Sequence of the *fog-3* transcript. The SL1 *trans*-spliced leader sequence is marked with a single underline, even-numbered exons with a double underline, and the polyadenylation signal sequence with black shading. Missense mutations are marked by an arrow, and deleted nucleotides are shaded gray. The *q520* deletion was induced with formaldehyde, the *q502* deletion with trimethyl psoralen and ultraviolet light, and the missense mutations with EMS (Ellis and Kimble, 1995). A BLAST search revealed that this transcript matches the cDNA clone yk464c9 (Kohara, 1996).

TABLE 4
Primers Used for Analyzing *fog-3*

Primer	Sequence
RE12	CAAACCTTCAGTGGCGTTGA
RE101	ATGTATACCGAAGTCCGCGAGC
RE102	AACTTGCTAGATCCGAAACGAG
RE104	GAACATCCCAGGTAGACGAGAA
RE105	AATCAGTCCCCGAGTACGTGGT
RE106	TTTTAAACTGTGGCTTATTTATTCC
RE107	ggatcctaatacgactcactatagggaggaCCAACAAG-AACCGATTTCCC
RE108	CGTTTTCGTCGTGGGTGGTCTC
RE109	TAACACAACCACTTCTTTATGC
SL1	GGTTTAATTACCCAAGTTTGAG
Q_0, Q_1, Q_T	Described by Frohman *et al.* (1988).

Note. RE107 includes the T7 promoter, shown in lowercase.

the nearby somatic gonad, we examined genetic mosaic animals in which all descendents of the founder cell MS were *fog-3*(−), but the germ cells carried an array with a wild-type copy of *fog-3*. Although their somatic gonads must have lacked *fog-3* activity, since these cells are descendents of MS, all six worms produced sperm and were self-fertile. These results, and the fact that *fog-3* mutants show defects only in the germ line, indicate that FOG-3 acts in germ cells to control their fates.

The Amino-Terminus of FOG-3 Is Similar to Those of the Tob Proteins of Vertebrates

Conceptual translation suggests that *fog-3* encodes a protein of 263 amino acids (Fig. 4). It does not contain obvious transmembrane domains or other known motifs. However, a BLAST search reveals similarities to both the Tob proteins of vertebrates and the related BTG proteins. Most significantly, the amino-terminal 116 amino acids of FOG-3 resemble the amino-termini of these proteins. Within this domain, 33% of the FOG-3 residues are identical to those found in at least one of the Tob proteins, and an additional 21% are similar; there is a single gap of 2 amino acids in the alignment (Figs. 5 and 6A). This same domain was originally identified in the BTG1 and BTG2 proteins (Figs. 5 and 6A), so we name it a "BTF" domain (for BTG, Tob, and FOG-3). Within this domain, FOG-3 is 23% identical to rat BTG1 and 22% identical to rat BTG2.

To elucidate the evolutionary relationships between these proteins, we used the ClustalX program to compare their BTF domains by bootstrap analysis. Our results suggest that FOG-3 is as closely related to the two BTG proteins as it is to the Tob family members (Fig. 6C). However, amino acids 198–223 of FOG-3 define a second domain that is shared with the Tob proteins, but missing from BTG1 and BTG2 (Fig. 6B). We name this region the TF domain because it is present in the Tob proteins and FOG-3.

The q520 Mutation Defines the Null Phenotype of FOG-3

To learn what regions of FOG-3 are essential, we identified the lesions associated with 10 *fog-3* mutations (Ellis and Kimble, 1995). To do this, we used the PCR to amplify the entire *fog-3* gene from the genomic DNA of homozygous mutants, cloned each fragment, and determined its sequence. To screen out errors introduced by *Taq* polymerase, we characterized two independent clones for each mutation.

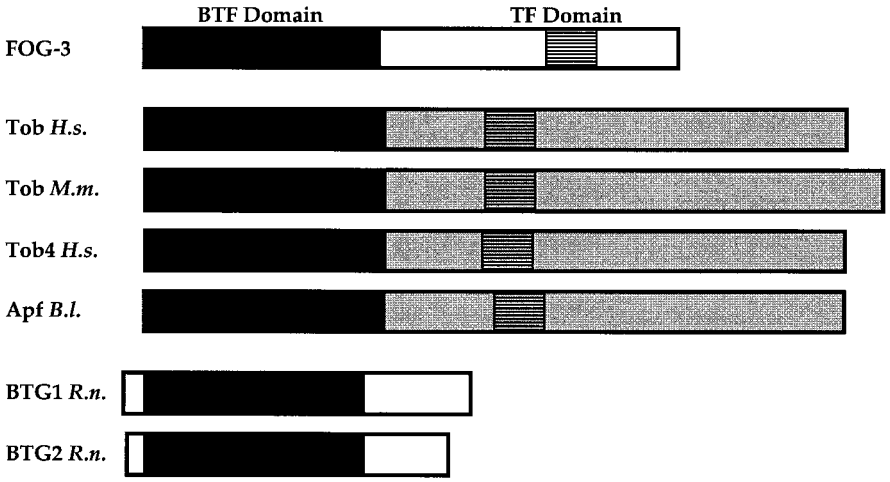

FIG. 5. Structural comparison of FOG-3 with the Tob and BTG proteins. The proteins of the Tob family shown are *Homo sapiens* Tob (Matsuda *et al.*, 1996), *Mus musculus* Tob (Yoshida *et al.*, 1997), *H. sapiens* Tob4 (pid g2493364), and Apf from *Branchiostoma lanceolotaum* (Holland *et al.*, 1997). The BTG proteins listed are *Rattus norvegicus* PC3/BTG2 (Bradbury *et al.*, 1991) and BTG1 (Rouault *et al.*, 1992). The BTF domain is black, the TF domain is striped, and the homologous carboxyl-termini of the Tob proteins are gray.

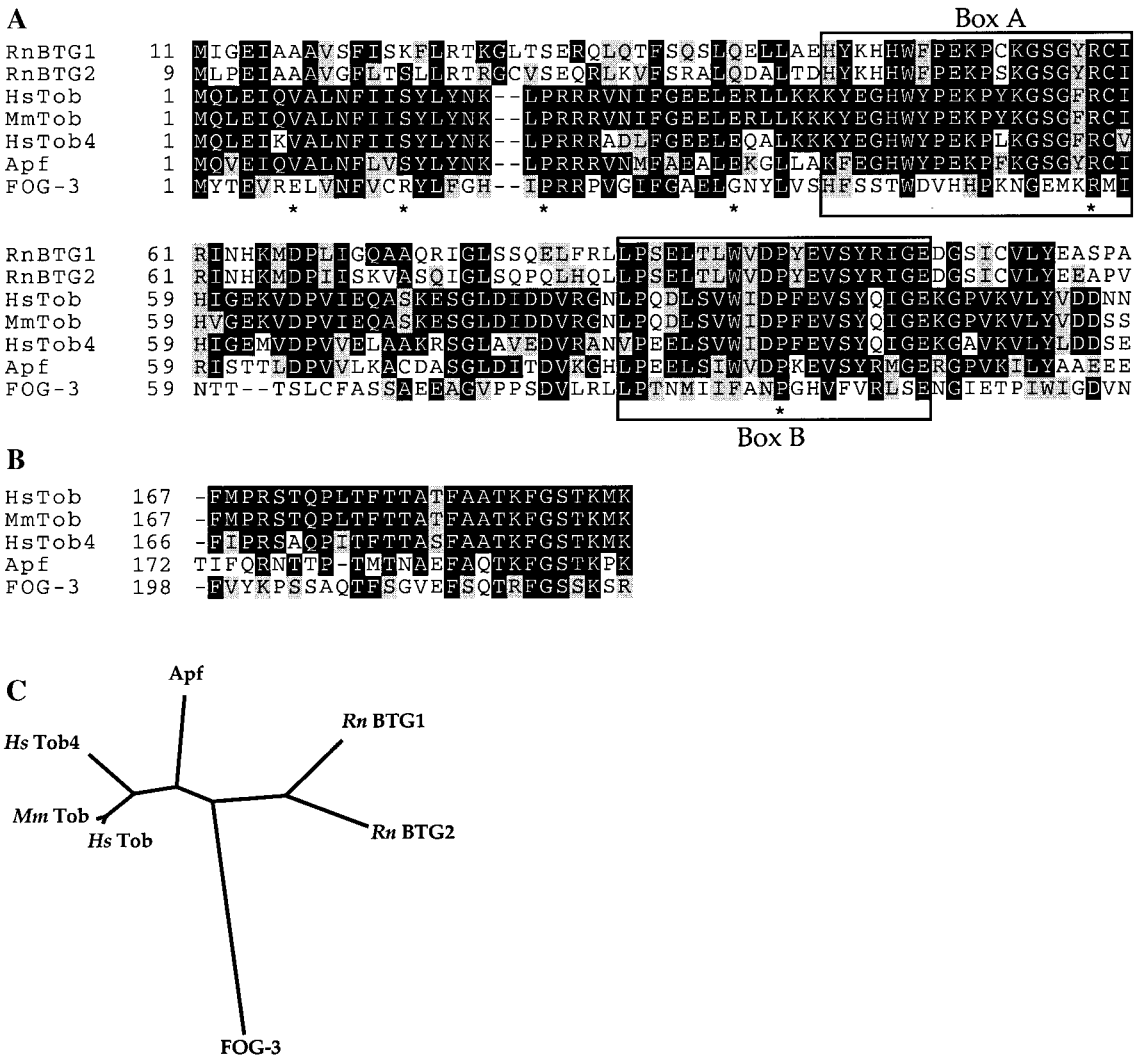

FIG. 6. Sequence comparison of FOG-3 with related proteins. This comparison was prepared using the Boxshade program. Identical residues are shown in black and similar residues in gray. (A) BTF domain. The numbers at the left indicate the starting amino acid. The sites of six *fog-3* mutations are shown below the FOG-3 sequence (indicated by asterisks), and the locations of two highly conserved regions defined by Guehenneux *et al.* (1997) are marked as Boxes A and B. (B) TF domain. (C) Unrooted tree showing the phylogenetic relationships between the BTF domains of FOG-3, the Tob proteins, and BTG1 and BTG2. The tree was prepared using a neighbor-joining bootstrap method with 10,000 trials (Felsenstein, 1996); the ClustalX program was used for all calculations.

In *fog-3(q520)* mutants, 4 nucleotides are deleted from the first exon (Fig. 4). This mutation was induced with formaldehyde (Ellis and Kimble, 1995), which often causes deletions (Moerman and Baillie, 1981). Since this deletion should destroy a *Sac*I site, we confirmed that *fog-3* genomic DNA from *q520* mutants is no longer sensitive to digestion with *Sac*I (data not shown). Because this deletion shifts the reading frame, it should abolish production of the FOG-3 protein. Analysis of *fog-3(q520)* mutants confirms our hypothesis that inactivation of *fog-3* causes germ cells to develop as oocytes rather than as sperm, but does not affect the development of other tissues (Ellis and Kimble, 1995; data not shown). In addition, *fog-3(q502)* causes a deletion of nucleotide 596 from the coding region, and should also shift the reading frame.

Missense Mutations in FOG-3 Affect the Amino-Terminal Domain

Eight of the *fog-3* alleles are missense mutations (Fig. 4). Of these alleles, six alter residues in the BTF domain (Figs. 5, 6A, and 6C). Furthermore, three of these missense mutations alter residues that are identical in FOG-3 and the

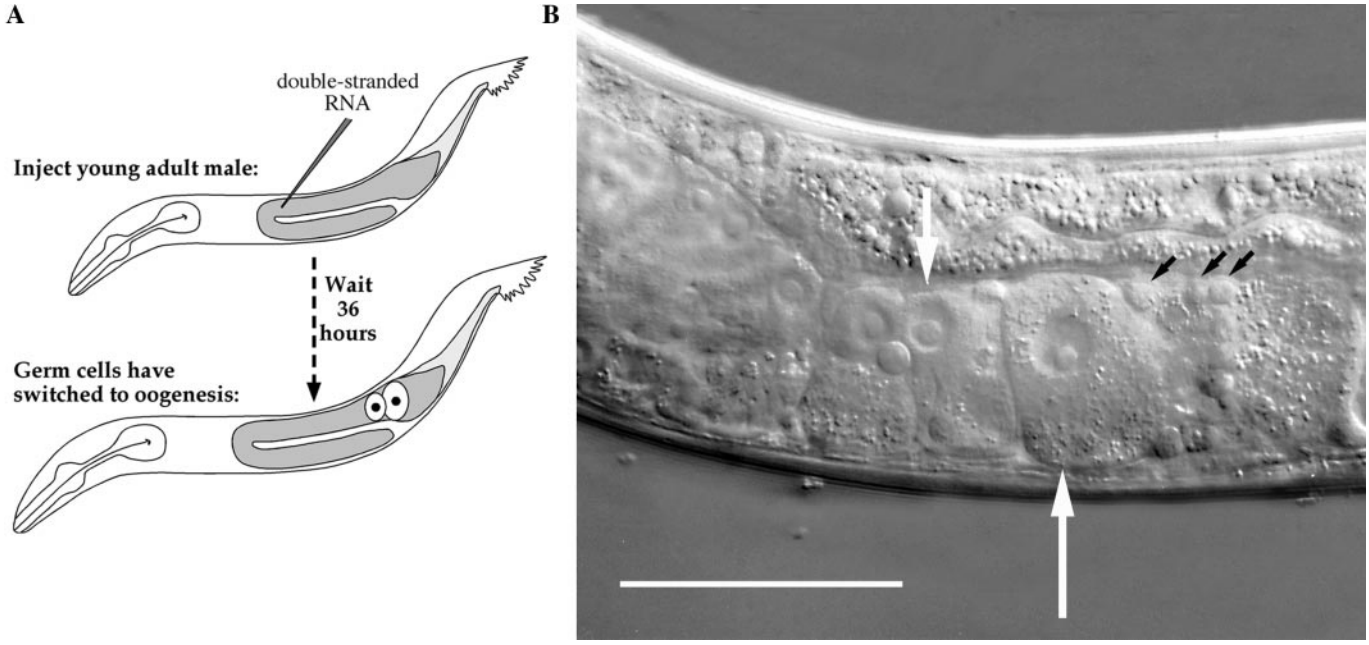

FIG. 7. Inactivation of FOG-3 causes germ cells to develop as oocytes. Nomarski photomicrograph of the germ line in a wild-type male that had been injected with double-stranded RNA corresponding to the *fog-3* transcript. The small black arrows mark sperm, the large white arrow indicates a mature oocyte, and the small white arrow indicates a developing oocyte. Anterior is to the left, and ventral is down. Scale bar, 30 μm.

Tob proteins. These results suggest that the BTF domain is essential for FOG-3 to function.

FOG-3 Is Required Continuously for Germ Cells to Adopt Male Fates

In principle, FOG-3 might be required for each germ cell to select a male fate, or it might be required only to initiate spermatogenesis in the germ line as a whole, which would then be maintained by other proteins. The first model implies that *fog-3* is required continuously for germ cells to become sperm, whereas the second model implies that *fog-3* is needed only in larvae. Because there are no temperature-sensitive mutations of *fog-3,* it has not been possible to distinguish between these models using genetic tests. However, while examining our *unc-29 fog-3(q504); vEx[rol-6(sd) fog-3(+)]* lines, we saw many hermaphrodites that were self-fertile, but produced small broods (data not shown). This observation suggested that the extrachromosomal array might produce enough wild-type FOG-3 to initiate spermatogenesis in hermaphrodites, but not enough to maintain spermatogenesis until the normal complement of 65–85 primary spermatocytes had been produced (Hirsh *et al.*, 1976).

To test the hypothesis that *fog-3* is required to maintain spermatogenesis, we selected young adult males that were producing sperm and injected them with double-stranded RNA corresponding to the 5′ half of the *fog-3* transcript (Fig. 7A). Within 36–48 h, germ cells in five of five injected animals began to differentiate as oocytes rather than as sperm (Fig. 7B). By contrast, all germ cells in five males injected with control dsRNAs developed normally. Because injection of double-stranded RNA can inactivate target messenger RNAs (Fire *et al.*, 1998), this result shows that *fog-3* activity is required continuously for spermatogenesis to occur. However, since the rate at which these messages are eliminated is not known, we cannot infer more precise details about when *fog-3* acts. Finally, this method for using RNA-mediated interference might prove helpful for study of other genes that lack temperature-sensitive alleles.

To learn if *fog-3* activity is required continuously in hermaphrodites, we used *fem-3(q96) dpy-20 XX* animals, raised at 25°C, since these worms normally produce sperm continuously. After soaking *fem-3(q96) dpy-20* larvae in 1 mg/ml *fog-3* double-stranded RNA for 24 h, we found that 17 produced only oocytes upon maturation, but 3 produced first sperm and then oocytes. Thus, *fog-3* is required in *fem-3(gf) XX* animals both for the initiation of spermatogenesis and for its maintenance.

DISCUSSION

FOG-3 Is a Novel Member of the Tob Family of Proteins

We cloned the *fog-3* gene, which regulates germ cell fate in *C. elegans.* Our data show that *fog-3* produces a single

major transcript, which encodes a protein of 263 amino acids. Furthermore, the *fog-3(q520)* mutation causes a frame shift that begins at the seventh codon of this transcript and is thus likely to be a null allele. Analysis of *q520* mutants confirms that *fog-3* is required for germ cells to develop as sperm rather than as oocytes, but not for the control of other sexual fates.

The amino-terminus of FOG-3 resembles that of the Tob, BTG1, and BTG2 proteins of vertebrates. This BTF domain appears to be an essential part of FOG-3, since it is altered by six of the eight missense mutations we identified. These mutations are likely to disrupt the structure of the BTF domain in FOG-3, since two alter conserved prolines, and three others alter the charge of an amino acid side chain (Fig. 4).

Tob and BTG Proteins Might Suppress Proliferation and Promote Differentiation

The BTF domain is the only feature shared by the Tob, BTG1, and BTG2 proteins, so it might mediate activities shared by these proteins. Tob protein was first identified by its ability to bind the receptor tyrosine kinase erbB-2 (Matsuda *et al.*, 1996). This binding requires the carboxyl half of Tob, which lies outside the BTF domain, but does not require the amino-terminal 19 amino acids of the BTF domain itself (Matsuda *et al.*, 1996). Thus, this interaction is not likely to reflect how the BTF domain functions.

However, several observations have been interpreted as evidence that the BTF domain can regulate cell proliferation. First, elevated expression of Tob can suppress the proliferation of NIH3T3 cells, but not that of NIH3T3 cells that have been cotransfected with *erbB-2* (Matsuda *et al.*, 1996). Thus, erbB-2 might promote proliferation by repressing Tob, as well as by regulating adaptor proteins in the Ras pathway. Second, transfection studies show that expression of either BTG1 or BTG2 can also suppress the growth and proliferation of NIH3T3 cells (Montagnoli *et al.*, 1996; Rouault *et al.*, 1992). Third, a disruption of BTG2 has the opposite effect on ES cells—they no longer arrest in the G2/M portion of the cell cycle in response to DNA damage (Rouault *et al.*, 1996). Because the BTF domain is the only feature shared by Tob, BTG1, and BTG2, it might mediate these effects on cell proliferation.

The expression patterns of these genes are consistent with the hypothesis that each acts to suppress proliferation. For example, quiescent NIH3T3 cells express BTG1 at high levels, as do cells in the early G1 stage, but these transcripts fall to a minimum during the S phase of the cell cycle (Rouault *et al.*, 1992). Furthermore, in PC12 cells, nerve growth factor induces BTG2 expression and also causes cells to cease proliferating and differentiate (Bradbury *et al.*, 1991). Finally, *in situ* hybridization of developing rats detects BTG2 in cells that are ceasing proliferation and beginning to differentiate as neurons, but these transcripts disappear after neurogenesis is complete (Iacopetti *et al.*, 1994). Similar studies reveal that the Tob family member Apf is expressed in a variety of tissues during *Amphioxus* development, most prominently in cells that are leaving the cell cycle and preparing to differentiate (Holland *et al.*, 1997). These studies are consistent with models in which the BTF domain suppresses proliferation but also raise the possibility that it helps cause cells to differentiate. However, these proteins do not act exclusively during development or differentiation, since high levels of BTG2 can be induced by the p53 transcription factor in response to DNA damage (Rouault *et al.*, 1996) or by the stress response during acute pancreatitis (Fiedler *et al.*, 1998).

Proteins That Interact with BTG1 and BTG2

The biochemical activities of the Tob and BTG proteins are not known, but the identification of proteins that interact with BTG1 and BTG2 has begun to provide clues to how they work. First, both BTG1 and BTG2 bind a protein-arginine *N*-methyltransferase (PRMT1; Lin *et al.*, 1996). This interaction was detected using the yeast two-hybrid system and confirmed by *in vitro* binding experiments using a GST–PRMT1 fusion protein. Furthermore, both BTG1 and BTG2 can regulate the ability of PRMT1 to methylate target proteins (Lin *et al.*, 1996). Although the role of PRMT1 in cells is not understood, one of its targets is hnRNP A1, which controls RNA splicing.

In mice, both BTG1 and BTG2 also interact with the CAF-1 protein (Rouault *et al.*, 1998). Furthermore, human BTG1 and CAF-1 bind each other (Bogdan *et al.*, 1998). These interactions were detected using the yeast two-hybrid system and confirmed either by coimmunoprecipitation or by *in vitro* experiments using GST–BTG fusion proteins. Deletion of Box B of the BTF domain abolishes this interaction, and in the yeast two-hybrid system, Box B alone can bind CAF-1 (Rouault *et al.*, 1998).

Why might this interaction with CAF-1 occur? CAF-1 is a conserved protein found in yeast (POP2; Sakai *et al.*, 1992) as well as in higher animals (Draper *et al.*, 1995). Its function has only been characterized in yeast, in which it is an important component of the CCR4 transcriptional regulatory complex (Draper *et al.*, 1994, 1995; Liu *et al.*, 1998). This complex promotes transcription of some genes and represses transcription of others. Mutations that inactivate *caf1* or *ccr4* not only alter transcriptional regulation, but also affect progression through the cell cycle (Liu *et al.*, 1997). Thus, one attractive hypothesis is that BTG1 and BTG2 interact with CAF-1 to regulate transcription; this complex might control progression through the cell cycle and perhaps regulate transcription of genes required for differentiation of some cell types.

What Is the Function of FOG-3?

FOG-3 is the only member of the BTF family that has been characterized genetically. We have shown that FOG-3 is needed for germ cells to become sperm rather than oocytes, but is not required for other aspects of develop-

ment (Ellis and Kimble, 1995). Furthermore, FOG-3 appears to function within germ cells to control their fates, since it is not expressed in animals that lack a germ line (Fig. 3) and is not needed in cells of the somatic gonad. Finally, tests of genetic epistasis (Ellis and Kimble, 1995), along with studies of *fog-3* expression in sex-determination mutants (Chen and Ellis, submitted for publication), indicate that FOG-3 acts at the end of the process of sex determination in the germ line. These results support the hypothesis that FOG-3, together with FOG-1, directly regulates germ cell fates.

What biochemical function might FOG-3 have? The decision of germ cells to undergo spermatogenesis or oogenesis affects the expression of many genes in *C. elegans*. For example, Northern analyses reveal that *spe-4*, *spe-26*, *spe-11*, and *fer-1* are expressed only during spermatogenesis (Achanzar and Ward, 1997; Browning and Strome, 1996; L'Hernault and Arduengo, 1992; Varkey *et al.*, 1995). Furthermore, *fer-1* is expressed early in this process, in primary spermatocytes (Achanzar and Ward, 1997). These results have been confirmed by *in situ* hybridization, which also revealed that many genes required for oogenesis or early embryonic development are expressed in oocytes, but not in spermatocytes (Jones *et al.*, 1996). Thus, the choice of germ cells to become sperm or oocytes involves either (1) transcriptional regulation or (2) differential stability of mRNAs. FOG-3 might control either one of these processes.

Because some of the homologs of FOG-3 are expressed in the testis, it is possible that one of these homologs promotes spermatogenesis in vertebrates. For example, Northern analyses show that human and mouse Tob are expressed both in the testis and in many other tissues (Matsuda *et al.*, 1996; Yoshida *et al.*, 1997). Furthermore, *in situ* hybridization reveals that amphioxus Apf is expressed in a wide variety of cell types in the developing embryo (Holland *et al.*, 1997). Most significantly, BTG1 is also expressed in a wide variety of tissues (Rouault *et al.*, 1992), but is found at particularly high levels in developing spermatocytes and sperm in rats (Raburn *et al.*, 1995). Thus, it is possible that BTG1, or perhaps one of the Tob proteins, regulates sexual fate in mammalian germ cells.

How Does the BTF Domain of FOG-3 Work?

Although more than 99% of the *C. elegans* genome has been sequenced by the Genome Sequencing Consortium (1998), the only nematode protein known to have a BTF domain is FOG-3. Because the *q520* mutation, which should eliminate FOG-3, affects only the decision of germ cells to become sperm or oocytes, FOG-3 cannot play a general role suppressing cell proliferation. Furthermore, FOG-3 is unlikely to determine if germ cells exit mitosis, since in *fog-3* mutants this decision is not affected. However, the BTF domain of FOG-3 might coordinate the exit of germ cells from mitosis with the determination of their sexual fates.

Alternatively, the BTF domain of FOG-3 and it homologs might regulate more general aspects of differentiation. For example, since BTG1 and BTG2 both interact with CAF-1, which forms part of a large transcriptional regulatory complex, it is possible that FOG-3 interacts with the nematode homolog of CAF-1, in order to regulate the transcription of genes required for spermatogenesis and oogenesis. One observation is consistent with this model—the *fog-3(q441)* mutation alters a conserved proline in Box B of the BTF domain, which is required for interaction between murine BTG1 and CAF-1.

Based on these observations, we are investigating two models that might explain how FOG-3 acts. In one, FOG-3 controls the activity of a transcriptional regulatory complex through an interaction with CAF-1, so as to promote expression of genes required for spermatogenesis and prevent expression of genes required for oogenesis. In the other model, *fog-3* coordinates the control of sexual fate with the exit of germ cells from mitosis, perhaps through an interaction with a protein-arginine *N*-methyltransferase. These models are not mutually exclusive. If either is correct, FOG-3 should be required continually for germ cells to adopt male fates and become sperm. We have used RNA-mediated inactivation to show that this is, indeed, the case.

These experiments indicate the importance of the BTF domain of FOG-3 in the control of germ cell fate in nematodes. To elucidate how it works, we are using the yeast two-hybrid system and genetic suppressor analysis to find proteins that interact with FOG-3 in the *C. elegans* germ line. Since the BTF domain has been conserved during evolution, our results might elucidate not only how germ cell fates are controlled, but also how the related Tob and BTG proteins function in other tissues.

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