Copyright © 2001 by the Genetics Society of America

Specification of Germ Cell Fates by FOG-3 Has Been Conserved During Nematode Evolution

Pei-Jiun Chen, Soochin Cho, Suk-Won Jin and Ronald E. Ellis

Department of Biology, University of Michigan, Ann Arbor, Michigan 48109

Manuscript received March 1, 2001
Accepted for publication May 4, 2001

ABSTRACT

Rapid changes in sexual traits are ubiquitous in evolution. To analyze this phenomenon, we are studying species of the genus Caenorhabditis. These animals use one of two different mating systems—male/hermaphroditic, like the model organism *Caenorhabditis elegans*, or male/female, like *C. remanei*. Since hermaphrodites are essentially females that produce sperm for self-fertilization, elucidating the control of cell fate in the germ line in each species could provide the key to understanding how these mating systems evolved. In *C. elegans*, FOG-3 is required to specify that germ cells become sperm. Thus, we cloned its homologs from both *C. remanei* and *C. briggsae*. Each species produces a single homolog of FOG-3, and RNA-mediated interference indicates that FOG-3 functions in each species to specify that germ cells develop as sperm rather than as oocytes. What factors account for the different mating systems? Northern analyses and RT-PCR data reveal that the expression of *fog-3* is always correlated with spermatogenesis. Since the promoters for all three *fog-3* genes contain binding sites for the transcription factor TRA-1A and are capable of driving expression of *fog-3* in *C. elegans* hermaphrodites, we propose that alterations in the upstream sex-determination pathway, perhaps acting through TRA-1A, allow spermatogenesis in *C. elegans* and *C. briggsae XX* larvae but not in *C. remanei*.

MOST animal species consist of two different sexes, whose body plans and behaviors are specialized to help them find and mate with each other. Despite the importance of sexual reproduction, all aspects of this process evolve rapidly (Marin and Baker 1998; Hansen and Pilgrim 1999; Schütt and Nöthiger 2000). In some cases this rapid evolution involves not only the relaxation of negative selective forces but also positive selection for new sexual traits (Wyckoff *et al.* 2000). These changes can occur in three different ways. First, the phenotypes of the sexes can change. For example, the genus Caenorhabditis contains nematodes with two different types of reproductive systems—*Caenorhabditis elegans* and *C. briggsae* have male and hermaphrodite sexes, whereas *C. remanei* has male and female sexes (Sudhaus 1974; Baird *et al.* 1992). Second, even if the phenotypes of the sexes remain constant, the underlying mechanisms that regulate sexual identity can change so that either the signal that controls sexual fate or the proteins that interpret this signal differ in closely related species (Bull 1983; Schütt and Nöthiger 2000). Third, even if the phenotypes of the two sexes and the basic mechanisms that determine them remain constant, the proteins that mediate these processes often undergo rapid divergence. For example, in nematodes, the crucial sex-determination protein TRA-1A shows only 44% amino acid identity between *C. elegans* and *C. briggsae* (Zarkower and Hodgkin 1992; de Bono and Hodgkin 1996).

Corresponding author: Ronald E. Ellis, Department of Biology, University of Michigan, Ann Arbor, MI 48109. E-mail: ronellis@umich.edu

To elucidate how these rapid changes occur, we are studying the control of germ cell fate in the genus Caenorhabditis. In particular, we want to understand what factors cause *XX* animals to become self-fertile hermaphrodites in some of these species but females in other closely related species. Our focus is the *fog-3* gene, which appears to directly control the production of sperm in both hermaphrodites and males.

Several factors make these nematodes particularly attractive. First, one member of this genus, *C. elegans*, is a leading model system for the study of sex determination (Meyer 1997; Ellis 1998). Second, the small genome size of these creatures and the complete genome sequence of *C. elegans* (*C. elegans* Genome Sequencing Consortium 1998) simplify the isolation of homologous genes. Third, molecular techniques such as RNA-mediated interference (Fire *et al.* 1998) allow genetic experiments to be carried out rapidly in all caenorhabditids. Fourth, the major difference between hermaphrodite and female nematodes is the ability of the hermaphrodites to produce sperm in an otherwise female body. Although this difference has a major impact on the animal's lifestyle, it involves a single cell fate decision—the control of how germ cells differentiate.

Several genes that regulate sexual identity and germ cell fate have been cloned from species related to *C. elegans*. These include the *C. briggsae* homologs of *tra-1* (de Bono and Hodgkin 1996), *tra-2* (Kuwabara 1996), *fem-2* (Hansen and Pilgrim 1998), and *her-1* (Streit *et*

al. 1999). However, none of these genes is likely to directly control the decision of germ cells to become sperm or oocytes. Instead, this decision is probably mediated by *fog-1* (Barton and Kimble 1990; Jin *et al.* 2001) and *fog-3* (Ellis and Kimble 1995; Chen *et al.* 2000). In *C. elegans*, these two genes act at the end of the sex-determination pathway and are required in both males and L4 hermaphrodites for germ cells to become sperm instead of oocytes.

FOG-3 is a member of the Tob family of proteins (Chen *et al.* 2000). These proteins are found throughout the animal kingdom, but their biochemical activities and biological functions in other animals remain unclear. To learn how the control of the sexual fate of germ cells has evolved in the caenorhabditids, we cloned the homologs of *fog-3* from *C. briggsae*, a species with *XX* hermaphrodites, and *C. remanei*, a species with *XX* females. We find that each species produces a single FOG-3 protein, that the structure and sequence of these genes is conserved, and that FOG-3 is required in each species for germ cells to become sperm rather than oocytes. Finally, the expression of these genes is correlated with periods of spermatogenesis in each species, and this expression is driven by promoters that are similar in sequence and function. These results show that the underlying mechanisms for controlling germ cell fates are similar in each species and imply that the crucial differences between the species lie in the regulation of genes that act upstream of *fog-3*.

MATERIALS AND METHODS

Genetic nomenclature: We use the genetic nomenclature described by Horvitz *et al.* (1979), with some modifications. First, we use "female" to designate an *XX* animal that makes oocytes but no sperm; by definition, female worms cannot self-fertilize. Second, we use capital letters to indicate the protein encoded by a gene. Thus, the protein produced by the *fog-3* gene is FOG-3. Third, we use *Cb-fog-3* and *Cr-fog-3* to refer to the *fog-3* homologs from *C. briggsae* and *C. remanei*, respectively.

Genetic methods: General techniques for culturing worms were described by Brenner (1974). Animals were raised at 20° unless otherwise indicated. Wild type refers to the Bristol strain N2 for *C. elegans* (Brenner 1974), AF16 for *C. briggsae* (Fodor *et al.* 1983), and EM464 for *C. remanei* (Baird *et al.* 1992).

Cloning *fog-3* homologs from *C. briggsae* and *C. remanei*: The central portions of the *fog-3* homologs from *C. briggsae* and *C. remanei* were obtained by PCR from genomic DNA, using degenerate primers RE301 and RE302 (Table 1). The PCR products were subcloned into the pGEM-Teasy vector (Promega, Madison, WI) and sequenced using the dideoxy nucleotide method (Sanger *et al.* 1977) with fluorescently labeled terminators (Halloran *et al.* 1993). We then used these sequence data to clone internal cDNA fragments for each gene by reverse transcriptase (RT)-PCR.

The 5′ end of the *C. briggsae* cDNA was obtained by rapid amplification of cDNA ends (RACE; Frohman *et al.* 1988), using primers Q_0 and RE303 for the primary amplification and Q_1 and RE304 for the secondary amplification. The 3′ end was also isolated by RACE using primers Q_0 and RE305 first and then primers Q_1 and RE306 for the secondary amplification.

Analysis of *C. remanei fog-3* was carried out using the same methods. We used primers RE307 and RE308 for the primary and secondary amplifications in 5′-RACE and primers RE309 and RE310 for the primary and secondary amplifications in 3′-RACE.

Northern analysis: Procedures for preparation of total RNA and Northern analyses were described by Chen *et al.* (2000). To obtain RNA from animals at specific stages, we grew large populations of worms in liquid culture and synchronized them by alkaline hypochlorite treatment. Pools of animals of the same age were harvested at each time point, and the ages of selected animals were confirmed by examining the development of the gonad and germ line with Nomarski optics. An anti-sense *C. briggsae fog-3* probe was prepared using the MaxiScript kit (Ambion, Austin, TX), with a DNA template generated by RT-PCR, using primers RE311 and RE312. As a loading control, the same blot was probed with RNA probes to the *C. briggsae* 18S ribosomal RNA. An anti-sense RNA probe for *C. remanei fog-3* was prepared using similar methods, with a DNA template generated using primers RE332 and RE313.

RNA mediated interference: To prepare double-stranded RNA, we used the PCR to generate templates flanked by T7 promoters at each end. A *C. briggsae* cDNA clone containing nucleotides 1–426 of the coding region was amplified using primers RE314 and RE312. A *C. remanei* cDNA clone containing nucleotides 1–362 of the coding region was amplified using primers RE315 and RE313. The double-stranded RNAs were prepared as described by Chen *et al.* (2000) and injected into adult hermaphrodites as described by Guo and Kemphues (1995) and Fire *et al.* (1998). To observe the phenotypes of *XO* animals, we injected either AF16 or EM464 *XX* animals, let them recover on plates overnight, and then crossed them with AF16 or EM464 *XO* males. The *XX* and *XO* progeny were characterized by Nomarski microscopy.

Panhandle PCR: The sequences upstream of the *C. briggsae* and *C. remanei fog-3* genes were obtained by Panhandle PCR, as described by Jones (1995). Briefly, *C. briggsae* genomic DNA was digested by selected restriction enzymes and ligated with primer RE316 using the bridging oligonucleotide RE317. The ligation mix was treated with 1 μl (20 units) of Exonuclease I at 37° for 30 min to remove unligated oligonucleotides, and then 1 μl of this mix was used as a template for PCR amplification, using primer RE318. Two more rounds of PCR amplification were done using primers RE319 and RE316. Finally, the PCR products were subcloned into the pGEM-Teasy vector (Promega) and sequenced. The sequences upstream of *C. remanei fog-3* were obtained using the same procedures, except that we used the RE320 oligonucleotide and primers RE321, RE322, and RE323 for the PCR reactions.

Construction of pRE70 and pRE71: To make pRE70, we used PWO DNA polymerase (Roche Molecular Biochemicals, Indianapolis) to amplify a 1.3-kb region of the *C. briggsae fog-3* promoter by PCR. The forward primer was RE324 and the reverse primer was RE325. These primers introduced a *Xho*I site into position −1344. This fragment was cut by *Xho*I and *Sac*I and used to replace the *Xho*I-*Sac*I fragment of pRE11, which contains the *C. elegans fog-3* gene (Chen *et al.* 2000). The relevant portions of the final plasmid were sequenced to confirm that no mutations had been introduced by this procedure.

We used a similar procedure to make pRE71. We amplified a 1.3-kb region of the *C. remanei fog-3* promoter using forward primer RE326, which introduced a *Xho*I site into position −1344, and reverse primer RE327. This fragment was cut by *Xho*I and *Sac*I and used to replace the *Xho*I-*Sac*I fragment of pRE11.

TABLE 1

Primers used in these experiments

RE301	TCAATTTTGTGTGCAGATACTTATTTGGACATAThCCnmGnAG
RE302	CATTGTCTGAAGATCCGGACGAGATTTGCTAGAnCCrAAnCGnGT
RE303	CAGCCGCGAAAATTCCGGTCTG
RE304	GACACTAATTGTTTTGTCTTTCAACTCACT
RE305	CGCAAAGCTGACTCCTGCAGC
RE306	GATTTCAGCTGTGCTCGAACTGGTC
RE307	CCCATGAGGACGAGAATTGGC
RE308	CTAGCTCCGAAGCAAAAATACC
RE309	CAGCCGGTGGCGGA
RE310	CGAGGTCCCAATATGGAATGG
RE311	ATGTACACGGAAGTAAAGGAGCTCG
RE312	GGATCCTAATACGACTCACTATAGGGAGCGTCTCCTTTCCAGATTGGAACATCGAC
RE313	GGATCCTAATACGACTCACTATAGGGAGCACTTCTCCATTCCATATTGGGACCTCG
RE314	GGATCCTAATACGACTCACTATAGGGAGATGTACACGGAAGTAAAGGAGCTCGTCAA
RE315	GGATCCTAATACGACTCACTATAGGGAGATGTACACCGAGGTCTTGTGAAGTCGTC
RE316	ACGAGGCAGTTTCCAAGCTCAGCCGCGA
RE317	CTAGTCGCGGCTGAGCTTGGAA
RE318	GATATCCCAAGAGGAGGAGAACTGCGCAAC
RE319	CCCAAGAGGAGGAGAACTGCGCAACGAGGC
RE320	gatcCGGAGCTAGCGAATTCTCTCGTC
RE321	AATGTCCCATGAGGACGAGAATTGGC
RE322	GAGGACGAGAATTGGCAGACGAGAG
RE323	GGCAGACGAGAGAATTCGCTAGCTCCG
RE324	AGCAGACTCGAGTACTTGCGAAATTCCG
RE325	AAAATTGACGAGCTCTTCTACTTCTGTGTACATTTTGACTCTAAAAATTAG
RE326	AGCAGACTCGAGTTATTTTCCTCACACAATGGC
RE327	AAAATTGACGAGCTCTTCTACTTCTGTGTACATTCAACCCTGAAAACTGAGTG
RE328	ATGTACACAGAAGTAAAGGAGCTCGTCAATTTTGTATGCCG
RE329	GTGGACAACAAACTAGTAATTGGAATAGATGGGCG
RE330	ATGTACACCGAGGTCTGTGAGCTC
RE331	GGAGCAGTGCCACTAGTAGTGCGAATAAATCATATG
RE332	ATGTACACCGAGGTCTGTGAAGTCGTC

Construction of pRE73 and pRE74: So that we could excise and replace the 1.3-kb coding region of *C. elegans fog-3* from pRE11, we introduced a *Spe*I site into the end of the coding region. To do this, the nucleotide sequence from position 1147 to 1151 of the *C. elegans fog-3* genomic sequence in pRE11 was changed from TTTAAT to ACTAGT. This construct is called pRE110. We then amplified the *C. briggsae fog-3* coding region from position 19 to position 1118, using PCR. The forward primer was RE328 and the reverse primer was RE329. This fragment was cut by *Sac*I and *Spe*I and used to replace the *Sac*I-*Spe*I fragment of pRE110, generating pRE73. Because we used the *Sac*I cloning site, the sequence of the first 24 nucleotides of pRE73 is derived from *C. elegans fog-3*. Thus, the first eight amino acids of the chimeric gene are MYTE V**R**EL (as in *C. elegans* FOG-3) instead of MYTEV**K**EL (as in *C. briggsae* FOG-3). Furthermore, to create a *Spe*I site in the *C. briggsae* sequence, the first nucleotide after the stop codon was changed from A to T.

We used a similar procedure to construct pRE74. The genomic region of *C. remanei fog-3* was amplified using forward primer RE330 and reverse primer RE331. This fragment was cut by *Sac*I and *Spe*I and used to replace the *Sac*I-*Spe*I fragment of pRE110. To use the *Sac*I site, the first eight amino acids of pRE74 are MYTEV**R**E**L** (as in *C. elegans* FOG-3) instead of MYTEV**CE**V (as in *C. remanei* FOG-3).

Transformation rescue: To assay these constructs for their ability to rescue *fog-3(q504)* mutants, we created simple extrachromosomal arrays as described by Chen *et al.* (2000). For each injection we used 100 ng/µl of pRF4 and 0.14 ng/µl of the relevant *fog-3* plasmid. After establishing stable lines of transgenic animals that were heterozygous for *unc-29(e1072) fog-3(q504)*, we measured the ability of each array to restore self-fertility to *unc-29 fog-3(q504) XX* homozygotes.

RESULTS

Both *C. briggsae* and *C. remanei* contain a single homolog of *fog-3*: We used RT-PCR and consensus degenerate oligonucleotides (Rose *et al.* 1998) to isolate internal portions of the *fog-3* transcripts of *C. remanei* and *C. briggsae* and then used RACE (Frohman *et al.* 1988) to isolate the 5′ and 3′ ends of each message. Next, we designed primers to the 5′ and 3′ ends of each gene and used them to clone complete cDNA and genomic DNA copies. Finally, we determined the intron/exon structures of each homolog by comparing their genomic and cDNA sequences. From these studies, we identified a single *fog-3* transcript in each species. To confirm that neither homolog of *fog-3* produced multiple transcripts, we probed Northern blots that contained *C. briggsae* RNA from animals of mixed ages and sexes and carried out similar tests for *C. remanei*. We observed only one

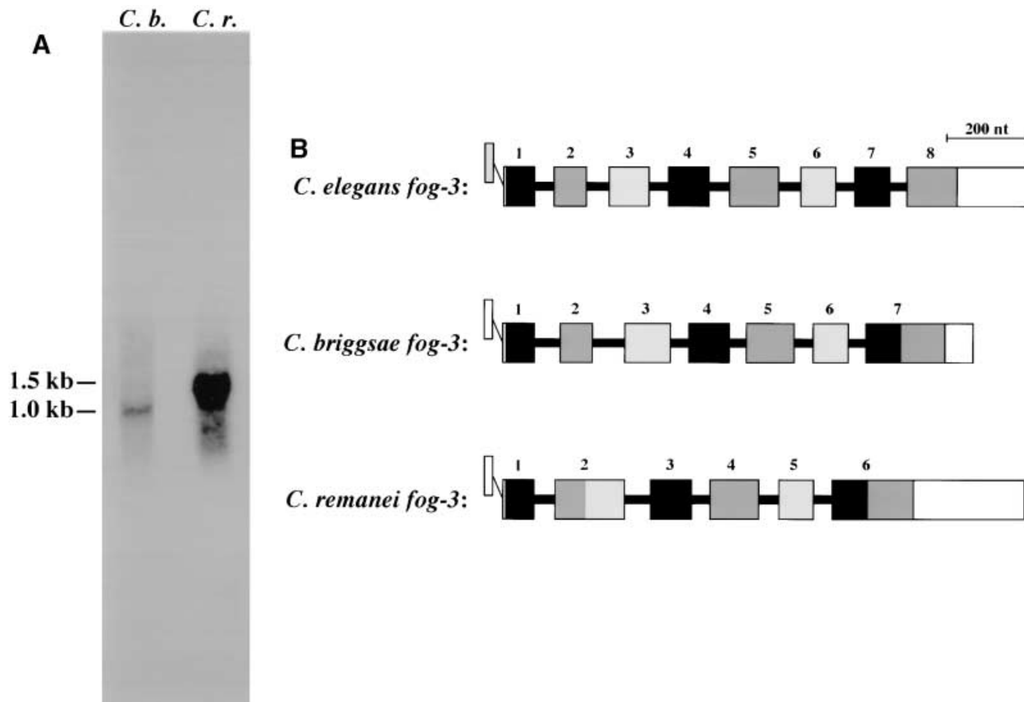


Figure 1.—The *fog-3* genes and transcripts. (A) Northern blots of *C. briggsae* and *C. remanei* RNA. *C. briggsae* total RNA was isolated from mixed-stage AF16 *XX* hermaphrodites. *C. remanei* total RNA was isolated from a mixed population of EM-464 *XX* and *XO* animals. We loaded 10 μg of total RNA in each lane but expected a higher level of *fog-3* transcripts in EM464, because its population contains 50% males, which produce sperm from the L4 larval stage through adulthood. By contrast, we expected lower levels of *C.b.-fog-3*, since only the L4 larvae, which constitute a small fraction of the population, are producing sperm. Probes are described in materials and methods. (B) Structure of the *fog-3* genes. The 5′ end is to the left and begins with the SL1 leader sequence, depicted as a rectangle above the line. Exons are shown as boxes and introns as lines connecting the boxes. Both exons and introns are drawn proportional to the scale bar. In the *C. elegans* gene, alternating black, dark gray, and light gray shading is used to label the different exons, and these shades are used to mark homologous sequences in the other two *fog-3* genes. White indicates the 3′-UTR.

fog-3 transcript in each species (Figure 1A). Furthermore, the size of each message corresponded to that of our cDNA clones. These transcripts are deposited in the GenBank database (accession nos. AF354169 for *C. briggsae* and AF354170 for *C. remanei*).

The three *fog-3* genes not only share similar sequences, but their structures also suggest a common evolutionary origin (Figure 1B). First, each *fog-3* gene is *trans*-spliced to the SL1 leader sequence. Second, the exons in each gene correspond to those of the others, with two exceptions—the second and third exons of the *C. elegans* and *C. briggsae* genes match exactly with the second exon of their *C. remanei* homolog, and the seventh and eighth exons of the *C. elegans* gene match with the seventh exon from the *C. briggsae* gene and the sixth exon from *C. remanei*. Third, the intron/exon boundaries are located at similar positions in each gene. One explanation of these results is that the ancestral *fog-3* gene had seven introns and the *C. elegans* gene has retained this ancestral structure, but the seventh intron was lost in the common ancestor of *C. remanei* and *C. briggsae*, and the second intron was lost in *C. remanei*. This model is consistent with the idea that *C. remanei* and *C. briggsae* are sister species, a possibility that we discuss in greater detail below.

Three domains of FOG-3 show a high degree of conservation: Previous comparisons between FOG-3 and other members of the Tob family revealed two conserved domains—the BTF domain and the TF domain (Chen *et al.* 2000). The BTF domain consists of the amino-terminal 116 amino acids of FOG-3 and is conserved between the FOG-3 proteins, the Tob proteins, and the BTG proteins. Among these three families of proteins, the Box A and Box B regions within the BTF domain appear more highly conserved than the remainder (Guehenneux *et al.* 1997). However, among the caenorhabditid FOG-3 proteins, the most highly conserved region is neither Box A nor Box B but the amino terminus (Figure 2A, Table 2). Furthermore, the amino terminus is affected by four of the eight known missense mutations that alter normal *fog-3* function (Chen *et al.* 2000), whereas Box A and Box B are each affected by only one. Taken together, these results suggest that the BTF domain contains three important regions rather than two—the amino terminus, Box A, and Box B. Although each of these regions is essential for activity, the amino terminus appears to have been subject to the most stringent selective constraints during nematode evolution.

The TF domain is found in both FOG-3 and the Tob proteins, though it is not present in either BTG1 or BTG2 (Chen *et al.* 2000). Our studies show that this TF domain is one of the most highly conserved regions in the three nematode FOG-3 proteins (Figure 2A; Table 2). For example, the *C. elegans* and *C. remanei* TF domains are 78% identical, and the *C. elegans* and *C. briggsae* domains are 82% identical. Despite these similarities, no mutations have been identified in the TF

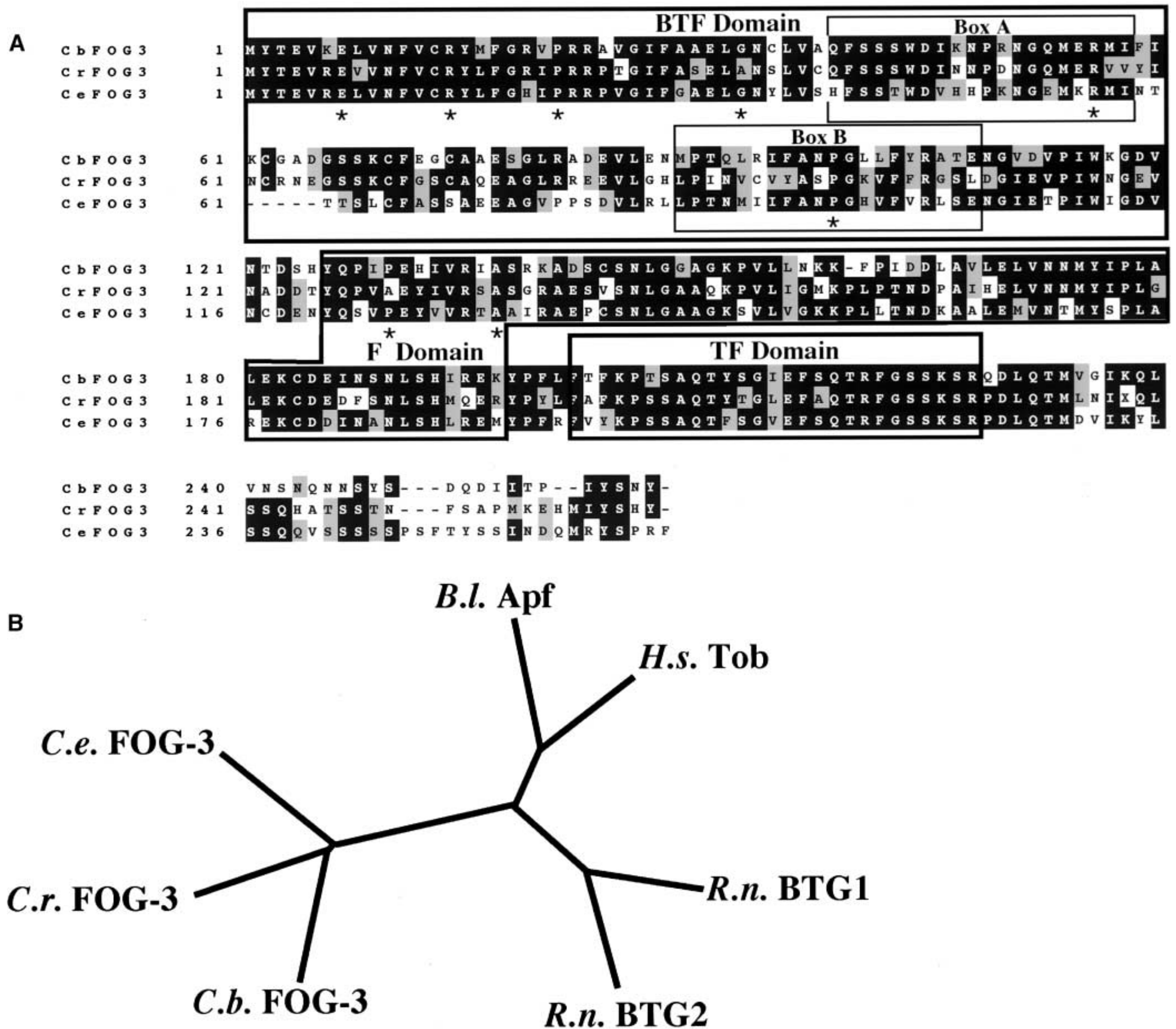


Figure 2.—The FOG-3 proteins define a new subgroup within the Tob family of proteins. (A) Boxshade alignment of *C.b.*-FOG-3, *C.r.*-FOG-3, and *C.e.*-FOG-3. Conserved domains are boxed and labeled. Residues that are identical are solid, and those that are similar are shaded. The alignment was prepared using ClustalX, and the figure was prepared using Boxshade. The locations of missense mutations in *C.e.*-FOG-3 are marked with asterisks below the affected residues (Chen *et al.* 2000). (B) Dendrogram of selected members of the Tob family of proteins. An unrooted tree was constructed using the neighbor-joining method, implemented by the ClustalX program, and is based on the amino termini of the seven proteins (ending at residue 120 of *C.e.*-FOG-3). The three members of the FOG-3 family of proteins were grouped together in 998/1000 bootstrap trials, and the *C.b.*-FOG-3 and *C.r.*-FOG-3 proteins were grouped together in 541/1000 bootstrap trials.

domain in *C. elegans* or in any other species, so we do not know if it is needed for activity.

The region between the BTF and TF domains is also highly conserved in the nematode FOG-3 proteins (Figure 2A; Table 2), although it is not found in other known proteins. In *C. elegans*, this region contains two missense mutations that inactivate the protein (Chen *et al.* 2000). Since this region is essential for activity, we named it the F domain (for FOG-3). It is important to note that the BTF, F, and TF domains together comprise most of FOG-3. Furthermore, the boundaries of each domain are based solely on blocks of amino acid conservation. We have no data that show that these domains function as independent units.

As one might expect from these results, phylogenetic analyses suggest that the FOG-3 proteins form a unique group within the Tob family (Figure 2B). Our results indicate that the *C. briggsae* and *C. remanei* proteins might be more closely related to each other than either is to that of *C. elegans* (Figure 2B). However, the level

TABLE 2

Percentage identity between domains of the different FOG-3 proteins

Conserved region: Length:	BTF: 120	Box A: 20	Box B: 20	F: 72	TF: 27	C terminus: 40	Total: 268
% identity							
Cb/Cr	61.7	80.0	30.0	63.9	81.5	32.5	59.7
Cb/Ce	55.8	60.0	50.0	59.7	81.5	32.5	56.0
Cr/Ce	56.7	50.0	50.0	61.1	77.8	40.0	57.1

These values are based on the alignment shown in Figure 2A. The BTF, F, and TF domains are defined in the text and in Figure 2A as are the Box A and Box B regions of the BTF domain. The C-terminal sequences are not highly conserved and are shown here to provide contrast to the other domains.

of identity between these three proteins is similar enough that we are hesitant to deduce a phylogeny of the caenorhabditids on the basis of these data alone.

We observed that two of the eight known missense mutations in *C. elegans* FOG-3 alter residues that are conserved in *C. briggsae* but not in *C. remanei* (Figure 2A; Chen *et al.* 2000). Previous studies showed that these mutations cause *XX* animals to develop as females (Ellis and Kimble 1995). Could these mutations define residues that are important for FOG-3 activity in *XX* animals but not in *XO* males? If so, changes in these residues might explain why *C. remanei* is a male/female species, whereas *C. elegans* and *C. briggsae* are male/hermaphroditic. To test this possibility, we generated *C. elegans* males homozygous for *fog-3(oz147)* [Pro125 → Leu] or *fog-3(q505)* [Gly33 → Arg] by standard crosses. These males produced oocytes but no sperm (data not shown). Thus, it seems unlikely that either of these residues plays a sex-specific role in the function of FOG-3. However, the *C. elegans* mutations introduce more dramatic substitutions than are found in the *C. remanei* FOG-3 protein (Arg instead of Gly33 *vs.* Ala instead of Gly33; Leu instead of Pro 125 *vs.* Ala instead of Pro125), and these changes could result in different biochemical properties.

FOG-3 is needed in each species for germ cells to become sperm rather than oocytes: Studies of *C. elegans* showed that mutations in *fog-3* or RNA-mediated interference produce the same phenotype—males and hermaphrodites make oocytes instead of sperm (Ellis and Kimble 1995; Chen *et al.* 2000). Since we do not yet have any mutations in *C. briggsae* or *C. remanei* that affect sex determination, we used RNA-mediated interference to determine what role *fog-3* plays in each species.

For *C. briggsae*, we found that RNA-mediated interference transformed the *XX* animals from self-fertile hermaphrodites into true females (Figure 3A). Furthermore, it caused the *XO* animals to produce oocytes instead of sperm (Figure 3B, Table 3) but did not alter any somatic sexual fates. In particular, the complex male tail and male somatic gonad appeared wild type (Figure 3C). These results show that FOG-3 plays the same role in *C. briggsae* as in *C. elegans*. When we carried out similar experiments with *C. remanei*, which is a male/female species, we obtained identical results—the *XO* animals produced oocytes instead of sperm (Figure 3D, Table 3) but still developed normal male bodies (Figure 3E). Thus, *fog-3* is required in each species to cause germ cells to initiate male development. In its absence, all germ cells become oocytes.

The *C. elegans* and *C. briggsae* FOG-3 proteins are functionally interchangeable: The similarities in sequence and function suggested that the three FOG-3 proteins might be interchangeable. To test this hypothesis, we constructed chimeric constructs in which the *C. briggsae* or *C. remanei* FOG-3 proteins were expressed under the control of the *C. elegans* promoter and 3′-un-translated region (UTR), created extrachromosomal arrays that carried these transgenes, and measured their ability to rescue *fog-3(q504)* mutants (Figure 4). In transgenic animals, the *C. briggsae* protein works almost as well as the *C. elegans* one itself (Table 4). This result shows that these FOG-3 proteins are indeed interchangeable. Furthermore, it implies that some regions of FOG-3, most notably the carboxyl terminus, can tolerate significant amino acid substitutions without affecting the activity of the protein.

By contrast, the *C. remanei* FOG-3 protein rescues poorly or not at all in this assay (Table 4), and the only two animals that it restored to self-fertility produced so few sperm that they did not establish stable lines. Although this result might be due to technical problems with the extrachromosomal arrays, it seems more likely that it reflects the inability of the *C. remanei* protein to function in *C. elegans*. This hypothesis is not implausible, since two residues that are essential in *C. elegans* FOG-3, as judged by analysis of mutants (Chen *et al.* 2000), were not conserved in the *C. remanei* protein (see above). However, it is also possible that the chimeric nature of this FOG-3 protein, whose first eight residues are derived from *C. elegans* sequences (see materials and methods), could lead to its inability to rescue *fog-3* mutants.

The expression of *fog-3* is correlated with spermatogenesis: If the functions of the FOG-3 proteins are conserved during nematode evolution, what factors can ac-

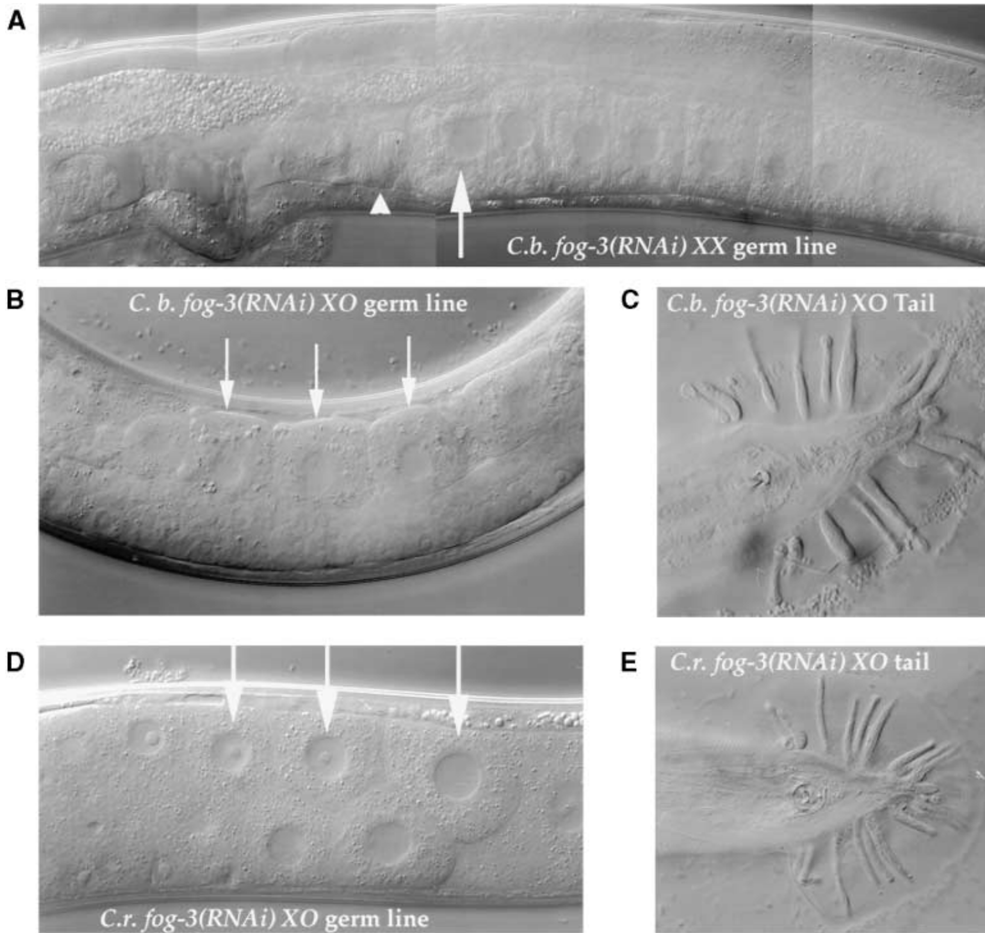


FIGURE 3.—FOG-3 is required for germ cells to initiate spermatogenesis in each species. Animals were injected with double-stranded RNA as described in MATERIALS AND METHODS, and their progeny were scored by Nomarski microscopy. In all cases, inactivation of FOG-3 caused all germ cells to differentiate as oocytes. (A) *C. briggsae fog-3(RNAi) XX* adult female. Anterior is to the left, and ventral is down. The white arrowhead marks the empty spermatheca, and the white arrow shows an oocyte. (B) *C. briggsae fog-3(RNAi) XO* adult male gonad. Anterior is to the left, and ventral is down. The white arrows mark oocytes. (C) *C. briggsae fog-3(RNAi) XO* adult male tail. Anterior is to the left, and ventral is up. (D) *C. remanei fog-3(RNAi) XO* adult male gonad. Anterior is to the left, and ventral is down. The white arrows mark oocytes. (E) *C. remanei fog-3(RNAi) XO* adult male tail. Anterior is to the left, and ventral is up.

count for the production of sperm by *XX* animals in *C. elegans* and *C. briggsae* and the lack of sperm production by *XX* animals in *C. remanei*? To address this problem, we began by studying the expression of *fog-3* transcripts in each species.

In both *C. briggsae* and *C. elegans*, the *XX* animals develop as hermaphrodites, which produce sperm during the fourth larval stage and oocytes as adults. In *C. elegans*, the expression of *fog-3* is correlated with this period of spermatogenesis (CHEN and ELLIS 2000). Is the same true for *C. briggsae*? To address this question, we used developmental Northern analysis to study the course of *fog-3* expression in a population of *C. briggsae*

TABLE 3

RNA-mediated inactivation of *fog-3* in *C. remanei* and *C. briggsae*

	% fog[a]	Number
C. briggsae XX	100	(>200)
C. briggsae XO[b]	92	(41)
C. remanei XO	97	(41)

[a] Animals were scored as Fog if they produced oocytes but no sperm.

[b] *C. briggsae XO* animals were the male progeny of *C. briggsae* hermaphrodites that had been injected with double-stranded RNA and then crossed with *C. briggsae* males. To ensure that the cross had been successful, we only scored broods in which approximately one-half of the progeny were male.

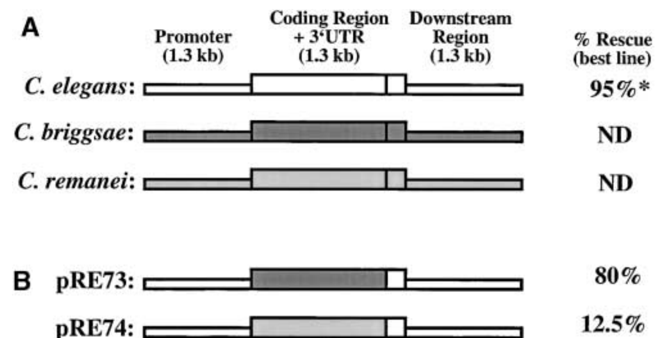


FIGURE 4.—The FOG-3 proteins are functionally interchangeable. (A) Diagram of the *fog-3* genes from three species of nematodes. (The coding regions contain the introns depicted in Figure 1A, but these are not depicted in the line drawing.) *, data by CHEN and ELLIS (2000). (B) Chimeric *fog-3* transgenes. The construction of these lines is described in MATERIALS AND METHODS. We prepared at least eight lines for each construct (Table 4). Because the structure of specific extrachromosomal arrays sometimes impairs the activity of genes they contain, we present the percentage rescue for the best line rather than an average of all.

TABLE 4

Rescue of *fog-3* phenotype by transgenes

A. Rescue for pRE11 (wild-type *C. elegans* gene)[a]													
Line no.:	1	2	3	4	5	6	7	8	9	10	11		
% rescue:	95	90	90	85	85	57	44	42	30	29	9		
N:	20	20	20	20	20	28	16	19	20	21	11		
B. Rescue for pRE73 (*C. briggsae* protein)													
Line no.:	1	2	3	4	5	6	7	8					
% rescue:	80	71	67	50	44	33	29	25					
N:	10	7	15	8	9	6	7	4					
C. Rescue for pRE74 (*C. remanei* protein)													
Line no.:	1	2	3	4	5	6	7	8	9	10	11	12	13
% rescue:	12.5	6.6	0	0	0	0	0	0	0	0	0	0	0
N:	8	16	6	6	6	4	5	25	8	13	15	12	18
D. Rescue for pRE70 (*C. briggsae* promoter)													
Line no.:	1	2	3	4	5	6	7	8	9	10			
% rescue:	60	50	29	18	0	0	0	0	0	0			
N:	5	14	7	17	17	12	17	7	8	6			
E. Rescue for pRE71 (*C. remanei* promoter)													
Line no.:	1	2	3	4	5	6	7	8					
% rescue:	82	74	37	19	13	11	7	0					
N:	22	23	30	21	24	28	15	24					

Percentage rescue reflects the percentage of *fog-3(q504) XX* animals that were restored to self-fertility by the transgene. These *fog-3* homozygotes were identified by a linked *unc-29* mutation, and fertile hermaphrodites were tested to see if they were recombinants. The line with the highest percentage rescue is underlined.

[a] Data from Chen and Ellis (2000).

hermaphrodites. We found that the expression of *fog-3* was highest during the L4 larval stage, when the animals produce sperm (Figure 5A), but that expression was low during adulthood, when the animals make only oocytes.

To analyze the expression of *fog-3* in *C. remanei*, we used quantitative RT-PCR to assay the levels of *fog-3* transcripts in individual male and female L4 larvae or adults. We found that both larval and adult males produce high levels of *fog-3*, but that neither larval nor adult females produce detectable levels of this message (Figure 5B). This result, in conjunction with our data showing that FOG-3 is necessary for spermatogenesis, is consistent with the idea that *C. remanei XX* animals fail to make sperm because they do not express *fog-3*.

Each *fog-3* promoter contains TRA-1A response elements: Since our results suggested that changes in the expression of *fog-3* might explain which animals produce sperm, we wanted to learn what factors regulate this expression pattern. Thus, we used panhandle PCR to clone the promoter regions from the *C. briggsae* and *C. remanei* genes (Figure 6A; Jones 1995). We found that *C. remanei* has two TRA-1A binding sites, each located close to the site for *trans*-splicing and thus probably near the transcriptional start site (Figure 6, A and B). In *C. elegans*, the single TRA-1A site located in this position is likely to mediate repression of *fog-3* by TRA-1A (Chen and Ellis 2000). Thus, in *C. remanei*, TRA-1A might bind to these sites in the *fog-3* promoter to repress transcription in *XX* animals. The *fog-3* promoter in *C. briggsae* also contains two TRA-1A binding sites located in a similar position (Figure 6, A and B). How could this promoter drive *fog-3* expression in *XX* larvae but not in adults? One possibility is that TRA-1A binds here to repress transcription of *fog-3* in *XX* animals but that upstream genes inactivate TRA-1A in L4 larvae to allow spermatogenesis during this stage of development.

We were surprised to find that the *C. briggsae* and *C. remanei* promoters contained significant similarities in sequence near these TRA-1A binding sites and these similarities were not shared with *C. elegans* (Figure 6B). In particular, each promoter contains several copies of a TTGCAG motif in this region. This result indicated that the *C. remanei* and *C. briggsae fog-3* promoters might not function like that of *C. elegans*. To test this possibility, we created chimeric constructs, in which the *C. elegans fog-3* gene was expressed under the control of either the *C. briggsae* or *C. remanei* promoters, and tested the ability of each to rescue a *fog-3* mutation when expressed from an extrachromosomal array. We found that both promoters function satisfactorily in *C. elegans* hermaphrodites (Figure 6C, Table 4). In particular, the *C. remanei* promoter is able to drive expression of *fog-3* so as to allow *C. elegans XX* larvae to produce sperm. This result implies that the differences in expression of *fog-3* be-

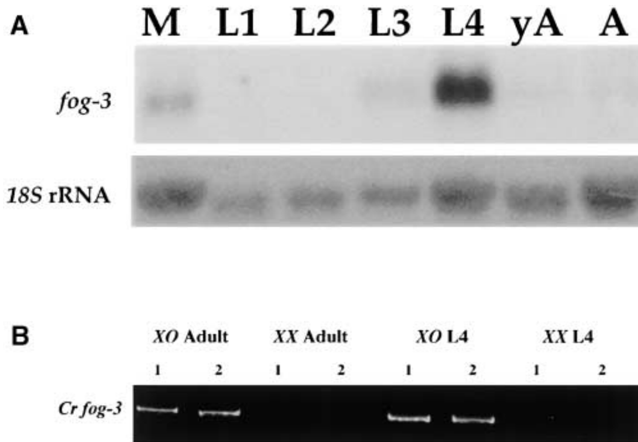


Figure 5.—Expression of each *fog-3* gene is correlated with spermatogenesis. (A) Developmental Northern blot of total RNA from populations of *C. briggsae XX* animals. M, mixed ages; L1 to L4, four larval stages; yA, young adults; A, adults. We used a probe for 18s ribosomal RNA on the same blot to control for variations in loading. The probes are described in materials and methods. (B) Quantitative RT-PCR analysis of *fog-3* expression in *C. remanei*. RNA was prepared from single worms of the indicated ages and used for RT-PCR analysis of *fog-3* expression. The number of cycles was set so that a twofold dilution of the male samples yielded a corresponding decrease in the intensity of the resulting band.

tween the species are probably caused by differences in the activity of upstream regulators like TRA-1A rather than by changes in the *fog-3* promoter itself.

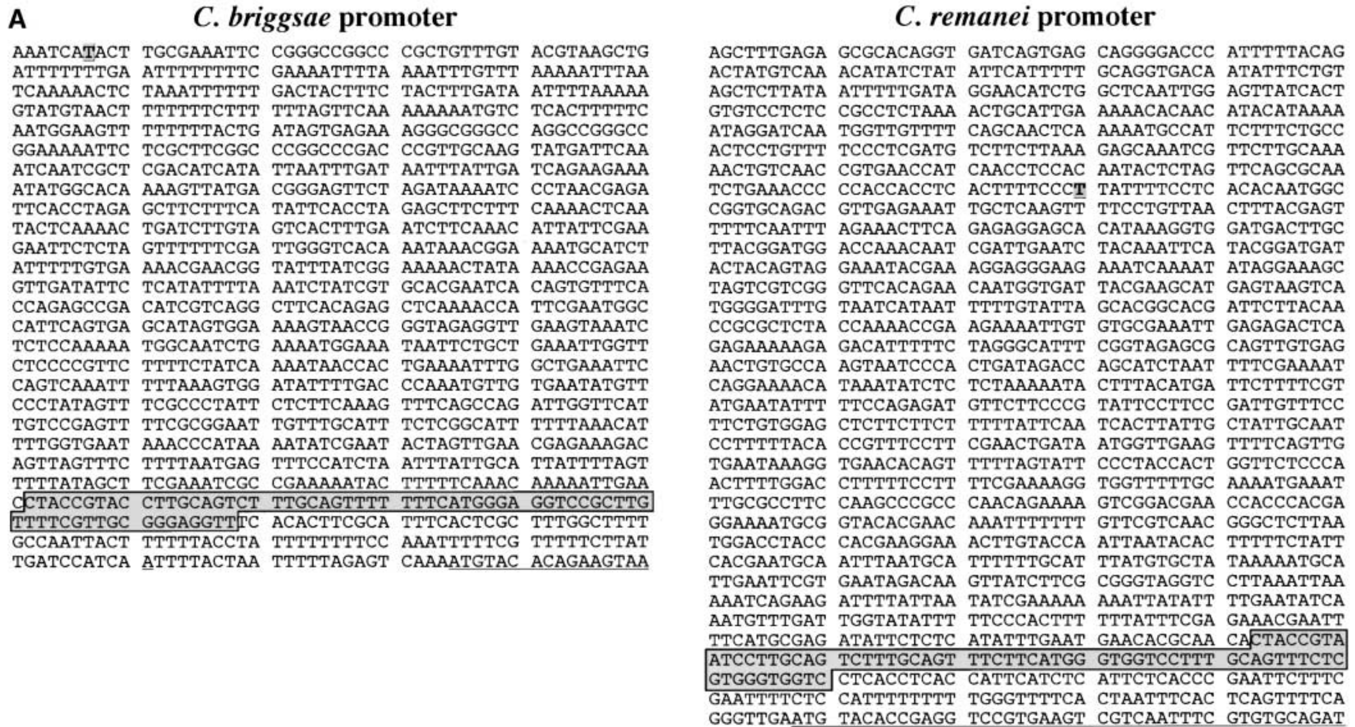
DISCUSSION

Conserved role for FOG-3 in regulating germ cell fates: Although proteins that contain BTF domains are expressed in a variety of tissues during vertebrate development, *C. elegans* contains only a single member of this family—FOG-3 (Chen *et al.* 2000). FOG-3 is expressed in the germ line, where it specifies that cells differentiate as sperm rather than as oocytes. Is this situation unique? We find that two other nematode species appear to produce a single BTF protein and these homologs of FOG-3 also specify that germ cells adopt male fates. Since some vertebrate homologs of FOG-3 are expressed in the male germ line, it is possible that some proteins with a BTF domain were conserved throughout evolution to regulate germ cell fates.

The conserved function of FOG-3 might allow rapid shifts in mating systems: Although proteins related to FOG-3 might play a role in the specification of germ cell fates in a broad range of species, the regulatory system that controls this decision appears unusually simple in nematodes. For example, no mutations that cause females to produce functional sperm or males to produce oocytes have been identified in other animals. By contrast, we know of two genes in *C. elegans* that appear to directly control this cell fate decision—*fog-1* and *fog-3* (Barton and Kimble 1990; Ellis and Kimble 1995). Mutations in these genes, or in many of the genes that regulate their activities, completely switch the sexual fates of germ cells without affecting other tissues (reviewed by Ellis 1998). Why is the regulatory machinery so simple and modular in nematodes? One possibility is that nematode germ cells adopt only a limited number of fates—proliferation, spermatogenesis, oogenesis, or cell death (reviewed by Ellis 1998; Ellis and Kimble 1994). By contrast, germ cells in other animals, such as flies and mammals, must be able to adopt a range of additional fates, such as nurse cells in female Drosophila (reviewed by McKearin and Christerson 1994) or types A_1, A_2, A_3, A_4, or B spermatogonia in male humans (reviewed by Sutton 2000).

By identifying homologs of *fog-3* from other caenorhabditids and demonstrating that these homologs are required for germ cells to become sperm rather than oocytes, we have shown that part of the simple regulatory machinery that controls germ cell fates in *C. elegans* might be a general feature of this group of nematodes. This result is particularly striking for *C. remanei*. We had suspected that nematodes that make *XX* hermaphrodites would need to have a modular system to control germ cell fates so that *XX* animals could produce first sperm and then oocytes. However, no such constraint need exist for a male/female species like *C. remanei*. Since *fog-3*, nevertheless, controls germ cell fates in *C. remanei*, and inactivation of *fog-3* in this species causes males to produce oocytes rather than sperm, we suspect that control of germ cell fates by the *fog* genes is a general feature of the caenorhabditids. If so, then perhaps one reason why different mating systems evolve so rapidly among these species is that any changes in the regulation of *fog-3* (and presumably in the regulation of its partner *fog-1*) could instantly and completely alter germ cell fates.

The regulation of *fog-3* expression changed during recent evolution: What regulatory changes might alter *fog-3* activity? Several observations suggest that changes in the mating systems of these nematode species might have been caused by changes in the upstream portions of the sex-determination pathway, which resulted in the expression of FOG-3 in *XX* hermaphroditic species but not in *XX* female species. First, FOG-3 is required for spermatogenesis in all three species. Second, the expression pattern for *fog-3* is correlated with spermatogenesis in each species. Third, the promoters from the three species are interchangeable, which implies that the differences in the expression patterns might be due to the altered activity of upstream regulatory genes. What types of changes could cause this effect? In *C. elegans*, mutations in *tra-2* can stop *XX* animals from producing sperm, effectively transforming them into females (Doniach 1986; Goodwin *et al.* 1993; Kuwabara *et al.* 1998). These changes in *tra-2* activity appear to act through the sex-determination pathway to regulate *tra-1*, which



B

C. b. CTACCGTA--CCTTGCAGTCTTTGCAGTTTTTTCATGGGAGGTCCGCTTGTTTCGTTGCGGGAGGTT

C. r. CTACCGTAATCCTTGCAGTCTTTGCAG-TTTCTTCATGGGTGGTCCTTTGCAGTTTCTCGTGGGTGGTC

C

	Promoter (1.3 kb)	Coding Region + 3'UTR (1.3 kb)	Downstream Region (1.3 kb)	% Rescue (best line)
C. elegans:				95%*
pRE70: *C. briggsae* fusion:				60%
pRE71: *C. remanei* fusion:				82%

FIGURE 6.—The *fog-3* promoters contain conserved TRA-1A binding sites. (A) Sequences of the *C. briggsae* and *C. remanei fog-3* promoter regions. These regions were cloned using panhandle PCR as described in MATERIALS AND METHODS. The shaded regions are shown in greater detail in B. The coding regions are underlined. The starting nucleotides for the chimeric constructs shown in C are boxed and shaded. Each occurs at position −1344 relative to the initiation codon. (B) Alignment of the TRA-1 binding sites in the *C. briggsae* and *C. remanei* promoters. TRA-1 binding sites are boxed, with conserved regions of the binding site light gray. The TTGCAG motif located upstream of these binding sites is dark gray. (C) Structure and activity of chimeric transgenes in which exogenous promoters are used to drive expression of *C. elegans* FOG-3 in *C. elegans fog-3* mutants. The construction of these lines is described in MATERIALS AND METHODS. We prepared at least six lines for each construct. Because the structure of specific extrachromosomal arrays sometimes impairs the activity of genes they contain, we present the percentage rescue for the best line rather than an average of all. *, data by CHEN and ELLIS (2000).

directly controls expression of *fog-3* and, perhaps, *fog-1* (CHEN and ELLIS 2000; JIN *et al.* 2001). However, biochemical experiments suggest that changes in the regulatory sites found in *tra-2* appear unlikely to explain the evolution of different mating systems in these nematode species (HAAG and KIMBLE 2000). It remains possible that genes that regulate *tra-2* activity might be involved in the creation of new mating systems. The simplest scenario involves *fog-2*, which is needed for spermatogenesis in *C. elegans* hermaphrodites (SCHEDL and KIMBLE 1988; CLIFFORD *et al.* 2000). If *C. remanei* animals had lost the activity of an ancestral *fog-2* gene, this change would have caused *XX* larvae to lose expression of *fog-3* and to make oocytes rather than sperm. These same regulatory changes might also affect the expression of FOG-1, which functions as partner of FOG-3 (BARTON and KIMBLE 1990; ELLIS and KIMBLE 1995), and which appears to be regulated in a similar manner (JIN *et al.* 2001).

By using the expression of *fog-3* as a molecular marker, we should be able to elucidate how specific changes in upstream regulatory genes like the *C. briggsae* homologs

of *tra-1* (de Bono and Hodgkin 1996), *tra-2* (Kuwabara 1996), *fem-2* (Hansen and Pilgrim 1998), and *her-1* (Streit *et al.* 1999) affect sex determination in germ cells. Furthermore, since all *fog-3* promoters contain TRA-1A binding sites, we can determine if the upstream sex-determination genes act through TRA-1A to control germ cell fates. These studies should allow us to compare, at a molecular level, how the control of *fog-3* expression differs between female and hermaphroditic nematodes.

Are downstream genes in sex determination pathways more highly conserved? Proteins that regulate sexual identity often diverge more rapidly than proteins that control other aspects of development. For example, when *C. elegans* is compared with *C. briggsae*, the TRA-1A proteins are only 44% identical, the TRA-2A proteins are 43% identical, the FEM-2 proteins are 63% identical, and the HER-1 proteins are only 57% identical. Furthermore, none of these proteins have homologs that regulate sexual identity in insects or vertebrates. By contrast, MAB-3, which acts downstream of TRA-1A (Shen and Hodgkin 1988), is homologous to DSX from Drosophila (Raymond *et al.* 1998) and regulates similar sexual characteristics. Since DSX acts at the end of the Drosophila sex determination pathway, this result raised the possibility that other downstream genes might also be more highly conserved than upstream ones. FOG-3 is the first downstream gene identified in the germ line and the first one for which homologs from other nematode species are available. However, we found that the three FOG-3 proteins are ~58% identical, so they appear to be diverging as rapidly as most other sex-determination genes. Since this divergence has not altered the function of these FOG-3 proteins, positive selection for an altered role in germ line development is not likely to be the cause of these changes.

Three regions of FOG-3 are defined by comparative analysis: Although there are few dramatic blocks of identity among the FOG-3 proteins, three regions appear important. First, the amino-terminal half of the BTF domain is highly conserved. This region is altered by four of the eight known missense mutations in the *C. elegans fog-3* gene. These results support the notion that the BTF domain is critical for FOG-3 function but point to its amino terminus, rather than Box A or Box B, as being least tolerant of amino acid substitutions. To date, the biochemical function of this portion of the BTF domain remains unknown. However, in BTG1 and BTG2, Box B is capable of interacting with mammalian CAF-1 (Bogdan *et al.* 1998; Rouault *et al.* 1998), a protein that is likely to form part of a transcriptional regulatory complex.

Second, the TF domain contains a region of high similarity among the FOG-3 proteins. Since this domain is also found in the Tob proteins of vertebrates, it is tempting to speculate that it interacts with or assists the BTF domain. Because none of the *C. elegans* mutations map to this domain we have not been able to test its function.

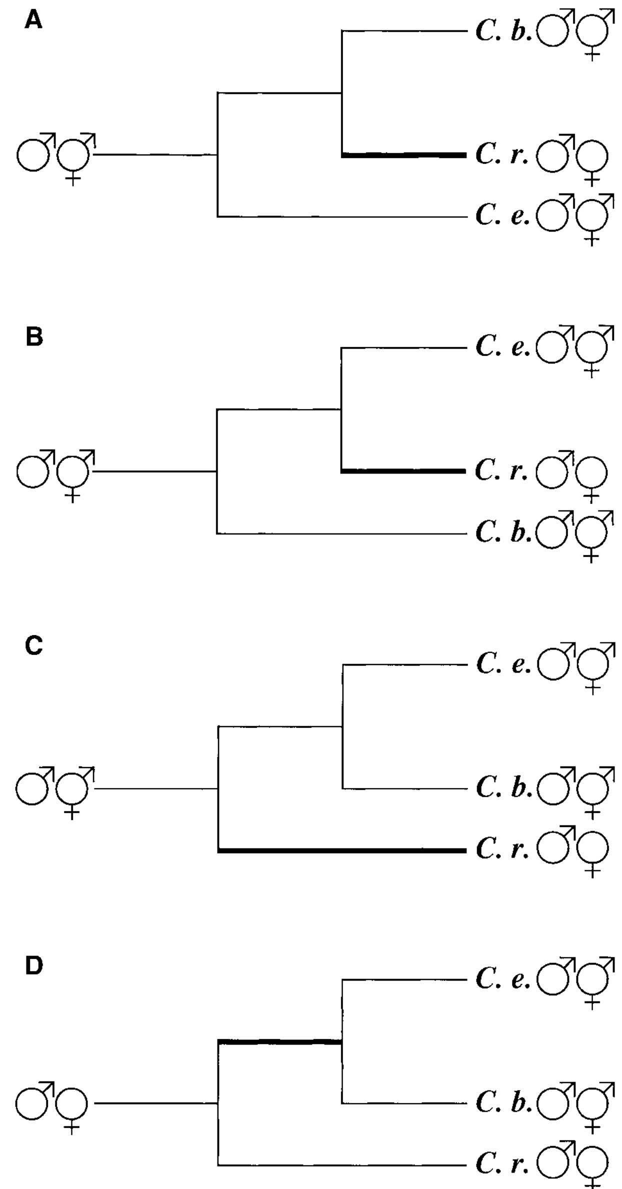


Figure 7.—Likely models for the evolution of mating systems in the caenorhabditids. The hypothetical status of the ancestral species is shown to the left, and the branch at which the mating system of one of the species switched its state is marked with a thick line. (A) Model in which *C.b.* and *C.r.* are sister species. Our data point in this direction but do not establish it conclusively. If true, the simplest model for the evolution of these mating systems is that *C.r.* switched from a male/hermaphroditic to a male/female system after it split from *C.b.* (B) Model in which *C.e.* and *C.r.* are sister species. (C) Model 1 in which *C.e.* and *C.b.* are sister species. Since this hypothesis groups the male/hermaphroditic species together, two different models can account for the evolution of these mating systems by positing that a switch in the trait occurred only once. This particular model assumes that the ancestral state was male/hermaphroditic. (D) Model 2 in which *C.e.* and *C.b.* are sister species. This model assumes that the ancestral state was male/female.

Third, two of the mutations in *C. elegans fog-3* map to

a region that lies between the BTF and TF domains. This region is conserved among the three FOG-3 proteins, although it is not found in other known proteins. We cannot tell if it serves as a functional domain in its own right or if it was conserved because of its position bridging the BTF and TF domains. Eventually, crystallographic analysis of FOG-3 and other Tob proteins might resolve this issue.

Phylogeny of the caenorhabditids: The phylogenetic relationship between *C. elegans*, *C. briggsae*, *C. remanei*, and other members of this genus has been hard to disentangle (Fitch *et al.* 1995). These three species are part of the Elegans subgroup within the caenorhabditids (Sudhaus and Kiontke 1996). However, we do not know the relationship between these three species, nor do we know if the ancestral caenorhabditid was a male/female species or a male/hermaphroditic one. To solve these problems, several genes from more distantly related caenorhabditids will have to be characterized to establish a reliable outgroup for the analysis of *C. elegans*, *C. briggsae*, and *C. remanei*.

Despite these concerns, our data suggest that *C. briggsae* (*C.b.*)-FOG-3 and *C. remanei* (*C.r.*)-FOG-3 are slightly more similar to each other than either is to *C. elegans* (*C.e.*)-FOG-3. Besides the overall percentage of similarity, four additional features of the alignment support this possibility. First, *C.e.*-FOG-3 contains a five-amino-acid deletion just after residue 60 that is not found in the other two proteins. Second, *C.e.*-FOG-3 contains a three-amino-acid insertion starting at residue 246 that is not found in the other two proteins. Third, the intron/exon structure of the three genes can be accounted for easily by assuming that *C. remanei* and *C. briggsae* are sister species. Fourth, the *C. remanei* and *C. briggsae fog-3* promoters are much more similar to each other than either is to that of *C. elegans*. Thus, comparative *fog-3* sequence data suggest that *C. briggsae* and *C. remanei* are sister species. Recent analyses of the *glp-1* genes of these three species support this conclusion (Rudel and Kimble 2001).

Evolution of mating systems in the caenorhabditids: The most parsimonious explanations for the evolution of the different mating systems in this group should assume only a single evolutionary change. For example, if *C. briggsae* and *C. remanei* were sister species, then the simplest model for the evolution of these mating systems would be that the ancestral caenorhabditid was male/hermaphroditic and that *C. remanei* became a male/female species through a change that caused the expression of *fog-3* to become repressed in *XX* animals (Figure 7A). This model could explain the evolution of a completely new mating system by a single mutation in an upstream regulatory gene such as *fog-2*. If *C. elegans* and *C. remanei* turn out to be sister species, a similar scenario works equally well (Figure 7B). However, if *C. elegans* and *C. briggsae* turn out to be sister species, it is much harder to predict the mating system used by their ancestor because the two alternatives appear equally likely (Figure 7, C and D). Thus, to elucidate how these mating systems evolved, we are now working to resolve the phylogeny of these nematodes and to assay the activity of upstream genes in the sex-determination pathway in the *XX* germ line. These studies could eventually provide a molecular explanation for the alteration of mating systems during evolution.

We thank Scott Baird, Scott Emmons, and the *Caenorhabditis* Genetics Center for providing strains; and Rolf Bodmer, Ken Cadigan, Kristin Douglas, and Priscilla Tucker for comments on this manuscript. Our work was funded by National Science Foundation grant MCB 9875398. R.E.E. was also funded by March of Dimes Basil O'Connor award 5-FY97-688.

LITERATURE CITED

Baird, S. E., M. E. Sutherlin and S. W. Emmons, 1992 Reproductive isolation in Rhabditidae (Nematoda:Secernentea); mechanisms that isolate six species of three genera. Evolution **46:** 585–594.

Barton, M. K., and J. Kimble, 1990 *fog-1*, a regulatory gene required for specification of spermatogenesis in the germ line of *Caenorhabditis elegans*. Genetics **125:** 29–39.

Bogdan, J. A., C. Adams-Burton, D. L. Pedicord, D. A. Sukovich, P. A. Benfield *et al.*, 1998 Human carbon catabolite repressor protein (CCR4)-associative factor 1: cloning, expression and characterization of its interaction with the B-cell translocation protein BTG1. Biochem. J. **336:** 471–481.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Bull, J. J., 1983 *The Evolution of Sex-Determining Mechanisms*. Benjamin/Cummings, Menlo Park, CA.

C. elegans Genome Sequencing Consortium, 1998 Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science **282:** 2012–2018.

Chen, P., and R. E. Ellis, 2000 TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*. Development **127:** 3119–3129.

Chen, P. J., A. Singal, J. Kimble and R. E. Ellis, 2000 A novel member of the tob family of proteins controls sexual fate in *Caenorhabditis elegans* germ cells. Dev. Biol. **217:** 77–90.

Clifford, R., M. H. Lee, S. Nayak, M. Ohmachi, F. Giorgini *et al.*, 2000 FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the C. elegans hermaphrodite germline. Development **127:** 5265–5276.

de Bono, M., and J. Hodgkin, 1996 Evolution of sex determination in Caenorhabditis: unusually high divergence of *tra-1* and its functional consequences. Genetics **144:** 587–595.

Doniach, T., 1986 Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. Genetics **114:** 53–76.

Ellis, R. E., 1998 Sex and death in the *C. elegans* germ line, pp. 119–138 in *Cell Lineage and Fate Determination*, edited by S. A. Moody. Academic Press, San Diego.

Ellis, R. E., and J. Kimble, 1994 Control of germ cell differentiation in *Caenorhabditis elegans*. Ciba Found. Symp. **182:** 179–188.

Ellis, R. E., and J. Kimble, 1995 The *fog-3* gene and regulation of cell fate in the germ line of *Caenorhabditis elegans*. Genetics **139:** 561–577.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811.

Fitch, D. H., B. Bugaj-Gaweda and S. W. Emmons, 1995 18S ribosomal RNA gene phylogeny for some Rhabditidae related to Caenorhabditis. Mol. Biol. Evol. **12:** 346–358.

Fodor, A., D. Riddle, F. Nelson and J. Golden, 1983 Comparison of a new wild-type *Caenorhabditis briggsae* with laboratory strains of *C. briggsae* and *C. elegans*. Nematologica **29:** 203–217.

Frohman, M. A., M. K. Dush and G. R. Martin, 1988 Rapid produc-

tion of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85:** 8998–9002.

Goodwin, E. B., P. G. Okkema, T. C. Evans and J. Kimble, 1993 Translational regulation of *tra-2* by its 3′ untranslated region controls sexual identity in *C. elegans.* Cell **75:** 329–339.

Guehenneux, F., L. Duret, M. B. Callanan, R. Bouhas, S. Hayette *et al.*, 1997 Cloning of the mouse BTG3 gene and definition of a new gene family (the BTG family) involved in the negative control of the cell cycle. Leukemia **11:** 370–375.

Guo, S., and K. J. Kemphues, 1995 *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611–620.

Haag, E. S., and J. Kimble, 2000 Regulatory elements required for development of *Caenorhabditis elegans* hermaphrodites are conserved in the tra-2 homologue of *C. remanei*, a male/female sister species. Genetics **155:** 105–116.

Halloran, N., Z. Du and R. K. Wilson, 1993 Sequencing reactions for the applied biosystems 373A automated DNA sequencer. Methods Mol. Biol. **23:** 297–315.

Hansen, D., and D. Pilgrim, 1998 Molecular evolution of a sex determination protein. FEM-2 (pp2c) in Caenorhabditis. Genetics **149:** 1353–1362.

Hansen, D., and D. Pilgrim, 1999 Sex and the single worm: sex determination in the nematode C. elegans. Mech. Dev. **83:** 3–15.

Horvitz, H. R., S. Brenner, J. Hodgkin and R. K. Herman, 1979 A uniform genetic nomenclature for the nematode *Caenorhabditis elegans.* Mol. Gen. Genet. **175:** 129–133.

Jin, S. W., J. Kimble and R. E. Ellis, 2001 Regulation of cell fate in *Caenorhabditis elegans* by a novel cytoplasmic polyadenylation element binding protein. Dev. Biol. **229:** 537–553.

Jones, D. H., 1995 Panhandle PCR. PCR Methods Appl. **4:** S195–201.

Kuwabara, P. E., 1996 Interspecies comparison reveals evolution of control regions in the nematode sex-determining gene *tra-2.* Genetics **144:** 597–607.

Kuwabara, P. E., P. G. Okkema and J. Kimble, 1998 Germ-line regulation of the *Caenorhabditis elegans* sex-determining gene *tra-2.* Dev. Biol. **204:** 251–262.

Marin, I., and B. S. Baker, 1998 The evolutionary dynamics of sex determination. Science **281:** 1990–1994.

McKearin, D., and L. Christerson, 1994 Molecular genetics of the early stages of germ cell differentiation during Drosophila oogenesis. Ciba Found. Symp. **182:** 210–219.

Meyer, B. J., 1997 Sex determination and *X* chromosome dosage compensation, pp. 209–240 in *C. elegans II*, edited by D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess. Cold Spring Harbor Laboratory Press, Plainview, NY.

Raymond, C. S., C. E. Shamu, M. M. Shen, K. J. Seifert, B. Hirsch *et al.*, 1998 Evidence for evolutionary conservation of sex-determining genes. Nature **391:** 691–695.

Rose, T. M., E. R. Schultz, J. G. Henikoff, S. Pietrokovski, C. M. McCallum *et al.*, 1998 Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. **26:** 1628–1635.

Rouault, J. P., D. Prevot, C. Berthet, A. M. Birot, M. Billaud *et al.*, 1998 Interaction of BTG1 and p53-regulated BTG2 gene products with mCaf1, the murine homolog of a component of the yeast CCR4 transcriptional regulatory complex. J. Biol. Chem. **273:** 22563–22569.

Rudel, D., and J. Kimble, 2001 Conservation of glp-1 regulation and function in nematodes. Genetics **157:** 639–654.

Sanger, F., S. Nicklen and A. R. Coulson, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

Schedl, T., and J. Kimble, 1988 *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans.* Genetics **119:** 43–61.

Schütt, C., and R. Nöthiger, 2000 Structure, function and evolution of sex-determining systems in Dipteran insects. Development **127:** 667–677.

Shen, M. M., and J. Hodgkin, 1988 *mab-3*, a gene required for sex-specific yolk protein expression and a male-specific lineage in *C. elegans.* Cell **54:** 1019–1031.

Streit, A., W. Li, B. Robertson, J. Schein, I. H. Kamal *et al.*, 1999 Homologs of the *Caenorhabditis elegans* masculinizing gene *her-1* in *C. briggsae* and the filarial parasite *Brugia malayi.* Genetics **152:** 1573–1584.

Sudhaus, W., 1974 Zur systematik, Verbreitung, Okologie und biologie neuer und wenig bekannter Rhabditiden (Nematoda). Zool. Jahrb. Syst. Bd. **101:** 417–465.

Sudhaus, W., and K. Kiontke, 1996 Phylogeny of Rhabditis subgenus Caenorhabditis (Rhabditidae, Nematoda). J. Zool. Syst. Evol. Res. **34:** 217–233.

Sutton, K. A., 2000 Molecular mechanisms involved in the differentiation of spermatogenic stem cells. Rev. Reprod. **5:** 93–98.

Wyckoff, G. J., W. Wang and C.-I Wu, 2000 Rapid evolution of male reproductive genes in the descent of man. Nature **403:** 304–309.

Zarkower, D., and J. Hodgkin, 1992 Molecular analysis of the *C. elegans* sex-determining gene *tra-1*: a gene encoding two zinc finger proteins. Cell **70:** 237–249.

Communicating editor: P. Anderson