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TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*

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SUMMARY

In *C. elegans*, the zinc-finger protein TRA-1A is thought to be the final arbiter of somatic sexual identity. We show that *fog-3*, which is required for germ cells to become sperm rather than oocytes, is a target of TRA-1A. First, northern analyses and RT-PCR experiments indicate that expression of *fog-3* is controlled by *tra-1*. Second, studies of double mutants show that this control could be direct. Third, the *fog-3* promoter contains multiple sites that bind TRA-1A in gel shift assays, and mutations in these sites alter activity of *fog-3* in vivo. These results establish *fog-3* as one of the first known targets of transcriptional regulation by TRA-1A. Furthermore, they show that *tra-1* controls a terminal regulator of sexual fate in germ cells, just as it is thought to do in the soma.

Key words: Germ line, Sex determination, *fog-3*, *tra-1*, Transcriptional regulation, *Caenorhabditis elegans*

INTRODUCTION

During animal development, many cells adopt one fate in males, and a different fate in females. To understand how sexual development is controlled, researchers have focused on several interrelated questions, which can be divided into three groups. First, what mechanism determines which animals will become male, and which female? Second, how does this mechanism control the many genes required for sexual differentiation. Third, how is this decision co-ordinated so that all tissues adopt the same sexual fate?

The nematode *Caenorhabditis elegans* is one of the leading models for study of how sexual fates are controlled (reviewed by Meyer, 1997; Ellis, 1998). These roundworms have two sexes – *XO* animals become males and *XX* animals become hermaphrodites. (Hermaphrodites are essentially females that produce sperm during larval development, which they use for self-fertilization). The initial signal that controls these sexual fates is the ratio of *X* chromosomes to autosomes (see Fig. 1; Madl and Herman, 1979). This signal regulates the activity of the *xol-1* gene, which acts through *sdc-1, sdc-2* and *sdc-3* to control transcription of *her-1*, which encodes a small, secreted protein produced only in males. Diffusion of HER-1 appears to co-ordinate the choice of sexual fate among different cells in the animal. Several studies suggest that HER-1 binds the TRA-2A protein in target cells, and that TRA-2A acts through FEM-1, FEM-2 and FEM-3 to regulate the activity of *tra-1*, the master switch for sexual fate. If *tra-1* is active, somatic cells adopt 'female' fates, whereas if *tra-1* is inactive they adopt male fates.

Molecular analyses indicate that *tra-1* encodes a large protein with five zinc-fingers and a smaller product of unknown function (Zarkower and Hodgkin, 1992; Hodgkin, 1993). The larger protein is known as TRA-1A; its zinc-fingers resemble those of the mammalian proteins GLI and GLI3, and of the *Drosophila* protein Cubitus interruptus (Ci). Furthermore, in vitro studies reveal that TRA-1A binds target sequences similar to those recognized by GLI, GLI3 and Ci, and has highest affinity for the sequence TTTTCnnnnTGGGTGGTC (Zarkower and Hodgkin, 1993). These results suggest that *tra-1* controls sexual fate by repressing the expression of genes required for male differentiation, or by promoting expression of genes required for female differentiation.

Several potential targets of *tra-1* are expressed in a sex-specific manner. In the soma these include *mab-3* and the yolk-protein genes. The promoters for the yolk-protein genes lack TRA-1A binding sites, and instead appear to be directly controlled by MAB-3 (Blumenthal et al., 1984; Zucker-Aprison and Blumenthal, 1989; Raymond et al., 1998; Yi and Zarkower, 1999). The *mab-3* gene regulates development of the male tail, and prevents expression of the yolk-protein genes in the male intestine (Shen and Hodgkin, 1988; Raymond et al., 1998). Genetic and molecular studies show that *mab-3* acts downstream of *tra-1* (D. Zarkower, personal communication). Furthermore, its promoter appears to contain TRA-1A binding sites (Clarke and Berg, 1998). However, it is not known if TRA-1A binds these sites and directly regulates expression of *mab-3*. Finally, the *egl-1* gene plays a general role regulating programmed cell deaths in both males and hermaphrodites (Conradt and Horvitz, 1998), and several dominant mutations in *egl-1* affect the survival of two neurons that control egg-laying in hermaphrodites, but which die in males (Trent et al., 1983; Ellis and Horvitz, 1986). This result raised the possibility that TRA-1A might regulate expression of *egl-1* in these

neurons, a hypothesis recently confirmed by Conradt and Horvitz (1999).

Although *tra-1* is clearly the master-control gene for sex determination in the soma, its role in the germ line has been elusive. Whereas gain-of-function mutations in *tra-1* cause all germ cells to differentiate as oocytes (Hodgkin, 1987), loss-of-function mutations cause some germ cells to develop as sperm and others as oocytes (Hodgkin, 1987; Schedl et al., 1989). These results indicate that *tra-1* is not required to specify either fate. Furthermore, analyses of double mutants suggest that *tra-1* either acts upstream of the *fem* genes in the germ line, or in parallel to them, although it acts downstream of the *fem* genes in the soma (Doniach and Hodgkin, 1984; Hodgkin, 1986). Thus, many researchers have suspected that *tra-1* plays a peripheral role in the control of germline fates.

How are germ cell fates controlled? Five genes are essential for germ cells to develop as sperm rather than as oocytes – *fem-1, fem-2, fem-3, fog-1* and *fog-3*. As with the *fem* genes, *fog-1* and *fog-3* appear to act downstream of *tra-1* in tests of genetic epistasis; however, although the *fem* genes also function in the soma, *fog-1* and *fog-3* control only germ cell fates (Barton and Kimble, 1990; Ellis and Kimble, 1995). Furthermore, northern analyses and studies of genetic mosaic animals suggest that *fog-3* functions only in the germ line (Chen et al., 2000). Thus, one simple hypothesis is that the FEM proteins regulate the activities of FOG-1 and FOG-3, which promote spermatogenesis and prevent oogenesis. We recently cloned the *fog-3* gene, and found that it encodes a homolog of the Tob, BTG1 and BTG2 proteins, which might suppress proliferation and promote differentiation in vertebrates (Chen et al., 2000). To learn how germ cell fates are specified, we analyzed the expression of *fog-3*, and found that it is one of the major targets of TRA-1A in *C. elegans*. This result reveals the underlying similarity between the control of somatic and germline sex in nematodes, and establishes the *fog-3* promoter as a model for understanding how TRA-1A regulates gene expression.

MATERIALS AND METHODS

Genetic nomenclature

The genetic nomenclature 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 cannot 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 methods

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), and contained one or more of the following sex-determination mutations: *fog-1(q253*ts*), fog-1(q187)* (Barton and Kimble, 1990), *tra-2(b202*ts*)* (Klass et al., 1976), *fog-3(q469), fog-3(q504)* (Ellis and Kimble, 1995), *fem-2(b245ts)* (Kimble et al., 1984), *fem-2(e2102), fem-2(e2105)* (Hodgkin, 1986), *tra-1(e1099)* (Hodgkin and Brenner, 1977), *tra-1(e1834)* (Hodgkin, 1993), *fem-1(hc17*ts*)* (Nelson et al., 1978), *fem-1(e2044)*(Hodgkin, 1986) *fem-1(e2382)* (Spence et al., 1990), *fem-3(e1996), fem-3(e2006),* (Hodgkin, 1986), *fem-3(q96)* (Barton et al., 1987) and *him-5(e1490)* (Hodgkin et al., 1979). A description of the marker mutations *unc-13(e1091), unc-29(e1072), unc-32(e189), mor-2(e1125)* and *dpy-20(e1282)* was prepared by Hodgkin (1997). Finally, *spf-1(q7)* causes abnormal gonadogenesis and sterility when homozygous (J. Miskowski, R. E. E. and J. Kimble, unpublished results).

To identify *tra-1(e1099); fem-1(hc17*ts*)* animals, we produced *tra-1/+; fem-1/+* heterozygotes at 15°C, and used replica plating to screen their progeny for *tra-1/+; fem-1* individuals. We let these individuals self-fertilize at 25°C, and identified homozygous *tra-1; fem-1* progeny by their male bodies.

The mutations *fem-1(e2044), fem-2(e2102)* and *fem-2(e2105)* all show a strong maternal effect – the *fem/fem* progeny of heterozygous mothers are self-fertile hermaphrodites (Hodgkin, 1986). Thus, to construct *tra-1(e1099); fem-1(e2044)* animals, we screened the self-progeny of *tra-1/+; fem-1/+* worms for self-fertile hermaphrodites that produced broods containing females rather than hermaphrodites; these broods were composed of homozygous *fem-1* animals born to a *fem-1* mother. From among these broods, we selected those containing *tra-1/tra-1* offspring, which have male bodies. Similar methods were used with both *fem-2* alleles. The *fem-2(e2102) tra-1(e1834)* animals were constructed similarly, but the *tra-1(e1834)* mutation was linked to the marker *unc-32*.

The *fem-3(e1996)* mutation was linked to the marker *dpy-20*. To identify *tra-1; fem-3(e1996) dpy-20* animals, we screened the self-progeny of *tra-1/+; fem-3 dpy-20* hermaphrodites for Dpy animals with male bodies.

Construction of transgenic animals

To make stable lines of transgenic animals, we injected *unc-29 fog-3(q504)/spf-1* animals with a solution containing a *fog-3* construct at 0.14 ng/μl, and the marker plasmid pRF4 [*rol-6(su1006*dm*)*] at 100 ng/μl (Mello et al., 1991). These relative concentrations were designed so that each extra-chromosomal array would incorporate only a few copies of *fog-3*. After identifying stable, transformed lines of worms that showed the phenotype of our marker, we tested homozygous *unc-29* progeny to see if the extra-chromosomal array restored self-fertility by allowing the *fog-3* animals to produce sperm. The progeny of fertile Unc Rol animals were tested to confirm that self-fertility had not been caused by the separation of *fog-3* from *unc-29* through recombination.

Northern analysis

Procedures for preparation of total RNA, northern analysis and preparation of *fog-3* probes are described by Chen et al. (2000). To detect the 26s ribosomal RNA, we used an antisense RNA probe; the template was prepared from reverse-transcribed RNA by PCR, using primers RE110 (CGGCGAGTGAAACGGG) and RE111 (GGATC-CTAATACGACTCACTATAGGGAGACCACTTTAGGCTGCACTT-TC), which contains the T7 promoter sequence.

Reverse-Transcriptase PCR analysis

To average out small variations in gene expression, we picked pools of 5 worms of identical age and genotype into a 2 μl drop of water, located in the lid of a micro-centrifuge tube. The worms were then transferred by centrifugation into a solution of 200 μl of TRI-Reagent plus 2 μl of Microcarrier Gel-TR (Molecular Research Center, Inc.). After freezing at −80°C, the worms were thawed and lysed by sonication at power 9 for 30 seconds, using a cup-horn probe on a Sonicator Xl (Misonix Inc.). The RNA was extracted following manufacturer's instructions (Molecular Research Center, Inc.) and resuspended in 25 μl of water. An 8 μl aliquot of each sample was reverse-transcribed using 100 units of MMLV-Reverse Transcriptase (Promega), 40 units of RNasin Ribonuclease inhibitor (Promega) and 25 μg/ml oligo-dT_{18} primer (Promega).

One-tenth of each reverse transcription reaction was tested by the PCR. In each series of reactions, a master mix containing 1× PCR buffer II and 0.5 units of AmpliTaq Gold (Perkin Elmer), 1.5 mM $MgCl_2$, 200 nM dNTPS, and 12.5 pmol each of primers RE101 (ATGTATACCGAAGTCCGCGAGC) and RE104 (GAACATCCCA-

GGTAGACGAGAA) was prepared and distributed among all samples. The polymerase was activated by heating to 95°C for 9 minutes, after which the samples were amplified during 33 cycles of [15 seconds at 95°C, 30 seconds at 62°C, 30 seconds at 72°C]. Control reactions using serial dilutions of a *fem-3(q96)* XX L4 sample showed that these conditions give a linear response to increasing amounts of *fog-3* transcripts (data not shown).

To detect partially processed transcripts, we prepared cDNA as described, but used 2.5 μM random hexamers (Ambion) to prime reverse transcription. For PCR, the primers were RE114 (GGTTTAATTACCCAAGTTTGAGGAGAA), which spans the SL1/exon#1 splice site, and RE115 (CTCGGGGACTGATTGGTAGCTGGA) from intron 4 (Chen et al., 2000). Samples were amplified for 40 cycles of [15 seconds at 95°C, 30 seconds at 62°C, 30 seconds at 72°C].

Many *tra-1(null)* mutants have abnormal gonads (Hodgkin, 1987). To avoid having differences in gonad morphology affect the levels of *fog-3* transcripts, all *tra-1* animals in these experiments were screened using Nomarski optics, and individuals with normal, reflexed gonads were selected for analysis.

Plasmid construction

Our wild-type *fog-3* plasmid was pRE11 (Chen et al., 2000). We built pRE21 by digestion of pRE11 with *Xho*I and *Bsr*GI, followed by re-ligation with a suitable linker, and pRE20 by digestion of pRE11 with *Xho*I and *Nsi*I, followed by re-ligation with a suitable linker. Finally pRE18 was constructed through a series of PCR reactions, in which we used primers containing the appropriate mutations to alter each TRA-1A binding site of pRE11 through splicing by overlap extension (reviewed by Vallejo et al., 1995).

Gel mobility shift assays

Procedures for analyzing the binding of TRA-1A fragment NF1-5 to DNA were developed by Zarkower and Hodgkin (1993), using methods described by Pollock and Treismann (1990). To make NF1-5 protein, we amplified the NF1-5 coding region from *C. elegans* cDNA using primers RE112 (ATGATGGCCCCCAGTACTGAGGA) and RE113 (CGCGGAATTCGACGATTCGAATAGTTTGCCGGA), and cloned the product into the pTOPO vector (Invitrogen). An *Eco*RI fragment that contained the TRA-1A sequences was isolated from this plasmid and ligated into the pRSET C vector (Invitrogen). We used sequence analysis to confirm that the *tra-1* sequences had inserted in the proper orientation. The TRA-1A fragment was expressed in *E. coli*, and whole cell lysates were used as a source of protein for mobility-shift assays.

RESULTS

fog-3 is highly expressed during periods of spermatogenesis

The *fog-3* gene appears to be expressed in germ cells, and is required for these cells to develop as sperm rather than as oocytes (Ellis and Kimble, 1995; Chen et al., 2000). To determine if expression of *fog-3* is correlated with spermatogenesis, we used northern analysis to examine pure hermaphrodite populations at different stages of development (Fig. 2A). High levels of *fog-3* expression are first seen in L3 larvae. These high levels continue through the L4 larval stage, when animals begin to produce sperm, and decline after young adulthood, when spermatogenesis stops. Similar studies using mixed populations of males and hermaphrodites suggest that males continue to express high levels of *fog-3* throughout adulthood, just as they continue to produce sperm (Fig. 2B).

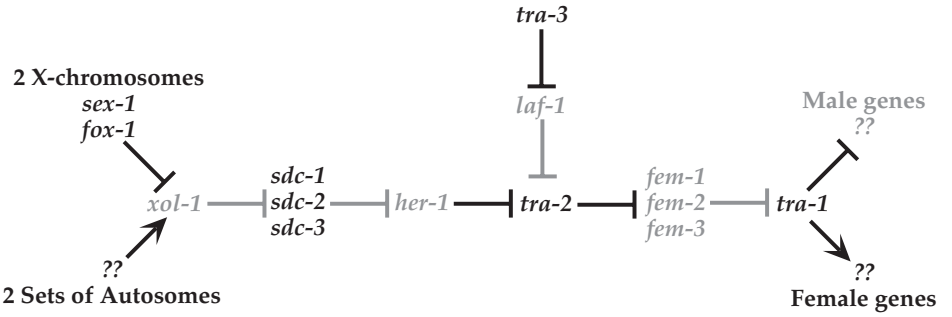


Fig. 1. Sex Determination in the soma of a *C. elegans* hermaphrodite. Active genes are shown in black, inactive genes in gray. Positive interactions are indicated by an arrow, and negative ones by a line with a bar. The regulatory relationships were inferred from analyses of double mutants (reviewed by Meyer, 1997; Ellis, 1998).

The sex-determination genes regulate expression of *fog-3*

To see if the genes that regulate sex-determination control expression of *fog-3*, we examined mutants using northern analysis. All RNA was prepared from pure populations of young *XX* adults. At this age, wild-type animals still contain a high level of *fog-3* transcripts (Fig. 3A). In addition, *tra-1(lf)* mutants, which make both sperm and oocytes, resemble the wild type. By contrast, the levels of *fog-3* transcripts are slightly higher in *tra-2(lf)* mutants and *fem-3(gf)* mutants, both of which produce only sperm, and are much lower in *fem-1(lf)* animals which make only oocytes. These results suggest that the sex-determination genes regulate the levels of *fog-3* transcripts. Furthermore, they show that *fem-1* and *fem-3* act

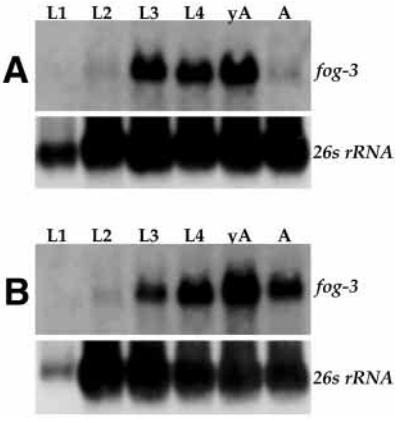


Fig. 2. The period of high *fog-3* expression coincides with spermatogenesis. (A) Northern blot of N2 total RNA, isolated from different developmental stages. L1, first larval stage; yA, young adult; A, adult. The blot was probed with an anti-*fog-3* probe (Chen et al., 2000) and then with a probe to the 26s ribosomal RNA, to test for loading errors. (B) Northern blot of *him-5(e1490)* total RNA, processed as described in A. These populations contain 30% males and 70% hermaphrodites (Hodgkin et al., 1979).

upstream of *fog-3*, which suggests that *fog-3* might directly control sexual fate in germ cells. By contrast, mutations in *fog-1* do not lower the levels of *fog-3* transcripts, although they do eliminate spermatogenesis (Fig. 3A). Thus, *fog-1* might act with *fog-3* or afterwards to help regulate germ cell fates.

Because mutations that alter sexual development generally cause sterility, northern analyses are only practical with temperature-sensitive mutants. To determine if null mutations in these genes had the same effect as the alleles described above, we developed a reverse transcriptase-polymerase chain reaction assay (RT-PCR) to measure the levels of *fog-3* transcripts (see Materials and Methods). In these experiments we analyzed *fog-3* transcripts produced by *XX* animals during the fourth larval stage, because at this stage the worms normally make sperm, the phenotype for which *fog-3* is required. We observed that null mutations in *fem-1*, *fem-2* or *fem-3* decrease or eliminate *fog-3* transcripts, whereas gain-of-function mutations in *fem-3* increase the levels of these transcripts (Fig. 3B-D). Furthermore, we found that mutations in *tra-1* had the opposite effect – null mutations increase the levels of *fog-3* transcripts, whereas a gain-of-function mutation decreases the amount of these transcripts (Fig. 3E).

These results support the conclusion implied by our northern analyses, that some sex-determination genes regulate *fog-3* transcript levels. However, we also found that *tra-1(lf)* mutations cause a dramatic increase in the expression of *fog-3* during larval development (Fig. 3E). Why do we not see a similar effect in adults? One possibility is that the RT-PCR experiments are more accurate, since the individual worms were each staged using Nomarski optics, whereas the *tra-1 XX* adults were staged in bulk and the males selected by size. However, since *tra-1(lf)* mutants produce sperm early in life, but often switch to oogenesis later on (Hodgkin, 1987; Schedl et al., 1989), our results might instead reflect a true decline in expression of *fog-3*. If so, then spermatogenesis would be caused by high levels of *fog-3* in *tra-1(lf)* larvae, and oogenesis by decreasing levels of *fog-3* as the animals age.

Northern analyses showed that a mutation in *fog-1* does not lower the levels of *fog-3* transcripts, although it does block spermatogenesis. To extend these studies, we used RT-PCR to measure levels of *fog-3* transcripts in *fog-1(q253*ts*)*, *fog-1(q187)* and *fog-3(q469)* mutants. Whereas loss-of-function mutations in the *fem* genes eliminated most *fog-3* transcripts, mutations in the *fog* genes either had no effect on the levels of *fog-3* transcripts, or caused them to increase slightly (data not shown). These results are consistent with our hypothesis that *fog-1* and *fog-3* behave differently than other genes in the sex-determination pathway.

The sex-determination genes might control the levels of *fog-3* transcripts by regulating either transcription of *fog-3*, or the stability of *fog-3* messages. To distinguish between these possibilities, we examined the levels of partially processed *fog-3* transcripts. To do this, we used primers to selectively amplify messages that had been trans-spliced to SL1, but which still contained intron 4. We found that partially processed *fog-3* messages were present at high levels in *fem-3(gf)* mutants, which also contain high levels of fully processed *fog-3* messages. By contrast, these partially processed messages are present at low levels in *fem-3(lf)* mutants or wild-type animals (Fig. 3F), and at intermediate levels in *tra-1(lf)* mutants (data not shown). If it is true that the regulation of mRNA stability occurs after processing is complete, these observations suggest that the sex-determination genes regulate transcription of *fog-3*, rather than stability of the *fog-3* messages.

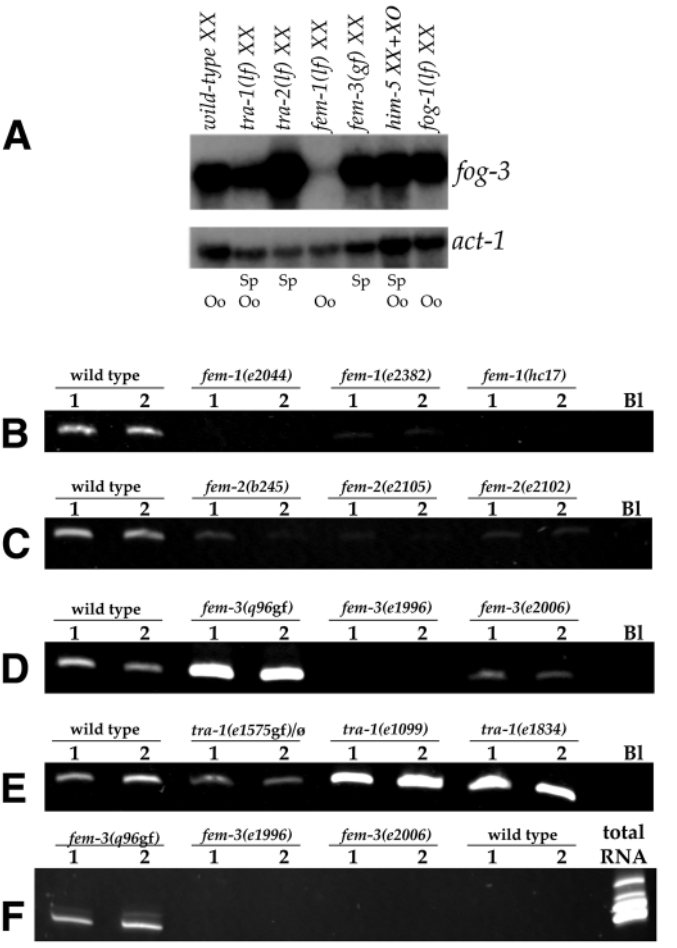
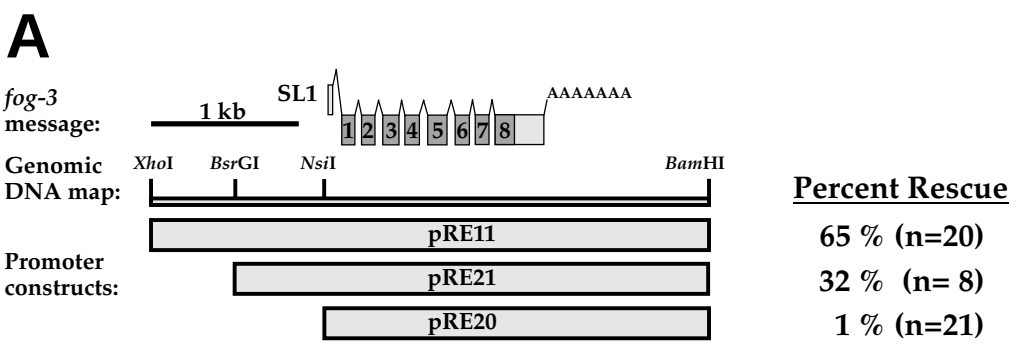


Fig. 3. Control of *fog-3* expression by the sex-determination genes. (A) Northern analysis of *fog-3* transcript levels in young adults. A Northern blot of total RNA was prepared from the following sex-determination mutants: purified *tra-1(e1099) XX* males, *tra-2(b202*ts*) XX* pseudomales, *fem-3(q96*gf,ts*) XX* Mog animals, *fem-1(hc17*ts*) XX* females, and *fog-1(q253*ts*) XX* females. Staged populations of mutants were prepared at permissive temperature, and shifted to restrictive temperature as embryos. RNA was harvested from adults. Sp, animals produce sperm; Oo, animals produce oocytes. (B-E) RT-PCR analysis of *fog-3* transcript levels in *XX* L4 larvae. Two independent samples of each genotype are presented as 1 and 2. Each lane represents RNA prepared from five pooled worms. The *fem-1(e2044)* and *fem-1(e2382)* mutants carried a *mor-2* mutation. The *fem-3(q96)* animals carried a *dpy-20* mutation, and the *fem-3(e1996)* animals also carried this marker mutation and were heterozygous for *tra-1(e1099)*. The *tra-1(e1575*gf*)/ϕ* worms were the female progeny of *tra-1(e1575gf)/tra-1(e1099*oc*)* females crossed with *tra-1(e1099*oc*)* males. Except for the *fem-1(e2382)* animals, all *fem* mutants were produced by homozygous *fem* mothers. With the exception of the *fem-3(e1996)* animals, all individuals in B, C, D and F were raised at 25°C; the remaining worms were raised at 20°C. The blank lane (Bl) contains samples prepared from *E. coli*. (F) RT-PCR analysis of partially processed *fog-3* transcripts. The total RNA was prepared from *fem-3(q96*gf*)* animals.

The *fog-3* promoter contains TRA-1A binding sites

To learn how transcription of *fog-3* is controlled, we analyzed a series of constructs to determine what upstream sequences are required for expression of a *fog-3* transgene (Fig. 4A; Table 1). Since we were unable to create a reporter construct that gave detectable expression of green fluorescent protein, we instead assayed the ability of transgenes encoding wild-type FOG-3 to restore self-fertility to *fog-3* mutants. Because the expression of transgenes can vary between different extra-chromosomal arrays, we studied several independent lines for each construct. Our data indicate that a region extending from a *Bsr*GI site at –771 to an *Nsi*I site at –165 (relative to site of trans-splicing) is essential for robust expression of *fog-3*. If *fog-3 XX* mutants contain a wild-type *fog-3* transgene driven by this region of the promoter, they often produce sperm as L4 larvae and then switch to oogenesis as adults, just like normal hermaphrodites (Table 1, pRE11, pRE21). Thus, the regulation of the transgene is approximately normal. (The total number of sperm produced by these animals is smaller than in the wild type, which is probably due to lower expression of *fog-3* because the transgenes are located on extra-chromosomal arrays.) By contrast, if the promoter lacks this region, the animals do not produce sperm (Table 1, pRE20). The region extending from the *Xho*I site at –1329 to the *Bsr*G1 site might also play a role in promoting *fog-3* expression, since lines containing this region show the highest frequency of rescue (Table 1, pRE11).

Because *tra-1* encodes a zinc-finger protein that can bind DNA in vitro (Zarkower and Hodgkin, 1992, 1993), we searched for TRA-1A binding sites in the *fog-3* promoter. Zarkower and Hodgkin (1993) showed that the amino-terminal half of TRA-1A binds sequences that contain three distinct elements – a TGGGTGGTC nonamer, which is recognized by fingers 3-5, a C residue five bases upstream of the nonamer, which is recognized by finger 1, and several T residues immediately upstream of the C, which are recognized by a domain amino-terminal to the zinc fingers. The *fog-3* promoter contains three perfect TRA-1A binding sites, two more sites that contain all three features, but lack a perfect nonamer, and an additional site that lacks a perfect nonamer and lacks the upstream C residue (Fig. 4B). This result suggested that TRA-1A might bind to the *fog-3* promoter to regulate transcription of *fog-3* in germ cells.



B

*Bsr*GI

TGTACACGAGCGTAAGTTATGAAAATTATCATAGACGTCTCGTAATAGAGGAATTATGGAAAGTGTAATT

TTAAAAAAAGTTTAATATATAAAACGAAACTCAACTCTATAAAGTCAGAAAATCTCAAGAATGTGCGAAT

GCTCA**TTTTC**ATCA**TGGGTGGTC**TTTCGTACAAGAACTGCCTAAAATTTTAAATTATTGTATAATTTGAT

TTTTCGAGTTATCGGTGTTTTCCTGTGATTCATGATTCTGAATTTCTCAAATAAATCTTACTGTAAACCG

TCTCACCAA**TTTC**TTCG**TGGGTGGTC**ATCAGGGTACTGTAGTTTCTGAATTGTGTGATGCTGGTGCGTAG

CTCAAG**TTTA**C**T**TCA**TGGG**C**GG**C**C**TATTCTTTTCGAATTTGCTTCATTATCATATTTTTAGCATATCTTC

CTCGATTTCCATTAAATTTACAATTAATTCAATGGTTTCTTCTTCGAATACTTTTTATAAGTTACTTTTT

CGTTAACATTTATTGAAAAAGAAGTACATTTTTCAA**TTATC**TTTGT**TGGTGGTC**CTCGG**TTTTGTCATGG**

GTGGTATTTACACTCTTCGCTCGCTCAATTTTACGTTCGCAATGCATAGATGAAATTTTTTAAACCGATA

TTGAGTTGATTTTGTCAGTATTTATGAACTCTTTTATTAAAATTAGAAGCTCGCACTTTCG**TTTTC**GTCG

TGGGTGGTCTCATATTTATTGTTTTATTGTTTAATTTGTATCTCTCACTATAATTAAATTTTCAGGAGAA

Aatgtataccgaagtccgcgagctcgtcaa

*Nsi*I

site 1 site 2 site 3 site 4 site 5 divergent site

Fig. 4. Identification and sequence of the *fog-3* promoter. (A) Percent rescue indicates the fraction of self-fertile hermaphrodites among *unc-29(e1072) fog-3(q504)* animals carrying the indicated transgene. The values represent the mean of all transgenic lines for each construct (see Table 1 and Materials and Methods). (B) Genomic sequence of the *fog-3* promoter was determined by the *C. elegans* Genome Sequencing Consortium (1998). The *Bsr*GI and *Nsi*I sites are boxed. Potential TRA-1A binding sites are underlined and nucleotides that match the consensus sequence are shown in black. The coding region of exon #1 is shown in lower case.

TRA-1A can bind the *fog-3* promoter in vitro

To begin testing this hypothesis, we used gel-shift assays to determine if TRA-1A can bind the *fog-3* promoter in vitro. Zarkower and Hodgkin (1993) showed that a fragment of TRA-1A that contains the amino terminus and the five zinc fingers (TRA-1A NF1-5) binds a 43 bp probe that contains a perfect consensus site (TBS1). However, this fragment of TRA-1A does not bind TBS2, a related sequence that contains a missense mutation at an essential residue of the nonamer. We prepared an 869 base-pair fragment of the *fog-3* promoter that contains all the potential TRA-1A binding sites, and a similar fragment in which the five

Table 1. The *fog-3* promoter contains essential TRA-1A binding sites

		Percentage self-fertile hermaphrodites											
Plasmid	Promoter	Mean	L 1	L 2	L 3	L 4	L 5	L 6	L 7	L 8	L 9	L 10	L 11
pRE11	wild type	62%	95% (20)	90% (20)	90% (20)	85% (20)	85% (20)	57% (28)	44% (16)	42% (19)	30% (20)	29% (21)	9% (11)
pRE21	Δ(*Xho*I-*Bsr*GI)	32%	88% (8)	37% (27)	32% (25)	0% (17)							
pRE20	Δ(*Xho*I-*Nsi*I)	1%	5% (21)	0% (24)	0% (21)	0% (18)	0% (12)						
pRE18	TRA-1A sites (−)(−)(−)(−)(−)	4%	13% (40)	8% (13)	3% (35)	3% (30)	0% (33)	0% (27)	0% (11)				

Activity of different *fog-3* promoters, measured in transgenic animals by the ability to restore spermatogenesis and self-fertility to homozygous *fog-3* mutants (see Materials and Methods). The Δ(*Xho*I-*Bsr*GI) and Δ(*Xho*I-*Nsi*I) deletions are described in Fig. 4, and the TRA-1(−)(−)(−)(−)(−) promoter in Fig. 5. Each L number describes the behavior of an independent line of transgenic animals, and the mean describes the average of all animals studied from all lines. The number tested for each line is given in parentheses.

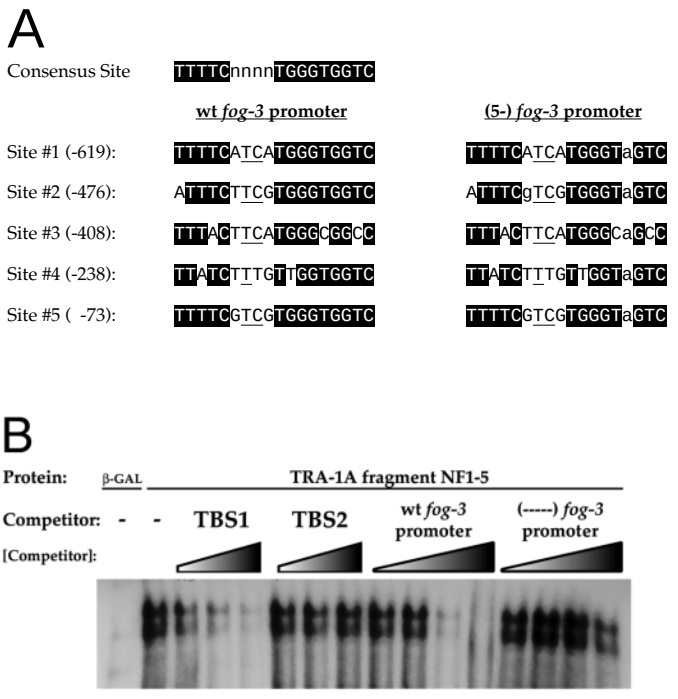


Fig. 5. The TRA-1A fragment NF1-5 binds the *fog-3* promoter in vitro. (A) The consensus TRA-1A binding site was determined by Zarkower and Hodgkin (1993) through in vitro studies. The sequence of five potential TRA-1A binding sites from the *fog-3* promoter is shown below it on the left, and the sequence of the altered sites in the mutant promoter on the right. Modified nucleotides are shown in lower case, and conserved residues with black shading. Conserved residues that are not part of the known TRA-1A target sequence are underlined. Binding sites are numbered as in Fig. 4A, and their position relative to the site of transplicing is shown in parentheses. In all five mutant sites, an essential 'G' was replaced with an 'A'; in site #2 an unselected change of 'T' for 'G' also occurred. (B) Gel-mobility-shift assay, using the radioactive 43-mer TBS1 (Zarkower and Hodgkin, 1993) as probe. The TRA-1/TBS1 complex runs as a doublet under these conditions; to save space, the free probe is not shown in the photograph. Lane 1 contains lysate from bacteria expressing β-galactosidase, and the other lanes contain lysate from bacteria expressing TRA-1A fragment NF1-5. The concentrations of TBS1 and TBS2 competitors were 0.5, 1.25 and 2.5 pmol/μl, and the concentrations of *fog-3* promoter were 0.1, 0.25, 0.5 and 2.5 pmol/μl.

best TRA-1A binding sites had been altered by mutation (Fig. 5A). The wild-type *fog-3* promoter competes successfully with TBS1 for binding to TRA-1A fragment NF-1-5 (Fig. 5B). By contrast, the mutant promoter is a poor competitor (Fig. 5B). These results indicate that TRA-1A is capable of binding the *fog-3* promoter in vitro, and that this interaction depends on one or more of the sites we had altered. The weak affinity for TRA-1A shown by the mutant *fog-3* promoter might be due to the unaltered sixth site, or to the fact that missense mutations lower but do not abolish the ability of the five primary sites to interact with TRA-1A (Zarkower and Hodgkin, 1993).

TRA-1A does not act through the FEM proteins to regulate expression of *fog-3*

At this point, we needed to reconcile our molecular data with previous genetic studies. Genetic tests had shown that *tra-1(lf); fem-1(lf)* double mutants, *fem-2(lf) tra-1(lf)* double mutants, and *tra-1(lf); fem-3(lf)* double mutants all produce oocytes rather than sperm (Doniach and Hodgkin, 1984; Hodgkin, 1986). These results suggested that *tra-1* acted through the *fem* genes to regulate spermatogenesis, or in parallel to them. We have established that *fog-3* acts downstream of the *fem* genes, that the *fog-3* promoter contains numerous TRA-1A binding sites, and that TRA-1A is capable of binding these sites in vitro. These observations imply that *tra-1* does not act through the *fem* genes, but instead directly regulates expression of *fog-3*. To further distinguish between these possibilities, we used RT-PCR to measure the levels of *fog-3* transcripts in *tra-1; fem* double mutants.

We find that null mutations in *tra-1* dramatically increase expression of *fog-3* in L4 larvae, even in animals also homozygous for a null mutation in *fem-1*, *fem-2*, or *fem-3* (Fig. 6). (In these experiments, we used *fem* mutants that were the offspring of homozygous *fem* mothers, since these genes each show maternal effects; Doniach and Hodgkin, 1984; Hodgkin, 1986.) Thus, *tra-1* does not act through the *fem* genes to regulate germ cell fate. Instead, our results are consistent with the hypothesis that TRA-1A binds directly to the *fog-3* promoter to repress transcription in these L4 larvae. Given the manner in which these genes are thought to interact in the soma, one simple model is that the FEM proteins prevent TRA-1A from binding to and repressing transcription of *fog-3*. This hypothesis would explain why mutations in the *fem* genes have

a strong effect on transcription of *fog-3*. Alternatively, TRA-1A and the FEM proteins might act independently on the *fog-3* promoter.

In this context, it is important to note that mutations in the three *fem* genes can affect the levels of *fog-3* transcripts, even when examined in a *tra-1(null)* mutant background. These effects vary from mild (Fig. 6A-C) to strong (Fig. 6D-F). Because independent samples give highly reproducible results, this variability is unlikely to be caused by the RT-PCR process itself. It might, however, be influenced by subtle changes in genetic background. To help control for this possibility, we used two different *tra-1* alleles in these studies (Fig. 6C-E) – *tra-1(e1099)* is a stop mutation located upstream of the region encoding the five zinc fingers, and *tra-1(e1834)* is a deletion of the 5′-half of the gene (Zarkower and Hodgkin, 1992; Hodgkin, 1993). The *fem* mutations can influence *fog-3* transcript levels in either case. This result implies that the FEM proteins regulate *fog-3* directly, and not just through TRA-1A.

Although many *tra-1(null); fem(null)* double mutants produce approximately wild-type levels of *fog-3* transcripts (Fig. 6), they make oocytes rather than sperm (our unpublished observations, Doniach and Hodgkin, 1984; Hodgkin, 1986). This fact implies that the *fem* genes play two essential roles in the germ line – in addition to regulating the expression of *fog-3*, they directly promote spermatogenesis. One possibility is that the FEM proteins activate either FOG-3 or FOG-1, enabling them to direct germ cells to become sperm.

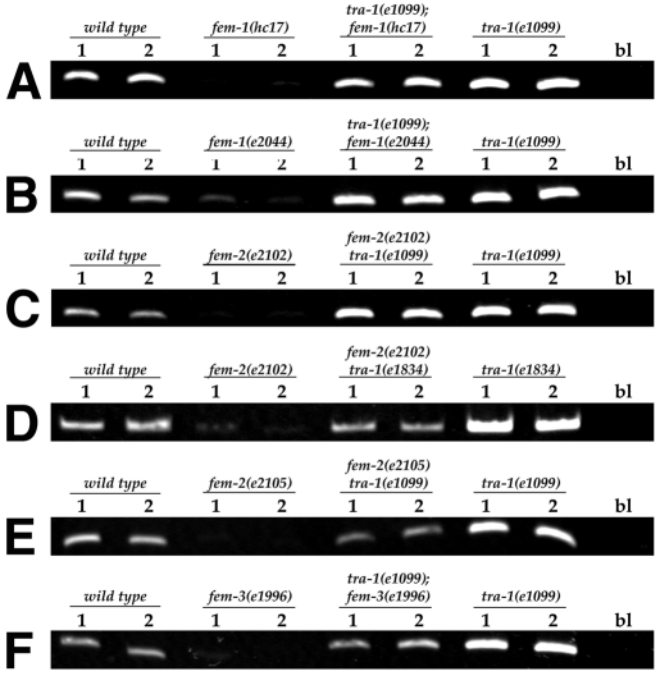


Fig. 6. TRA-1A does not required the *fem* genes to control transcription of *fog-3*. (A-F) RT-PCR analysis of *fog-3* transcript levels. Experimental conditions and marker mutations are described in the legend to Fig. 3. Animals shown in A, C, D and E were raised at 25°C, all other animals were raised at 20°C. For a description of the strain constructions, see Materials and Methods.

The TRA-1A binding sites are required for regulation of transgenenic *fog-3*

To determine if mutations in these TRA-1A sites altered regulation of *fog-3* in living animals, we compared the activity of a wild-type promoter with that of a promoter in which all five binding sites had been altered by a point mutation. To assay activity, we measured the ability of each promoter to drive a wild-type copy of *fog-3*, and thereby restore spermatogenesis to a *fog-3* mutant. We found that simultaneous disruption of the five TRA-1A binding sites abolished the activity of the *fog-3* transgene (Table 1, pRE18). Thus, some of these sites might activate transcription of *fog-3*, rather than repress it. We used sequence analysis to confirm that the only changes to the mutant transgene were the six shown in Fig. 5A. Furthermore, we have been unable to detect any *fog-3* transcripts produced from this mutant transgene by RT-PCR (unpublished data). Thus, these results suggest that one or more of the TRA-1A sites are required for *fog-3* activity.

Two models seem plausible: these sites might bind an activator that competes with TRA-1A, or TRA-1A itself might function as both an activator and a repressor. Because *tra-1(null)* mutants produce *fog-3* transcripts and sperm, TRA-1A is not needed for *fog-3* to be expressed in its normal chromosomal context. However, mutations in *tra-1* cause a smaller increase in both sperm production (Hodgkin, 1987; Schedl et al., 1989) and in the levels of *fog-3* transcripts (Fig. 3A), than those produced by other mutations that promote male development. These results are consistent with the possibility that TRA-1A both activates and represses *fog-3*. Since the transgenic copies of *fog-3* are located on extra-chromosomal arrays, this unusual environment might exacerbate the need for activation by TRA-1A.

We have done two kinds of experiments to explore this possibility. First, to determine if *tra-1* is required to activate a *fog-3* transgene, we crossed an extra-chromosomal array containing a *fog-3* transgene with a wild-type promoter into an *unc-29 fog-3(q504); tra-1(e1099)/+* genetic background. In *unc-29 fog-3; +/+* or *unc-29 fog-3; tra-1/+ XX* animals, the transgene restored the ability to produce sperm in 8/20 gonad arms, as scored by Nomarski microscopy. Control crosses showed that this array also restored sperm production to 6/9 *unc-29 fog-3(q504) XO* males. However, we could not find any sperm in the gonads of 25 *unc-29 fog-3; tra-1/tra-1 XX* males. Thus, *tra-1* is necessary for expression of our wild-type *fog-3* transgene. This result is consistent with models in which TRA-1A activates transcription of *fog-3* under certain circumstances, and represses it in others.

Second, we studied the possibility that some of these TRA-1A sites might be needed for activation, and others for repression. To do this, we created transgenic animals in which only one of the five TRA-1A sites in the promoter had been altered. We found that the third site was required for activation of the transgene, and that the fifth site appeared completely dispensable (Table 2). This fifth site is located so close to the start of transcription that TRA-1A might be able to bind here and block access by RNA polymerase. The other three sites appear to be partially required for full activity of the transgene. Thus, these TRA-1A binding sites are essential for regulation of *fog-3* in living animals, and appear to have distinct functions.

Table 2. Some of the TRA-1A binding sites are essential for activation

Plasmid	Promoter	Mean	L 1	L 2	L 3	L 4	L 5	L 6	L 7
pRE46	(–)(+)(+)(+)(+)	20%	68% (25)	32% (19)	24% (21)	10% (20)	0% (22)	0% (21)	0% (20)
pRE47	(+)(–)(+)(+)(+)	11%	25% (16)	12% (17)	0% (22)				
pRE48	(+)(+)(–)(+)(+)	3%	8% (13)	0% (28)	0% (12)				
pRE49	(+)(+)(+)(–)(+)	31%	33% (27)	29% (21)					
pRE50	(+)(+)(+)(+)(–)	69%	75% (28)	69% (29)	68% (25)	66% (29)			

Activity of different *fog-3* promoters, measured in transgenic animals by the ability to restore spermatogenesis and self-fertility to homozygous *fog-3* mutants (see Materials and Methods). Each L number describes the behavior of an independent line of transgenic animals, and the mean describes the average of all animals studied from all lines. In the promoter column, (+) indicates a wild-type TRA-1A binding site, and (–) indicates a site that has been altered from by changing the 'G' at position 7 of the nonamer to an 'A'. These sites are listed with the most upstream at the left. The number tested for each line is given in parentheses. In these experiments, we used the PCR to confirm that each extra-chromosomal array contained at least part of the *fog-3* plasmid.

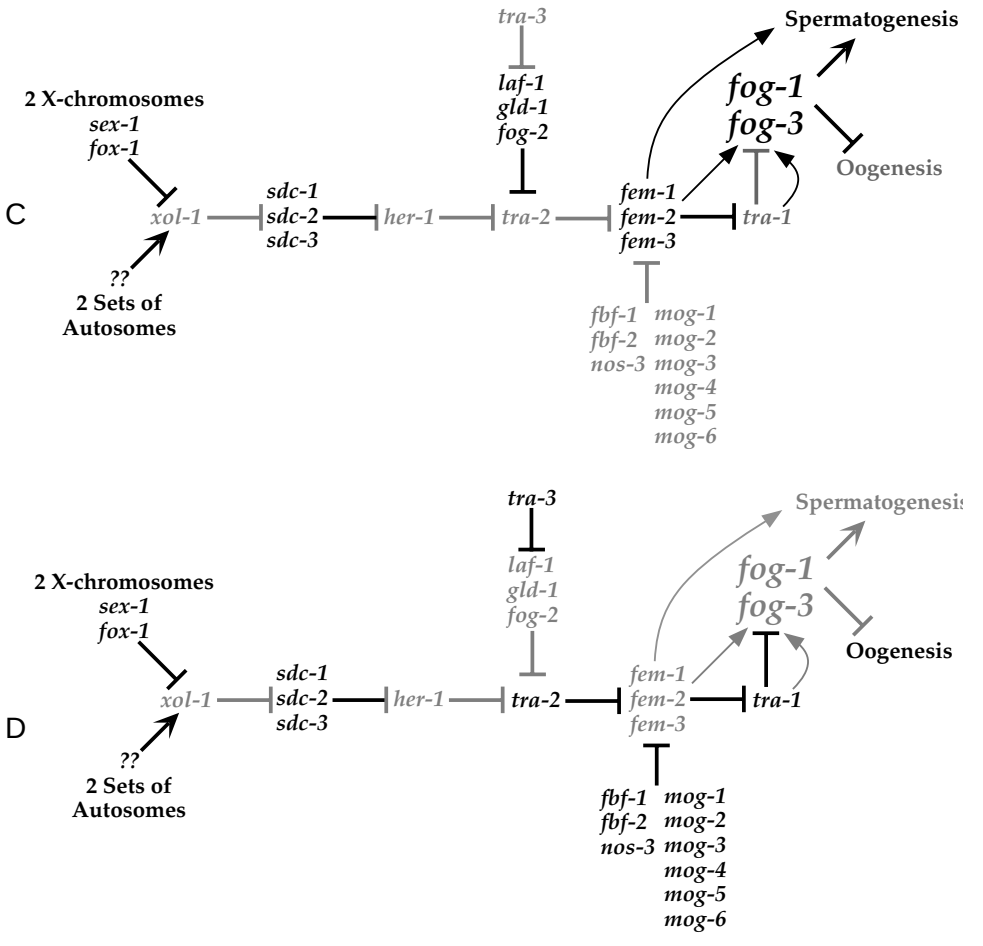


Fig. 7. Regulation of sexual fate in the germ cells of larval hermaphrodites. Active genes are shown in black, inactive genes in gray. Positive interactions are indicated by an arrow, and negative ones by a line with a bar. (A,B) Two possible models for the way in which the FEM proteins and TRA-1A interact to control *fog-3*. Current data cannot distinguish between these possibilities, but only the latter is shown in C and D. (C) Summary of how the sexual fate of hermaphrodite germ cells is controlled during larval development. Although the *fem* genes are required for spermatogenesis, we do not know if they work through the FOG-1 or FOG-3 proteins, or independently of them; in this model, we have presented the latter possibility. For the roles of the *fog-2* and *fbf* genes in regulating the limited production of sperm by these animals, see Ellis (1998). (D) Summary of how the sexual fate of hermaphrodite germ cells is controlled in adults. Note that we do not yet know which genes cause the switch from spermatogenesis to oogenesis, so we have presented a model in which all of the genes that act in this process switch activities when the animals enter adulthood.

DISCUSSION

fog-3 is a terminal regulator of sexual fate in germ cells

Genetic analyses indicated that *fem-1, fem-2, fem-3, fog-1* and *fog-3* were essential for germ cells to initiate spermatogenesis (reviewed by Ellis, 1998), but could not determine the order in which these genes acted. Our molecular data suggest that *fog-3* is a terminal regulator of sexual fate, and that the three *fem* genes act upstream of *fog-3* to regulate its expression (Fig. 7A,B). Thus, the sexual fate of germ cells is in part determined in a modular fashion. First, genes such as *fem-1, fem-2, fem-3* and *tra-1* choose the sexual fate of the entire animal; they accomplish this in part by regulating tissue-specific genes like *fog-3* and *fog-1*. Second, these tissue-specific genes control specific cell fates, such as spermatogenesis or oogenesis.

However, this cell fate decision is not completely modular. To account for all of the data, our model requires one other feature – in addition to regulating transcription of *fog-3*, the FEM proteins must play a second, essential role in spermatogenesis. This activity is revealed by the fact that *tra-1; fem-1* double mutants produce oocytes, even though they make wild-type levels of *fog-3* (Fig. 6).

TRA-1A regulates sexual fate by controlling transcription of target genes like *fog-3*

How is the expression of *fog-3* controlled? Several lines of evidence show that TRA-1A is an important regulator of *fog-3* transcription. First, loss-of-function mutations in *tra-1* increase the levels of *fog-3* transcripts in L4 animals, whereas gain-of-function mutations decrease them (Fig. 3). Second, analyses of double mutants show that TRA-1A does not act through the FEM proteins, but is instead likely to directly regulate these transcript levels (Fig. 6). Third, the *fog-3* promoter contains at least five potential TRA-1A binding sites (Fig. 4). In fact, computer analysis by Clarke and Berg (1998) shows that this is the largest concentration of potential TRA-1A binding sites near any of the 19,000 *C. elegans* genes. Fourth, the *fog-3* promoter binds a TRA-1A fragment containing the zinc fingers in a gel mobility-shift assay, and this binding depends on one or more of these five target sites (Fig. 5). These results establish *fog-3* as a target of TRA-1A, and support the hypothesis that *tra-1* regulates cell fate by controlling the transcription of genes that act in specific tissues or regions of the animal (Zarkower and Hodgkin, 1992; Zarkower and Hodgkin, 1993).

To date, the only other potential target of TRA-1A that has been experimentally confirmed is *egl-1* (Conradt and Horvitz, 1999). Analyses of *fog-3* and *egl-1* confirm that the TRA-1A binding sites characterized by Zarkower and Hodgkin (1993) from in vitro studies play an important role in regulating actual target genes in developing worms. In particular, each of the TRA-1A sites near *fog-3* or *egl-1* contains the 'TGGGTGGTC' nonamer or a close variant, and each site contains a C residue five nucleotides upstream of the nonamer. Finally, the four nucleotides upstream of the C residue are predominantly composed of T residue residues in the five *fog-3* sites. There are potential TRA-1A binding sites near *mab-3* (Clarke and Berg, 1998; Raymond et al., 1998), *lin-31* (Clarke and Berg, 1998) and *tra-1* itself (Zarkower and Hodgkin, 1993) that might also be targets of TRA-1A regulation, so we expect that the group of known target genes should rapidly expand.

TRA-1A appears to both repress and activate expression of *fog-3*

Two genetic observations suggested that TRA-1A might repress transcription of genes needed for spermatogenesis. First, *tra-1(null) XX* animals often produce sperm during adulthood, whereas wild-type *XX* animals make oocytes. Second, *tra-1(gf)* mutants make oocytes rather than sperm. Our data show that these *tra-1* mutations alter expression of *fog-3* in L4 hermaphrodites exactly as one would predict if TRA-1A were a repressor of transcription (Fig. 3).

Is TRA-1A only a repressor? Both the Ci protein of *Drosophila* and GLI of mammals are homologs of TRA-1A (Zarkower and Hodgkin, 1992). Ci has been shown to activate transcription of *patched* and *wingless* (Alexandre et al., 1996; Von Ohlen et al., 1997). In each case, it acts through multiple Ci binding sites located at least 600 bp upsteam of the transcriptional start site. Similarly, GLI has been shown to activate transcription of HNF-3β (Sasaki et al., 1997). Can TRA-1A also function as an activator, or is its sole function to repress genes required for male development? Our data suggest that TRA-1A is able to repress transcription of *fog-3* during larval development, and Conradt and Horvitz (1999) have shown that TRA-1A is likely to repress *egl-1*. Furthermore, genetic and molecular data strongly suggest that if TRA-1A binds to the potential target sites near *mab-3*, it should repress transcription (Shen and Hodgkin, 1988; Raymond et al., 1998).

However, we found that a point mutation in the third TRA-1A site of the promoter abolishes activity of a *fog-3* transgene, and that the first, second and fourth sites play a minor role in activation. Thus, TRA-1A might bind these sites to promote expression of transgenic *fog-3*. One line of genetic experimentation supports this model: *tra-1* acts in both *XX* and *XO* adults to maintain spermatogenesis and prevent oogenesis (Hodgkin, 1987; Schedl et al., 1989), which suggests that it could promote transcription of *fog-3* during adulthood. Furthermore, although TRA-1A is not needed for expression of chromosomal copies of *fog-3* in larvae, it seems to be required for activation of *fog-3* transgenes in larvae. Perhaps this requirement is due to the location of these transgenes on an extra-chromosomal array, rather than at their normal position on the chromosome. It is also possible that a more distant regulatory element is missing from the *fog-3* transgenes, and that its absence exacerbates the need for activation by TRA-1A.

Alternatively, the point mutations we introduced into these TRA-1A sites might also disrupt binding of a transcriptional activator. If this latter possibility were true, then TRA-1A might repress transcription by competing for access to these binding sites. One observation supports this model: four of the binding sites in the *fog-3* promoter contain a TC sequence in a region not conserved in other TRA-1A targets (Fig. 4; Zarkower and Hodgkin, 1993; Conradt and Horvitz, 1999), and the fifth site contains a TT at this position. These sequences might be part of the recognition site for a protein that activates transcription of *fog-3*.

Transcription of *fog-3* is also controlled by other factors

Our results also show that *tra-1* is not the only gene capable

of regulating the expression of *fog-3*. In *tra-1(null)* mutant animals, mutations in the *fem* genes can decrease the level of *fog-3* transcripts (Fig. 6). These results suggest that the FEM proteins either directly regulate the levels of *fog-3* transcripts, or regulate these levels through proteins other than TRA-1A. Current molecular data point towards the second possibility. FEM-1 contains ankyrin repeats, and so is likely to interact with other proteins in order to control cell fate (Spence et al., 1990). FEM-2 is a protein phosphatase (Pilgrim et al., 1995; Chin-Sang and Spence, 1996). Finally, FEM-3 is a novel protein that contains a potential nuclear localization signal, but no known DNA-binding motifs (Ahringer et al., 1992). Thus, if the FEM proteins regulate expression of *fog-3*, they are likely to do so by controlling the activity of another protein, such as a transcription factor.

Since the FEM proteins and TRA-1A have at least partially independent effects on the expression of *fog-3*, there are two ways to summarize the relationships between these genes (Fig. 7A, B). In both models, the FEM proteins promote transcription of *fog-3*, but in the second model they also inhibit TRA-1A, just as the FEM proteins are suspected to do in the soma. We favor the second model because mutations in the *fem* genes dramatically affect transcription of *fog-3* when TRA-1A is present, but have only a mild effect in *tra-1* mutants, just as if they were acting through TRA-1A. Furthermore, mutations that inactivate one of the *fem* genes cause a much greater decrease in expression of *fog-3* than the strong gain-of-function allele *tra-1(e1575gf)*. This result suggests that the FEM proteins might regulate transcription of *fog-3* by acting through TRA-1A, and also through a mechanism that is independent of TRA-1A.

TRA-1A functions similarly in the soma and germ line

One major question researchers have struggled with in analyzing the control of germ cell fates is whether regulatory genes function similarly in the soma and germ line. For example, in fruit flies the *Sex lethal* gene is required at an early time for female somatic development and dosage compensation, but appears to act at a later stage in germ cells, possibly after their sex has been determined (Nothiger et al., 1989; Oliver et al., 1993; Steinmann-Zwicky, 1994). The reason for this difference is not understood. Furthermore, in nematodes, genetic assays had suggested that *tra-1* played a different role in germ cells than it did in the soma, where it acts as the terminal regulator for sexual fate (Doniach and Hodgkin, 1984; Hodgkin, 1986). Our analyses, in conjunction with those of Conradt and Horvitz (1999) suggest that TRA-1A acts as a terminal regulator of sexual fate in both tissues, by repressing transcription of genes in *XX* animals that are designed to function in males. However, one major difference between the tissues is that the FEM proteins have two detectable functions in the germ line, but only one in the soma.

Since *tra-1* appears to play a similar role in the soma and germ line, our results raise the possibility that the FEM proteins directly regulate downstream genes in the soma, just as they do in germ cells. Furthermore, the possibility that the FEM proteins do not directly inhibit TRA-1A exists equally for both tissues (Fig. 7A).

How does TRA-1A repress transcription?

TRA-1A might repress transcription by any of several mechanisms. One of the simplest methods would be to bind and block sequences required for the initiation of transcription. The following observations suggest that this mechanism could explain in part how TRA-1A regulates *fog-3*. First, the *fog-3* message is one of many trans-spliced *C. elegans* genes. In vitro studies by Conrad et al. (1995) suggest that the region upstream of the splice site must be at least 50 nucleotides long for efficient trans-splicing to occur. Second, trans-splicing of *fog-3* to SL1 is very efficient, relative to the removal of intron #4, since most partially processed transcripts that contain intron #4 have already been trans-spliced (unpublished data). Third, the fifth TRA-1A binding site lies between nucleotides −73 and −56 (counting backwards from the site of trans-splicing). Thus, transcription of *fog-3* either begins adjacent to the fifth TRA-1A binding site, or perhaps upstream of it. In either case, TRA-1A bound at this site might hinder assembly of the transcriptional apparatus. One result is consistent with this model. This TRA-1A site is the only one that plays no detectable role in the activation of *fog-3*, so it might function purely to repress transcription. Recent results suggest that an excellent TRA-1A binding site also lies 33 nucleotides upstream of the site of trans-splicing in the *fog-1* gene (S.-W. Jin and R. E. E., unpublished data), where it might repress transcription by a similar mechanism.

However, several observations suggest that TRA-1A need not directly block a transcriptional start site to repress transcription. The strongest evidence is that Conradt and Horvitz (1999) have shown that the TRA-1A target site near *egl-1* lies downstream of the gene, rather than near the site where transcription begins. In addition, the other TRA-1A binding sites in the *fog-3* promoter lie much farther upstream. Furthermore, the potential binding site near *tra-1* (Zarkower and Hodgkin, 1993) also lies farther upstream, and potential TRA-1A binding sites near *mab-3* and *lin-31* (Clarke and Berg, 1998) lie either far upstream or downstream of the transcription start sites (unpublished observations). Thus, TRA-1A might be able to repress transcription in more than one manner.

Transcriptional regulation is essential for control of germ cell fates in *C. elegans*

Our results suggest that genes of the sex-determination pathway regulate targets such as *fog-3* by acting through the zinc-finger protein TRA-1A. This regulatory system appears to be used in both soma and germ line. For hermaphrodite germ cells to adopt male fates, additional genes act in the germ line to modulate the activity of the *fem* genes and *tra-1* (reviewed by Ellis, 1998). Combining these results, we propose that germ cell fates are regulated in L4 hermaphrodites as shown in Fig. 7C and D. These models include *fbf-1*, *fbf-2*, *nos-3* and the *mog* genes, which repress translation of *fem-3* messages in adult hermaphrodites, to prevent spermatogenesis from continuing indefinitely (reviewed by Ellis, 1998). As indicated in this figure, our studies support the hypothesis that *fog-3*, along with *fog-1*, directly controls the decision of germ cells to differentiate as sperm or as oocytes; this conclusion indicates that understanding *fog-3* is essential for learning how this conserved fate decision is carried out. Finally, the compact size of the *fog-3* promoter, along with the large number of TRA-1A binding sites it contains, establish it as an excellent model for characterizing how proteins like TRA-1A, Ci and GLI interact with target promoters to regulate transcription.

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