Genetic and Molecular Analysis of *fox-1*, a Numerator Element Involved in *Caenorhabditis elegans* Primary Sex Determination

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ABSTRACT

fox-1 was previously identified as a candidate numerator element based on its overexpression phenotype. FOX-1 is an RRM-type RNA-binding protein, which can bind RNAs *in vitro*. Western analysis detects FOX-1 throughout development. *fox-1::lacZ* comes on ubiquitously early during embryogenesis. Postembryonically, *fox-1::lacZ* is expressed sex specifically in a subset of cells in the head and tail. We describe a Tc1-derived deletion allele [*fox-1*(Δ)] that removes the RRM domain. *fox-1*(Δ) confers no phenotype in *XX*s, but can rescue *XO*-specific lethality and feminization caused by duplications of the left end of the *X*. *fox-1*(Δ) synergizes with putative numerators, resulting in abnormal *XX* development. Genetic analysis indicated that *fox-1*(Δ) leads to a slight increase in *xol-1* activity, while *fox-1*(*gf*) leads to partial loss of *xol-1* activity, and *xol-1* is epistatic to *fox-1*. RNase protection experiments revealed increased levels of the 2.2-kb *xol-1* message in *fox-1*(Δ) animals, and reduced levels in *fox-1*(*gf*) animals. Additionally, *fox-1*(Δ) impairs male mating efficiency, which, we propose, represents another function of *fox-1*, independent of *xol-1* and its role in sex determination.

THE first step toward sex determination in *Caenorhabditis elegans* involves evaluating the ratio of the number of *X* chromosomes to the number of sets of autosomes (the *X*:*A* ratio) (Madl and Herman 1979). *C. elegans* exists in populations of hermaphrodites (*XX*), with males (*XO*) arising only infrequently (0.2%) as a result of meiotic nondisjunction of an *X* chromosome. Hermaphrodites can be regarded as modified females, which, for a limited period in their lives, produce sperm that are then used exclusively for self-fertilization. Diploid animals with two *X* chromosomes develop into hermaphrodites (*X*:*A* ratio = 1); those with one *X* chromosome develop into males (*X*:*A* ratio = 0.5).

To control sexual phenotype, the *X*:*A* primary signal is transduced through a cascade of negatively regulated genes, which are either in high or low activity states in *XX* or *XO* animals (for review see Hodgkin 1992; Parkhurst and Meneely 1994; Cline and Meyer 1997; Meyer 1997; Figure 1). The cascade terminates with *tra-1*, for which the high activity state promotes female sexual differentiation, while the low activity state promotes male sexual differentiation.

In addition, the *X*:*A* ratio controls dosage compensation: this is a process whereby the expression of the two hermaphrodite *X*s is equalized to the level of one male *X*. In *C. elegans*, dosage compensation and sex determination are coordinately controlled by four early genes: *xol-1* and *sdc-1*, *sdc-2*, and *sdc-3*. The *sdc* genes, together with products of several other genes, achieve dosage compensation by downregulating transcription of the two hermaphrodite *X*s (for review see Hsu and Meyer 1993). A lack of dosage compensation is lethal to *XX* animals, while active dosage compensation is lethal to *XO* animals. The *sdc* genes also control sex by repressing (directly or indirectly) the transcription of *her-1*.

Although much has been learned about many individual components involved in *C. elegans* sex determination, until recently little was known about the nature of the very first step, the *X*:*A* primary signal. Early observations indicated that the primary signal is not equivalent to the absolute number of *X* chromosomes, but rather to the *X*:*A* ratio (Madl and Herman 1979), suggesting the existence of numerator elements located on the *X* and autosomal denominator elements, which together contribute to the *X*:*A* ratio. Numerators can be seen as feminizing elements, since increasing their dose leads to *XO*-specific feminization and lethality (dosage compensation effect), whereas decreasing their dose leads to *XX*-specific masculinization and lethality. Primary sex determination, in this model, would resemble that in Drosophila. Original experiments directed at finding numerator elements concluded that such elements may be numerous and scattered all along the *X* (Madl and Herman 1979). More recent experiments suggested that there may be only a few elements with numerator activity (Akerib and Meyer 1994; Hodgkin *et al.* 1994).

Identification of the first candidate numerator locus, *f*eminizing *o*n *X* (*fox*), was described by Hodgkin *et al.* (1994). *fox-1* encodes an RNA-binding protein with a 90-amino-acid RNA recognition motif (RRM) domain.

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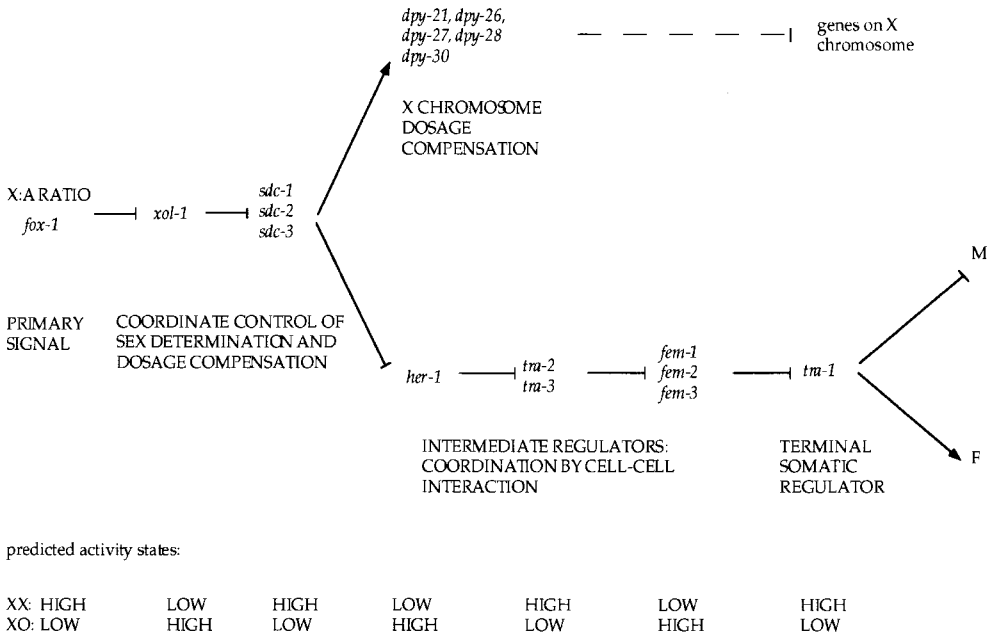


Figure 1.—Somatic sex determination pathway in *C. elegans*. The pathway involves a series of negative interactions, which begin with the assessment of the *X*:*A* ratio and end with the putative transcription factor *tra-1*, a promoter of female-specific fates and a repressor of male-specific fates. Genes and interactions required for germline sex determination as well as other minor interactions have been omitted for simplicity.

The locus was identified following a detailed analysis of a novel duplication of the *X*, *eDp26*, which showed *XO* lethal and feminizing properties. *fox-1* was found within the *unc*-2 *lin*-32 interval, a region of the *X* previously unduplicated. Microinjections of extra copies of *fox-1* were found to be almost completely *XO* lethal and feminizing, exactly mimicking the lethal and feminizing effects of *eDp26*.

The left end of the *X*, corresponding to *eDp26*, was also analyzed in detail by Akerib and Meyer (1994). As a result of combinations of smaller deletions and duplications, the region was subdivided further into three parts, each with distinct but additive numerator activities. The numerator elements are not equally potent, because disturbing a dose of one is not equivalent to disturbing another (Akerib and Meyer 1994). *fox-1* lies in region three, the region with the strongest effects on sex determination. Nicoll *et al.* (1997) showed more recently that region 1 and another putative numerator element, *sex-1*, are able to affect *xol-1::lacZ* expression, implying their involvement in transcriptional regulation of *xol-1*. Regions 2 and 3 show no effect on *xol-1::lacZ* expression but are able to downregulate *xol-1::GFP* transgenes, implying their involvement in post-transcriptional regulation of *xol-1*. *xol-1* is the earliest acting gene in the sex determination cascade and is therefore likely to be the target of the primary signal.

The analysis of numerator function can be approached from two different angles. One can analyze the numerator dosage effects on sex determination and dosage compensation. An alternative approach involves the predicted downstream target of the primary signal, *xol-1* (Miller *et al.* 1988; Rhind *et al.* 1995). *xol-1* is expressed at high levels in *XO*s and its main function is to specify male development. Therefore a high *X*:*A* ratio is predicted to downregulate *xol-1* in *XX*s, while a low *X*:*A* ratio permits its expression in *XO*s. The *XO*-specific masculinizing, function of *xol-1* is carried out at very early stages in embryogenesis. Paradoxically, *xol-1* also has a minor feminizing role in *XX* animals during L2 and L3 larval stages (Rhind *et al.* 1995). The *XX*-specific feminizing role of *xol-1* can be seen in a *tra-2* and *her-1* background (Miller *et al.* 1988). *tra-2* loss-of-function mutations transform *XX* animals into males. This transformation is incomplete, resulting in nonmating males with some morphological abnormalities in the copulatory structures (Hodgkin and Brenner 1977). A complete transformation toward a male indistinguishable from a wild type can be achieved when *xol-1* function is removed. Enhancement of the partial masculinization of *XX* animals caused by a gain-of-function mutation in *her-1* (Trent *et al.* 1988) can also be seen in the absence of *xol-1*.

We present here a genetic and molecular analysis of *fox-1*. We investigated *fox-1* expression using Western analysis and lacZ reporter transgenes and examined the ability of FOX-1 to bind RNA *in vitro*. The effects of *fox-1* overexpression in *XO* animals have been partly described elsewhere (Hodgkin *et al.* 1994); here we report the results of genetic analyses aimed at revealing *fox-1* interactions with other genes involved in sex determination. We describe construction and characterization of a transposon-mediated *fox-1* deletion, and we demonstrate that this deletion behaves as a biological null, able to synergize with other putative numerator elements. We also use genetic analysis of *fox-1* deletion and *fox-1* overexpression (*gf*) to investigate the numerator properties of *fox-1* and to demonstrate its involvement in the regulation of *xol-1*. Molecular analysis of a 2.2-kb *xol-1* transcript in *fox-1(gf)* and *fox-1(lf)* genetic

backgrounds presented here provides a confirmation of the genetic data. Furthermore, we uncover two potential late functions for *fox-1*, which we propose are distinct from its involvement in the *X*:*A* ratio assessment.

MATERIALS AND METHODS

General genetic methods, genes, alleles, and strains used: Worms were cultured under standard conditions at 20° (Sulston and Hodgkin 1988). Nomenclature is standard (Horvitz *et al.* 1979), and the *fox-1* deletion allele (*e2643*) is referred to as *fox-1(*Δ*)*. The following strains and mutations were used: N2 Bristol strain, MT3126 (*mut-2*), CB4852, CB4932, RC301, KR314, CB4855, CB4858, CB4857, CB4854, CB4856, AB1, and AB3 (Hodgkin and Doniach 1997); Linkage Group (LG) I, *dpy-5(e61), fer-1(hc1*ts*)*; LG II, *bli-2(e768), tra-2(e1095, q276)*; LG III, *unc-32(e189), tra-1(e1099, e1076)*; LG IV, *him-8(e1489), unc-5(e53), dpy-9(e12), tra-3(e1767), fem-1(hc17*ts*)*; LG V, *dpy-11(e224), unc-51(e369), her-1(y101*sd*)*; LG X, *lon-2(e678), xol-1(y9), dpy-3(e27), unc-1(e1598*dm*, e719), unc-2(e55), dpy-18(e81)*. Duplications, *eDp26* (*X*;*X*), *yDp13* (*X*;*f*); deficiencies, *meDf6 X*.

Detection of Tc1 insertions: All procedures were performed as described in Zwaal *et al.* (1993), except that the first and the second PCR series were increased from 30 to 35 cycles. The following primers were used:

Tc1 specific:

right 1 (GCTGATCGACTCGATGCCACGTCG)
right 2 (GATTTTGTGAACACTGTGGTGAAG)
left 1 (TGTTCGAAGCCAGCTACAATGGC)
left 2 (TCAAGTCAAATGGATGGATGCTTGAG).

fox-1-specific primers:

JDZ8 (GCGACCAAGAAGAGATTGT)
MSE (GAAGTTGTGCCAGCGGATTGCC).

Once the positive address was identified, a corresponding pool of worms was thawed and worms were singled and allowed to lay eggs. PCR was then performed on the mothers to identify individuals carrying the insertion. Single-worm PCR was performed as described in Williams *et al.* (1992).

Detection and isolation of a deletion mutant: Screening for Tc1-derived deletions was done as described in Zwaal *et al.* (1993), with some minor modifications. A total of 100 cultures of 10 worms each were set up. After two to three generations half of each culture was lysed by 2-hr incubation in single worm (SW) buffer (50 mm KCl, 10 mm Tris-HCl pH 8.3, 2.5 mm $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin) with proteinase K 100 (g/ml), at 65°. Lysate (2.5 μl) was used as a template for the first round of PCR, performed as above. The primers used were gene specific and flanked the Tc1 insertion site:

SKIP2 (TTCGACGAATGGCTCGGCGGC)
SKIP3 (AGCCCGATCCCAGCACTAG)
SKIP7 (GCATTCTTGCTCATTCACCAC)
SKIP8 (CAAAACATGTTAAGAAATCATTC).

All PCR reactions were done in duplicate. Once a positive was found, the remaining half of the culture was divided into eight subcultures, and the procedure was repeated. The culture was abandoned unless at least four out of eight subcultures were positive following the next round of selection. The deletion mutant was outcrossed six times in the first instance, using an *unc-2* marked strain, before any further genetic analysis.

Sequencing: PCR deletion products were gel purified and subcloned into a modified pBluescript II SK+ Phagemid vector (Stratagene, La Jolla, CA). The vector had been digested with *Eco*RV (Biolabs, Inc.) restriction endonuclease; poly(Ts) [Pharmacia (Piscataway, NJ) dTTPs] were transferred onto blunt ends by incubating the cleaved vector in the presence of dTTPs, Taq polymerase buffer, and Taq Polymerase (Promega, Madison, WI) for 1 hr at 72°. The inserts were sequenced by the dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase Version 2.0 DNA Sequencing Kit (Amersham Life Science) with T7 (AATACGACTCACTATAG) and T3 (ATTAACCCTCAC TAAAG) primers. All primers were custom made.

Construction of *fox-1(gf)* strains: Transgenic lines containing extrachromosomal copies of RO4B3, a cosmid containing the *fox-1* genomic region and a *rol-6* marker plasmid were subjected to X-ray mutagenesis to integrate the cosmid arrays. A total of 50 young adult hermaphrodites from each strain were irradiated with a dose of 3500 rads (dose rate 1.35 rad/sec) using a Torrex 150 X-ray machine. F_1 hermaphrodites were transferred singly to fresh plates and allowed to self; their broods were examined for 100% rolling progeny. Three independent integrated lines were obtained: *eIs25(V), eIs26(IV),* and *eIs27(V)*. They were mapped using standard marked strains (data not shown).

Construction of double mutants: Where possible, double mutants were obtained by using *fox-1(*Δ*) XO* males. In all genetic experiments involving *fox-1(*Δ*)* its presence was confirmed by single-worm PCR. *fox-1(gf)* was tracked by *rol-6* marker. A *fox-1(*Δ*); tra-2(q276)* strain was constructed by mating *q276 XX* males with *fox-1(*Δ*)* hermaphrodites and double mutants were isolated from the F_2 generation. *fox-1(*Δ*) xol-1*; *tra-2(e1095*sd*)* triple mutant was made by mating *xol-1*; *tra-2 XX* males with *fox-1(*Δ*) unc-18* hermaphrodites. Non-Unc F_2 hermaphrodites were isolated and selfed. The mothers were tested for the presence of the deletion by PCR, and their broods were examined for the presence of *XX* males. *fox-1(*Δ*); tra-3* and *fox-1(gf); tra-3* were constructed by mating *tra-3 XO* males with *fox-1(*Δ*)* and *fox-1(gf)* hermaphrodites, respectively. F_3 cross-progeny were picked individually and allowed to self. *fox-1(*Δ*); tra-3* and *fox-1(gf); tra-3* daughters of *tra-3* homozygous mothers give entirely masculinized broods. *dpy-3 fox-1(*Δ*); yDp13 XO* males were obtained as the non-dumpy F_1 male cross-progeny from mating *meDf6*; *yDp13* males with *dpy-3 fox-1(*Δ*)* hermaphrodites. *fox-1(*Δ*)* was crossed into 11 different wild-type strains by mating *fox-1(*Δ*) XO* males with wild-type hermaphrodites from the strain of interest. F_1 cross-progeny were transferred singly to fresh plates and allowed to self. The presence of *fox-1(*Δ*)* was confirmed by PCR. The F_2 broods from these animals were examined for any signs of masculinization. *fox-1(gf); tra-2(q276)* was made by mating *q276 XX* males with *fox-1(gf)* hermaphrodites; double mutants were recovered from the F_2 generation. *fox-1(gf); tra-2(e1095*sd*)* and *fox-1(gf); tra-1(e1076)* were made by mating *XO* males heterozygous for *tra-2* or *tra-1* with *fox-1(gf)* hermaphrodites. Double mutant cross-progeny were identified from the F_2 generation. *fox-1(gf); tra-1(e1099)* was isolated following a cross between *e1099 XX* males and *fox-1(gf)* hermaphrodites. *fox-1(gf); her-1(y101*sd*)* was made from a cross between *y101*sd *XO* males and *fox-1(gf)* hermaphrodites.

Outcrossing of the deletion: *dpy-3 unc-2* hermaphrodites were crossed with *fox-1(*Δ*) XO* males to construct *dpy-3 fox-1(*Δ*)* and *fox-1(*Δ*) unc-2* strains. *dpy-3 fox-1(*Δ*) XO* males were mated with *fox-1(*Δ*) unc-2* hermaphrodites to construct *dpy-3 fox-1(*Δ*) unc-2*. *dpy-3 fox-1(*Δ*) unc-2* hermaphrodites were crossed with N2 males to break up the triple mutant and recover *dpy-3 fox-1(*Δ*)* and *fox-1(*Δ*) unc-2*, now outcrossed on the right and left of the *fox-1* locus, respectively. *fox-1(*Δ*) unc-2* hermaphrodites obtained in this way were mated with *tra-2(q276) XX* males to construct a *fox-1(*Δ*) unc-2*; *tra-2(q276)*/+ strain. *fox-1(*Δ*) dpy-3*; *tra-2(q276)* was constructed in an analogous way. *dpy-3*; *tra-2(q276)*

XX males were mated with *fox-1(Δ) unc-2*; *tra-2(q276)*/+ hermaphrodites. F_1 *dpy-3*/*fox-1(Δ) unc-2*; *tra-2(q276) XX* males were mated with *dpy-3 unc-2* hermaphrodites. Rare non-Dpy and non-Unc F_1 hermaphrodites were isolated. They must have been of one of the following genotypes: *fox-1(Δ)*/*dpy-3 unc-2*; *tra-2(q276)*/+ or +/*dpy-3 unc-2*; *tra-2(q276)*/+. Animals of the former type were identified by PCR and selfed for succeeding generations until no Dpy, Uncs, or males were segregated.

Mating efficiency tests: Single L4 males were mated at 20° with four young *fem-1(hc17*ts) females (raised at 25°) until the females laid no more eggs. Fertilized females were transferred daily onto fresh plates, and their progeny were counted.

Expression analysis: Two *fox-1::lacZ* reporter constructs were made and their expression was analyzed *in vivo*. Both constructs are translational fusions that include the first 800 bp of *fox-1* genomic sequence. CB#1505 was made by subcloning an *Eco*RI-*Pst*I 5.5-kb fragment into the pPD89.20 lacZ vector; CB#1504 was made by subcloning a *Xho*I-*Pst*I 4-kb fragment into the pPD89.20 lacZ vector. The plasmids were coinjected with a pRF4, a *rol-6* marker plasmid, at a concentration of 50 μg/μl each, into young N2 hermaphrodites as described in Fire (1986). lacZ staining of embryos and mixed-stage worms was performed as described in Fire (1986).

RNase protection assay (RPA): Total RNA was isolated from embryos of *fox-1(gf)*; *him-8*, *xol-1*; *him-8*, *fox-1(Δ)*; *him-8*, and *him-8* strains using Trizol Reagent (GIBCO BRL, Gaithersburg, MD). The embryos were obtained from worm cultures grown on 9-cm plates (Lewis and Fleming 1995). ^{32}P-labeled RNA probes for RPA were *in vitro*-transcribed using a MaxiScript kit (Amicon, Beverly, MA). The *xol-1*-specific probe was designed to correspond to a 287-bp fragment unique for the 2.2-kb message. The fragment was PCR amplified from the WOE7E cosmid and subcloned into pBlueScript SK+ (Stratagene). The following PCR primers were used: 22MS1 (TAGCTATTGCTACTGAATCAAGG) and 22MS2 (TCACTCTTCATCCTCATCATACG). A 250-nucleotide *act-1* probe, 90 nucleotides of which are protected in RPA, was used as a control (Pulak and Anderson 1993). Full-length labeled RNA probes were gel purified. RPA was performed using an RPA II kit (Amicon). Hybridization was performed at 45° for 14 hr. In each reaction 20 μg of total RNA, 1×10^4 cpm of *act-1* probe, and 1×10^5 cpm of *xol-1*-specific probe were used. Results were visualized on a polyacrylamide gel, which was subsequently exposed to film over several days. RPA results were scanned using a Densitometer and analyzed using ImageQuant (Aladdin Systems).

***In vitro* RNA binding assays:** To create the *fox-1* expression vector pT7TSCB, an *Eco*RI to *Nde*I fragment from CBH4E1 (Hodgkin *et al.* 1994), which contained the entire ORF of the 454-aa *fox-1* product, was blunt ended with Klenow and placed into the *Eco*RV site of pT7TS (Zorn and Krieg 1997) such that the 5′ end of the gene was adjacent to the T7 promoter. The luciferase expression vector was supplied by Promega. ^{35}S Met-labeled FOX-1 and luciferase protein were produced *in vitro* from their respective expression vectors using a Promega TNT *in vitro* transcription-translation kit according to the manufacturer's guidelines. RNA binding was tested essentially as described in Swanson and Dreyfuss (1988). Briefly, 2 μl of labeled protein was added to 25 μl of a 50% slurry of Sepharose-bound RNA homopolymers [Sigma (St. Louis); 0.25–1.5 mg polyribonucleotide per milliliter of resin as indicated] in a binding buffer consisting of 10 mm Tris (pH 7.4), 2.5 mm $MgCl_2$, 100 mm NaCl, 0.5% Triton-X 100, and 1 mg/ml heparin. The final volume was brought to 50 μl with RNA-binding buffer. After a 15-min incubation at 23° the beads were washed four times with 1 ml of wash buffer [100 mm Tris (pH 7.4), 2.5 mm $MgCl_2$, 1 m NaCl, 0.5% Triton-X 100]. The bound material was eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer. The products were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

Western blot analysis: Mixed-stage populations of worms were collected in 1.5 ml of M9 buffer from a 4.5-cm plate that was just clearing and spun in a 2-ml tube at 6500 rpm for 2 min. Pelleted worms were washed once in M9 buffer and then resuspended in 150 μl of SDS sample buffer. The worms were boiled for 10 min and then loaded immediately onto a 10% SDS-polyacrylamide gel. The protein was transferred from the gel to Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a standard Western blotting procedure (Harlow and Lane 1988). Western blots were probed with the affinity-purified rabbit polyclonal antibody directed against bacterially expressed FOX-1 protein at a 1/100 dilution. The protein was detected using enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions.

For staged Westerns large populations of gravid adults from liquid culture were bleached to release embryos that were then allowed to hatch overnight in M9 buffer as described in Wood (1988). Embryos were harvested fresh by bleaching gravid adults at the same time as the other stages were collected.

Pure populations of males were generated as in Zarkower and Hodgkin (1992).

Construction of the *fox-1* genomic construct expressing only the second exon start: A 16-kb *Kpn*I-*Stu*I genomic fragment containing *fox-1* and its 5′ and 3′ flanking regions was subcloned from cosmid R04B3 into pBluescriptII KS$^-$ to create pCMFG1. Deletion of the *fox-1* genomic coding sequence within exon 1 was achieved by PCR using the overlap extension procedure described by Ho *et al.* (1989) and Higuchi (1990). Amplification of a region upstream of the first exon was performed with primer A 5′-GGCGCCATGGCAGCTGCTCCC-3′ (*Nco*I site underlined) and primer B 5′-GTAAGTTACCCTCAATTGG*TCTCTAATTTTGGCAACAC*CCGAAT-3′. Amplification of the region downstream of exon 1 was achieved with primer C 5′-CCCACCTGCAGAGACAGCGAGCT-3′ (*Pst*I site underlined) and primer D 5′-GTGTTGCCAAAATTAGAGA*CCAATTGAGGGTAACTTAC*TTTTTTTTT-3′. The underlined region of primer B is an add-on sequence, which is complementary to the italicized sequence in primer D. Similarly, the underlined sequence in primer D is complementary to the italicized sequence in primer B. The two PCR products were gel purified, mixed, and subjected to four PCR cycles to allow extension of heteroduplexes formed between the overlapping sequences. The extended heteroduplexes were then amplified using primers A and C. The resulting product was gel purified, digested with *Nco*I and *Pst*I, and subcloned into PRS314 (Sikorski and Hieter 1989). After sequencing confirmed the correct introduction of the deletion, the *Nco*I-*Pst*I fragment was used to replace the corresponding fragment of pCMFG1 to create pCMFGexΔ.

pCMFGexΔ was used to transform *C. elegans* homozygous for the *fox-1* deletion. pCMFG1 was also transformed into the *fox-1* deletion strain.

RESULTS

Analysis of *fox-1* gene products: Sequencing of the complete *fox-1* genomic region by the sequencing consortium predicts a gene spanning 5 kb and containing six putative exons. The open reading frame (ORF) created by joining all six of these exons would contain 1368 bp and produce a protein of 454 amino acids. The

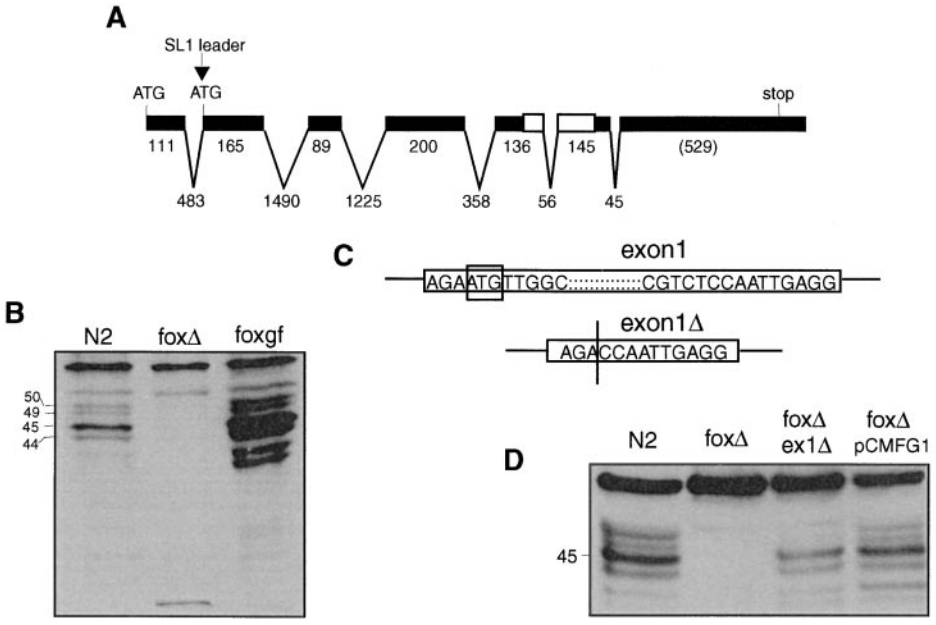


Figure 2.—Description of *fox-1* genomic region and expected products. (A) The genomic structure of the *fox-1* region. Exons are indicated by the black boxes while introns are shown as black lines. The size of each in base pairs is indicated below. The position of the RRM-type RNA-binding domain is indicated by the white box. The translation start sites of each of the two different transcripts are represented by an ATG above the exons. The start of the shorter transcript is spliced to an SL1 leader sequence as indicated. (B) A Western blot of extracts from mixed-stage populations of worms. The first lane contains wild-type extract (N2), the second lane is extract from homozygous *fox-1* deletion mutants (foxΔ), and the third lane is extract from the *fox-1* overexpressing strain with an integrated array of cosmid R04B3 (foxgf). The blot has been probed with affinity-purified rabbit polyclonal antibody against bacterially expressed FOX-1. The sizes of the *fox-1*-specific bands of the wild-type strain are indicated to the left in kilodaltons. The top band is not FOX-1 specific and serves as a loading control. (C) The extent of the deletion in the exon 1 deletion construct pCMFGexΔ. The top part of the figure represents the undeleted exon (:::: is used to represent the bulk of the sequence in the middle of the exon). The ATG is boxed. In the bottom of the panel the remainder of the exon in the deletion construct is shown in its entirety. The ATG and the coding sequence have been removed. The site of the junction is shown by the vertical line. (D) Mixed stage extracts from *fox-1* deletion worms containing an extrachromosomal array of pCMFGexΔ (foxΔ ex1Δ) are compared to extracts from the *fox-1* deletion strain with a wild-type pCMFG1 extrachromosomal array (foxΔ pCMFG1), the *fox-1* deletion strain on its own (foxΔ), and wild-type (N2) in a Western blot probed with affinity-purified rabbit polyclonal antibodies against bacterially expressed FOX-1. The position of the 45-kD band is indicated on the left.

RNA-binding motif is split between exons 5 and 6 (Figure 2).

The cDNA initially identified by Hodgkin *et al.* (1994) as the putative agent of *fox-1* numerator activity contains an ORF of 1248 bp and encodes a 415-amino-acid protein. Sequence alignment indicates that this cDNA corresponds to a transcript that begins at the second exon predicted from the genomic sequence. To determine if this cDNA is complete at the 5′ end and to see if a transcript containing the first exon exists we used rapid amplification of cDNA ends (RACE) analysis (Frohman *et al.* 1988). Because ~70% of *C. elegans* transcripts are trans-spliced to one of two leader sequences (SL1 or SL2) we used these as primers for cDNA amplification as well as standard RACE primers and primers specific to the first and second exons of *fox-1*. These studies revealed that there are two alternatively spliced forms of *fox-1*. The first contains all six exons and is not trans-spliced to a leader sequence. The second corresponds to the originally isolated cDNA. It is trans-spliced to an SL1 leader sequence and begins at exon 2 (data not shown; Figure 2). The significance of the two different starts is unclear. The additional 39 amino acids in the longer product have no obviously remarkable features.

To investigate the *fox-1* products at the protein level, polyclonal antibodies were raised in rabbits against the 415-amino-acid FOX-1 protein. The resulting affinity-purified antisera recognized FOX-1-specific bands. Western blots of extracts from mixed-stage populations of a wild-type strain, a *fox-1* deletion strain (see below), and a *fox-1* overexpressing strain were probed with the affinity-purified antibody. Proteins detected in extracts from the wild-type N2 strain are clearly absent in the *fox-1* deletion strain (Figure 2). Four *fox-1*-specific products can be seen in the wild-type extract. They run at apparent molecular weights of 44,000, 45,000, 49,000, and 50,000. The expected sizes of the proteins arising from the two alternatively spliced *fox-1* transcripts are 44,300 and 49,450 D. The antibody also detects a 27,000-D band in the extract from the deletion strain. This corresponds to the size predicted for the remainder of the coding region in the *fox-1* deletion mutant and indicates that the truncated fragment is expressed. This fragment does not contain the RNA-binding motif (see below).

To determine which of the two different transcripts might be responsible for the four *fox-1*-specific products observed, we generated a genomic *fox-1* clone that is deleted for the coding region of the first exon (Figure 2). This construct was transformed into *fox-1* deletion animals so that, when expressed, it would provide the shorter transcript as the only available form of

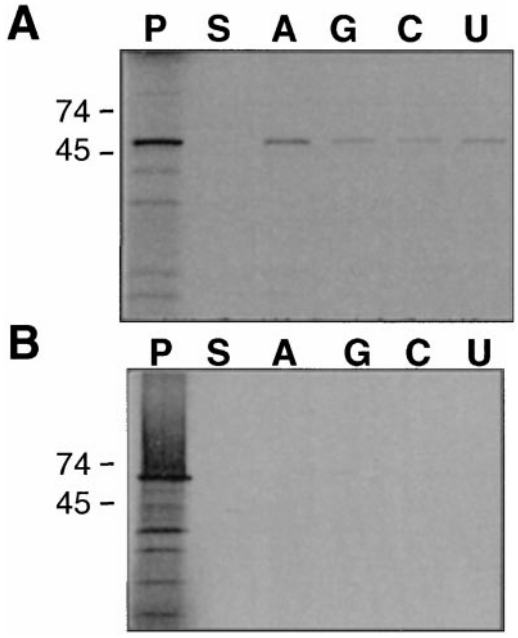


Figure 3.—*In vitro* RNA-binding analysis of *fox-1*. ^{35}S Met-labeled *in vitro* transcribed and translated FOX-1 [(A), P] and luciferase [(B), P] were incubated with sepharose-bound poly(A) (A), poly(G) (G), poly(C) (C), poly(U) (U) RNA, or sepharose only (S). After several washes protein still bound to the RNA homopolymers was eluted by boiling in an SDS sample buffer and run out on an SDS polyacrylamide gel. The proteins were visualized by autoradiography. Sizes indicated on the left are in kilodaltons.

FOX-1. A wild-type version of the *fox-1* genomic region was also transformed into the *fox-1* deletion strain for comparison. The results of this experiment show that the transcript beginning with the coding region of the second exon provides the two smaller *fox-1* products at 44 and 45 kD but not the 49- and 50-kD forms (Figure 2).

FOX-1 binds RNA with some sequence preference: Both transcripts of the *fox-1* gene encode proteins with a centrally located RRM-type RNA-binding motif. The presence of this well-studied motif suggests that FOX-1 may bind RNA as part of its role as a numerator element. To determine if FOX-1 is capable of binding RNA we used *in vitro*-synthesized FOX-1 protein for *in vitro* RNA-binding experiments. ^{35}S-labeled *in vitro* transcribed and translated FOX-1 was mixed with Sepharose-bound poly(A), poly(G), poly(C), or poly(U) ribonucleotide. The results of this experiment indicate that FOX-1 is capable of binding RNA and shows some sequence preference (Figure 3). FOX-1 binds poly(A), poly(G), and poly(U) RNA to some degree and may show some preference for poly(A) RNA. Luciferase, a non-RNA-binding protein used as a control, does not bind any of the polyribonucleotides in similar experiments.

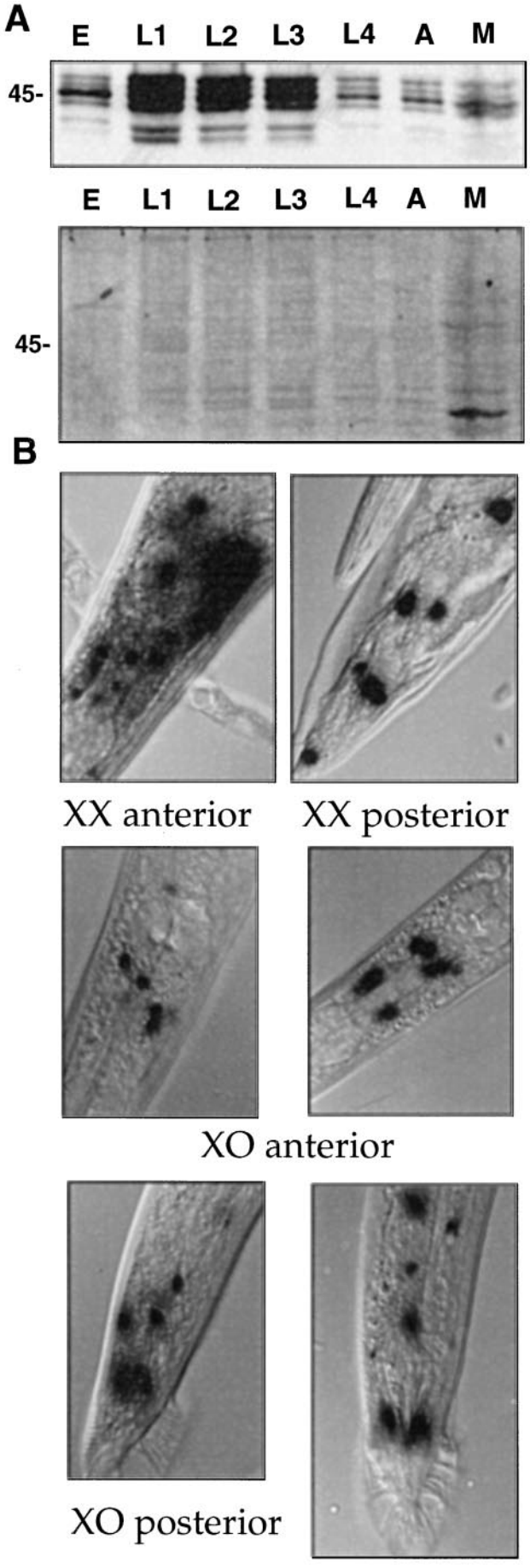
***fox-1* has a ubiquitous early embryonic expression that gives way to a more restricted postembryonic expression pattern:** We examined *fox-1* expression by means of Western blot analysis and *fox-1::lacZ* reporter constructs expression *in vivo*. After embryogenesis, *C. elegans* goes through four larval stages before reaching adulthood. Extracts collected from individual stages of development were probed on a Western blot with the FOX-1 antibody to determine the protein expression profile throughout the life of the animal. FOX-1 can be detected at some level throughout all stages of development (Figure 4). The predominant form of the protein observed in embryos is the 45,000-D protein. All of the larval stages appear to have all four forms present, as does the adult. All forms are also present in a pure population of adult male animals.

lacZ driven from the *fox-1* promoter is ubiquitously expressed in the embryo from at least the 18–20 cell stage up to the threefold stage (data not shown). The expression of transgenes becomes much more restricted in postembryonic life (Figure 4). The staining can only be detected in a small subset of cells in the head and the tail of both hermaphrodites and males, although the expression pattern differs between the sexes. We suggest that postembryonic *fox-1* expression is limited to a small subset of neurons within the soma. Since lacZ transgenes are not normally expressed in the germline we cannot assess expression there.

Construction of *fox-1(gf)*: To achieve stable overexpression of *fox-1*, extrachromosomal arrays of RO4B3 and pRF4 were integrated into the chromosomes and mapped (data not shown). Dot blot analysis of the three integrated lines and a wild-type strain, followed by a quantitation of the hybridization signal, allows one to estimate the number of extra copies of *fox-1*. Because the arrays have likely undergone rearrangements and recombination, the estimate corresponds to the maximum number of copies present. The strain selected for all subsequent analysis (*eIs26*) was estimated to contain 42 extra copies of *fox-1*.

Isolation of Tc1 insertions: A Tc1 transposable element insertion in *fox-1* was obtained from the insertion library generated by Zwaal *et al.* (1993). A total of 10 pairs of *fox-1*-specific primers were used in combination with Tc1-specific primers in the screen. Two independent insertions were recovered, one located in the second intron, IS1(*e2641*), and the other in the fourth intron, IS2(*e2462*), just upstream of the RRM domain (Figure 5). Observation of nonspecific and transient PCR products seen in the deletion screens suggested a difference in the levels of somatic excision between the two insertion mutants. The more active insertion, IS2(*e2462*), was subsequently chosen for further deletion screens. Note that the apparent excision frequency can be influenced by the choice of PCR primers used or by a genuine difference in transposition activity between the two regions.

No phenotype was detected in the *XX* or *XO* worms for either of the insertion mutants. This is not unexpected because both insertions are within introns and are most likely removed in hnRNA processing. Cases of efficient



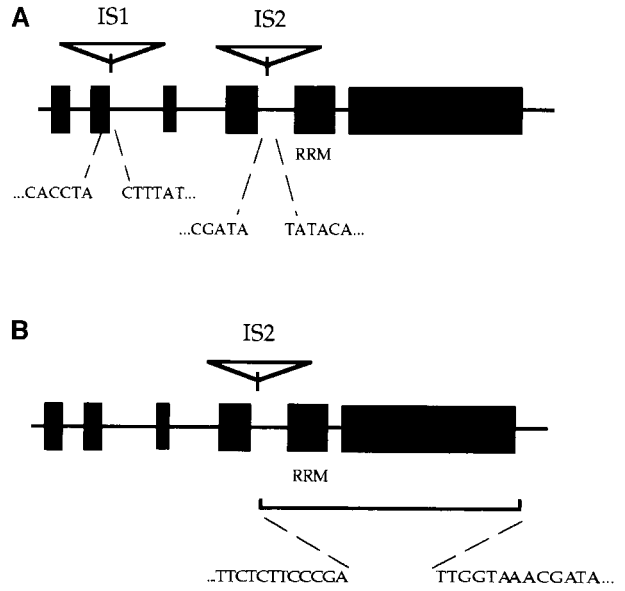


Figure 5.—Identification of a Tc1-dependent deletion within *fox-1* ORF. (A) Two Tc1 insertions obtained (IS1 and IS2) were intronic, located in introns 2 and 4, respectively. The sequence immediately around the insertions is shown. (B) IS2 gave rise to a 1.2-kb deletion as a result of an imprecise, one-sided deletion event. The extent of the deletion is indicated by a square bracket and the sequence around the deletion is shown.

Tc1 removal from both introns and exons, resulting in a wild-type phenotype, have been reported previously (for review see Plasterk 1992).

Isolation of the deletion: IS2 was chosen for further deletion screens for reasons of its genomic location and the higher somatic excision activity. A deletion mutant was isolated following a screen of at least 7000 initial polyclonal worm cultures. The choice of primers biased the screen toward a recovery of one-sided deletions, where the left end of Tc1 excised more or less precisely and the right end imprecisely (see Figure 5). The recovered transposon-mediated deletion removes ~1.2 kb of *fox-1* genomic sequence at the 3′ end of the ORF. Significantly, it completely removes the RRM domain, the only functional domain predicted at the sequence level. Despite this, no obvious phenotype was observed in *fox-1(Δ) XX* animals.

***fox-1(Δ)* rescues *XO*-specific lethality caused by dupli-**

Figure 4.—*fox-1* expression analysis. (A) A Western blot with extracts collected from a pure population of L4/adult males (M) and N2 animals at the different stages of development from embryo (E), L1, L2, L3, L4, to young adult (A), was probed with affinity-purified rabbit polyclonal antibody against bacterially expressed FOX-1. The Ponceau-stained blot is shown below for loading control. The position of the 45-kD FOX-1-specific band is indicated to the left. (B) *fox-1::lacZ* expression in adult *XX* and *XO* animals is confined to small subsets of cells in the head and the tail. The expression pattern in *XX*s is distinct from that in *XO*s.

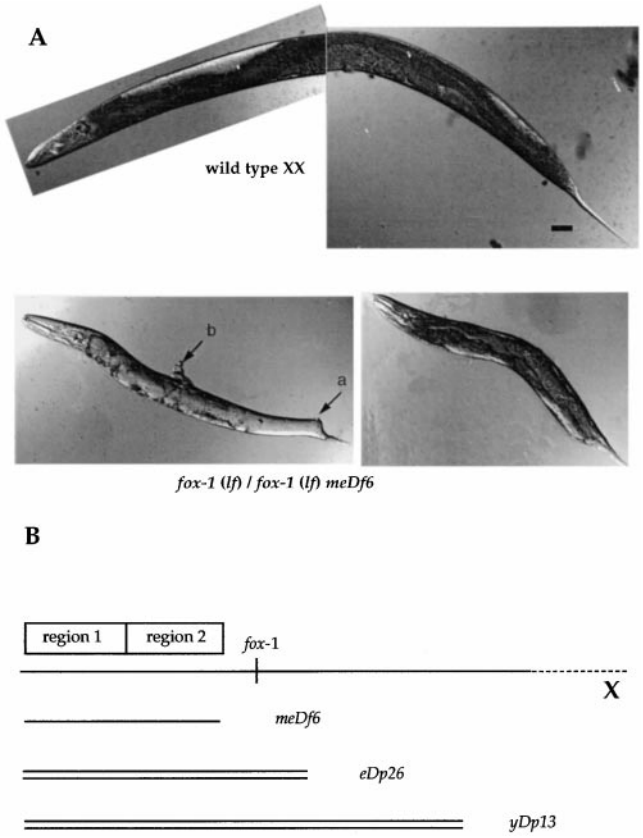


Figure 6.—*fox-1(Δ)* synergizes with *meDf6* and results in varying degrees of masculinization. (A) Wild-type *XX* and *fox-1(Δ)* comparison. *fox-1(Δ) XX* hermaphrodites are morphologically wild type, as are *XX* hermaphrodites carrying *meDf6*. However, *fox-1(Δ)/meDf6 XX* animals are dumpy, which is characteristic of a dosage compensation defect. Also, a proportion of animals have abnormal vulvas (b) and severely truncated tail spikes (a), a sign of masculinization. Bar, 20 μm. (B) A schematic representation of duplications and deficiencies of the *X* chromosome used in this study. A single bar represents a deficiency, while a double bar represents a duplication. Note that *eDp26* is attached to the left end of the *X* in an inverse orientation (Hodgkin *et al.* 1994). Regions 1 and 2 refer to regions of the *X* that have been shown by Akerib and Meyer (1994) to exhibit numerator properties.

cations of the left end of the X: *fox-1(Δ)* removes the RRM domain, the only functional domain predicted at the sequence level, and yet *fox-1(Δ) XX* animals show no phenotype. To establish if the deletion represented a null mutation, we tested the ability of *fox-1(Δ)* to rescue the *XO*-specific lethality caused by the duplications of the left end of the *X*.

fox-1 was identified as a result of analysis of *eDp26*, an *XO* lethal and feminizing duplication that increases the dose of the numerators (Hodgkin *et al.* 1994). Overexpression of *fox-1* effectively mimics the *XO*-specific lethality caused by *eDp26*. One would predict that if *fox-1(Δ)* is a biological null, then males carrying *fox-1(Δ)* and *eDp26* should be viable. Unexpectedly, the desired recombination event, which would put *fox-1(Δ)* and *eDp26* on the same chromosome, was never achieved despite very extensive screens. Snapback pairing of *eDp26*, which is attached in inverted orientation to the left end of the *X*, probably leads to a complete suppression of recombination in this region. Another duplication, *yDp13*, slightly larger than *eDp26* but otherwise equivalent, was used in the same experiment (see Figure 6). Unlike *eDp26*, *yDp13* is a free duplication that makes genetic manipulation easier. The results are presented in Table 1. *XO* males that are *fox-1(Δ)* and carry *yDp13* are ~95% viable, in contrast with *yDp13 XO* males that are ~4% viable. Therefore *fox-1* is wholly or largely responsible for *XO*-specific lethality caused by *yDp13*, since *fox-1(Δ)* is capable of rescuing *XO*-specific effects of this duplication. This finding is consistent with the deletion being a loss-of-function allele.

***fox-1(Δ)* synergizes with putative numerator elements:** *fox-1(Δ)* hermaphrodites appear morphologically and behaviorally wild type. Because there are at least four putative numerator elements in the worm (Akerib and Meyer 1994; Nicoll *et al.* 1997), it is likely that they are partially redundant. To test whether *fox-1* synergizes with other numerator elements, *fox-1(Δ)* males were crossed with hermaphrodites carrying *meDf6*, a deletion of the left end of the *X* that removes the putative numerator elements from regions 1 and 2. *fox-1(Δ)* and *meDf6 XX* hermaphrodites appear wild type, but *XX* hermaphrodites that are hemizygous for the two putative elements, *i.e.*, *fox-1(Δ)/meDf6*, are often masculinized and dumpy (Figure 6). The masculinization manifests itself through a truncated tail spike (hermaphrodites have a long, pointy tail spike), deformed gonad, and vulval abnormalities. There also appear to be germline problems, although these were not extensively investigated. The animals are often constipated, sometimes severely, resulting in the tail end bursting open as a result of the pressure in the gut. The dumpiness is assumed to be a result of inappropriate dosage compensation. *XX* animals normally downregulate expression from both *X*s to a level equivalent to that of a single male *X*. Masculinization results in a reduced or a complete lack of dosage compensation. Reducing the numerator dose further by removing both copies of *fox-1* (*fox-1(Δ)/fox-1(Δ) meDf6*) does not appear to exacerbate the above phenotype. These results are in agreement with those of Nicoll *et al.* (1997), who used *meDf6* and point mutations in *fox-1*; they also showed strong synergy with *sex-1*.

fox-1 and *meDf6* results show that, just as in Drosophila, in *C. elegans* the numerator function is partially redundant. It has been reported in Drosophila that the strength of the individual numerator elements can vary in different wild-type genetic backgrounds (Cline 1988). In an attempt to find a genetic background in which *fox-1(Δ)* had a phenotype in *XX* animals, *fox-1(Δ)* was crossed into 11 different wild-type strains of *C. elegans* (see materials and methods) and 50 F_2 populations were examined for each strain. In all strains tested *fox-1(Δ)* was compatible with normal *XX* hermaphrodite development.

TABLE 1

fox-1(Δ)* is capable of rescuing *XO*-specific lethality caused by *yDp13

Genotype of mother	No. of broods scored	No. of individuals counted	No. of wt F_1 males/ no. of wt F_1 hermaphrodites × 100	No. of Dpy F_1 males/ no. of wt F_1 hermaphrodites × 100
dpy-3; *yDp13*	3	199	4% (2–5%)	NA
dpy-3	3	1375	NA	101% (96–104%)
fox-1(Δ) dpy-3; *yDp13*	12	827	100% (78–177%)	NA

NA, not applicable.

Genetic analysis of *fox-1(Δ)*: *xol-1* is the predicted downstream target of *fox-1*. It is difficult to study this epistatic relationship directly because *fox-1* and *xol-1* exert their influence on opposite sexes (*fox-1* in hermaphrodites and *xol-1* in males). Therefore we examined the minor, *XX*-specific function of *xol-1*. This way the phenotypic effects of *fox-1* and *xol-1* can both be analyzed in *XX* animals. The *xol-1 XX*-specific feminizing role can be seen in *tra-2*, *tra-3*, and *her-1* backgrounds (Miller *et al.* 1988). *tra-2 (lf)* mutations transform *XX* animals into incomplete, nonmating males (Hodgkin and Brenner 1977). A complete transformation toward a male fate can be achieved when the wild-type function of *xol-1* is removed. Similarly, masculinizing effects of *her-1* (Trent *et al.* 1988) can be enhanced in the absence of *xol-1*. We reasoned that if there were a subtle phenotype associated with *fox-1(Δ)* it might become more prominent in *tra-2*, *tra-3*, and *her-1 XX* animals. Moreover, because the role of *fox-1* is opposite to that of *xol-1*, the effects of *fox-1(Δ)* should be comparable to *xol-1* overexpression and vice versa. The details of the double mutant analysis for *fox-1(Δ)* and *tra-2*, *tra-3*, and *tra-1* are shown in Table 2. The effects of *fox-1(Δ)*; *tra-2 XX* double mutant combinations on the *XX* male tail morphology can be seen in Figure 7. Sexual transformation in *tra-2* and *tra-3 XX* animals, which also carry a deletion at the *fox-1* locus, is poorer than in *tra-2* or *tra-3 XX* animals alone. The effect is particularly pronounced within the copulatory structures of the male tail. There is a marked reduction in the fan size, and the continuity of the fan is often broken. Ray morphology is variable (often short and stumpy), with frequent reduction in ray number. In most cases the whole fan structure is almost completely absent and there is no appreciable regression of the cytoplasm from the distal regions of the tail. The animals are also often severely constipated. Constipation is probably due to a defect in the anatomy of the cloaca, a side effect of the morphological abnormalities of the tail.

To test whether the feminization of *tra-2 XX* animals caused by *fox-1(Δ)* is dependent on the wild-type function of *xol-1*, we examined *fox-1(Δ)* xol-1(y9); *tra-2(e1095) XX* animals. *xol-1(y9)* completely removes *xol-1* activity (Rhind *et al.* 1995). *fox-1(Δ) xol-1(y9)*; *tra-2(e1095) XX* animals are no longer feminized. They are phenotypically indistinguishable from *xol-1(y9)*; *tra-2(e1095) XX*. This observation confirms that *xol-1* is epistatic to *fox-1* and that feminization caused by *fox-1(Δ)* requires wild-type function of *xol-1*.

The effects of *fox-1(Δ)* were also examined in the unusual *tra-2* allele, *q276* (P. E. Kuwabara and T. Schedl, unpublished results). Unlike *tra-2(e1095) XX*, which do not mate, *q276 XX* animals are mating males,

TABLE 2

Effects of *fox-1(Δ)* on sexually transformed *XX* animals

Genotype	Phenotype	
	fox-1(+)	*fox-1(Δ)*
tra-2(e1095) XX	Nonmating pseudomales	Weak masculinization
tra-2(q276) XX	Mating males	Nonmating males
tra-3(e1767) XX	Nonmating pseudomales	Weak masculinization
tra-1(e1099) XX	Mating males (gonad often defective)	Mating males (gonad often defective)
tra-1(e1076) XX	Weak masculinization	Weak masculinization
tra-2(e1095); xol-1(y9) XX	Many mating males	Many mating males
tra-2(q276); xol-1(y9) XX	Many mating males	ND
tra-3(e1767); xol-1(y9) XX	Many mating males	ND
tra-1(e1099); xol-1(y9) XX	Mating males (gonad often defective)	ND
tra-1(e1076); xol-1(y9) XX	Weak masculinization	ND

Phenotypes described in this table are highly penetrant (a majority of animals of a given genotype exhibit the phenotype described). See Figure 7 for representative examples. ND, not determined.

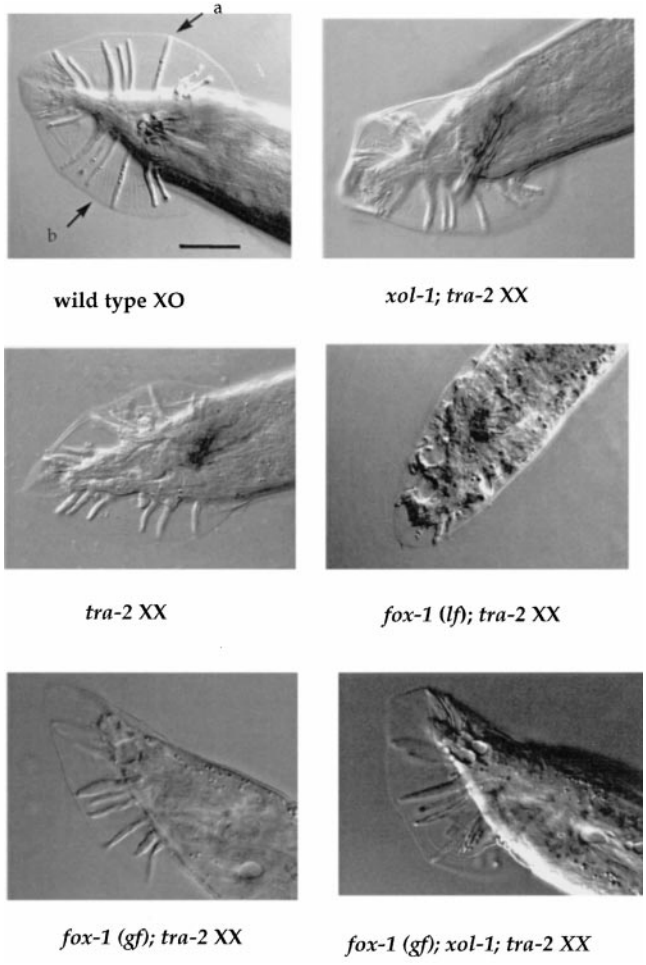


Figure 7.—Phenotypic analysis of *fox-1(Δ)*; *tra-2(e1095)* and *fox-1(gf)*; *tra-2(e1095) XX* animals. *tra-2(e1095) XX* animals are transformed into incomplete males with reduced rays (a) and fan (b). *xol-1(y9)*; *tra-2(e1095) XX* animals, however, are morphologically indistinguishable from wild-type *XO* males. *fox-1(Δ)* has the opposite effect to that of *xol-1(y9)* in *tra-2(e1095) XX* animals, in that it results in a poorer sexual transformation than *tra-2(e1095)* alone. Note the severely reduced rays and an almost complete loss of the fan. *fox-1(gf)*; *tra-2(e1095) XX*s have a morphology comparable with that of *xol-1*; *tra-2*; *i.e.*, they are more transformed than *tra-2* alone, but they do not show mating behavior. Therefore, *fox-1(gf)* is only partially able to phenocopy *xol-1(lf)*. Bar, 20 μm.

although the mating efficiency is lower than that of the wild type. *fox-1(Δ)*; *tra-2(q276) XX* males are more similar to *tra-2(e1095)* in a behavioral sense. *fox-1(Δ)*; *tra-2(q276) XX* males show very little interest in hermaphrodites. Occasionally, one will pause by a hermaphrodite and initiate the typical mating behavior of tracking along the hermaphrodite body. The male almost invariably falls off the head or tail and loses contact with the hermaphrodite body. In most cases tracking is not reinitiated. Such behavior is assessed as poor according to the Loer and Kenyon assay (Loer and Kenyon 1993). The males were individually tested for their ability to sire progeny, and the results can be seen in Table 3.

Genetic analysis of *fox-1(gf)*: Genetic analysis of *fox-1(gf)* and *tra-1*, *tra-2*, *tra-3*, and *her-1* yielded reciprocal results to those obtained from *fox-1(Δ)* analysis (Table 4). The effects of *fox-1(gf)* in *tra-2* and *tra-3* animals are to shift the phenotype toward more complete masculinization (Figure 7). However, *fox-1(gf)*; *tra-2 XX* animals are not transformed into complete males, as seen in *xol-1*; *tra-2 XX*s. Despite their almost wild-type morphology, they do not show mating behavior. The alteration in brood profile in *fox-1(gf)*; *her-1 XX*s (see Table 4) is probably not significant; however, it is consistent with the mild masculinizing effects seen in *tra-2* and *tra-3* animals. The shift toward stronger masculinization of *tra-2*, *tra-3*, and *her-1* animals by *fox-1(gf)* is comparable with that of weak *xol-1* alleles, *e.g.*, *y70* (Miller *et al.* 1988, and Table 2). The difference between *fox-1(gf)*; *tra-2* and *xol-1*; *tra-2 XX* phenotypes is likely to be due to the existence of additional downregulators of *xol-1*. There is good evidence for strong transcriptional regulation of *xol-1* (Rhind *et al.* 1995; Nicoll *et al.* 1997) and some evidence of additional regulation at both transcriptional and post-transcriptional levels (Cline and Meyer 1997; Nicoll *et al.* 1997). To account for our genetic results we suggest that levels of functional XOL-1 are reduced, but not completely absent, in *fox-1(gf)* animals. This reduction is sufficient to cause *XO*-specific lethality, but not sufficient to remove feminizing effects of *xol-1* in *XX* animals. It is possible that *XO* levels of *xol-1* are close to a critical threshold and any reduction in *xol-1* activity will result in the expression of the *sdc*s, resulting in feminization and aberrant dosage compensation expression.

The same overall phenotypic tendency seen in sexually sensitized backgrounds is weakly detectable in *XX* animals that are only mutant at the *fox-1* locus. *fox-1(Δ)* hermaphrodites have reduced brood size (mean = 256 ± 36, N = 10 broods, range 213–329; wild-type figures, mean = 329 ± 32, N = 12 broods, range 274–374). The brood sizes of additionally outcrossed *fox-1(Δ)* hermaphrodites are also reduced (mean = 254 ± 28, N = 12, range 211–309). Broods for *fox-1(Δ) xol-1*, however, are close to wild type (mean = 310 ± 22, N = 6, range 288–339).

***fox-1* expression levels influence the levels of *xol-1* 2.2-kb message:** Our genetic analysis suggests that *fox-1* overexpression leads to downregulation of *xol-1*, while removing *fox-1* leads to *xol-1* upregulation. We investigated the possibility that *xol-1* transcript levels may be altered in *fox-1* mutant backgrounds. FOX-1 is an RNA-binding protein; therefore its involvement in post-transcriptional regulation of its target would not be unexpected. Furthermore, Nicoll *et al.* (1997) showed that the expression of a translational reporter fusion of *xol-1* and GFP was affected when FOX-1 levels were altered. *xol-1* is alternatively spliced to produce three transcripts. The 2.2-kb transcript of *xol-1* was shown to be both necessary and sufficient for the wild-type function of *xol-1* (Rhind *et al.* 1995). Using an RNase protection

TABLE 3

Male mating efficiency

Genotype	*X* chromosome complement	Progeny sired
fox-1(Δ)	*XO*	437.3 ± 256.4 (99–947), $N = 11$
fox-1(Δ) outcrossed	*XO*	455.45 ± 205.9 (238–949), $N = 11$
N2	*XO*	1046.6 ± 153.24 (820–1294), $N = 5$
fox-1(Δ) xol-1(y9); tra-2(e1095)	*XX*	327.2 ± 146.7 (122–671), $N = 19$
xol-1(y9); tra-2(e1095)	*XX*	761 ± 124.2 (557–975), $N = 10$
fox-1(Δ); tra-2(q276)	*XX*	2 ± 0.6 (0–2); $N = 10$
tra-2(q276)	*XX*	181 ± 254 (0–728); $N = 9$

assay we looked at the levels of 2.2-kb transcripts in four strains: (1) *fox-1(gf); him-8*, (2) *fox-1(Δ); him-8*, (3) *him-8*, and (4) *xol-1*; *him-8*. *xol-1* messages are 10-fold more abundant in *XO*s than in *XX*s; therefore, to facilitate *xol-1* message detection we used *him-8* strains to enrich the population in *XO* animals. Broods of *him-8* hermaphrodites are 38% male, as a result of increased incidence of *X* chromosome nondisjunction (Hodgkin *et al.* 1979). *xol-1* is also most abundant in early embryogenesis, and for that reason total RNA was prepared from embryos. Moreover, populations of *xol-1*; *him-8* and *fox-1(gf); him-8* will no longer be enriched in *XO* animals once embryogenesis is over, due to *XO*-specific lethality caused by *xol-1 (lf)* or *fox-1(gf)*. RNase protection assay results reveal that in the *fox-1(gf); him-8* strain the levels of the 2.2-kb *xol-1* message are significantly reduced as compared to wild type, but they are not removed completely. Conversely, in the *fox-1(Δ); him-8* strain the level of the same *xol-1* message is increased (Figure 8).

***fox-1* has a late function, distinct from its role as a numerator:** The function of a numerator element in *C. elegans* is presumed to be over by the time dosage compensation becomes activated, which corresponds to the 30-cell stage of embryogenesis. It is intriguing to find *fox-1* expressed beyond embryonic development. This phenomenon could be either fortuitous or indicative of a late requirement for *fox-1* in some aspect of worm development. It would not be without precedent. In Drosophila, for example, there is evidence for the involvement of some numerator elements in neural development. Further characterization revealed that the mating efficiency of *fox-1(Δ) XO* males is lower than wild type. Total progeny sired by single males from *fox-1(Δ)* and wild-type strains were counted (Table 3). The aver-

TABLE 4

Effects of *fox-1(gf)* on sexually-transformed *XX* animals

Genotype	Phenotype	
	fox-1(+)	*fox-1(gf)*
tra-2(e1095) XX	Nonmating pseudomales	Nonmating males, very good morphological masculinization
tra-2(q276) XX	Mating males	Mating males
tra-3(e1767) XX	Nonmating pseudomales	Nonmating males, good morphological masculinization
tra-1(e1099) XX	Mating males (gonad often defective)	Mating males (gonad often defective)
tra-1(e1076) XX	Weak masculinization	Weak masculinization
tra-2(e1095); xol-1(y9) XX	Many mating males	ND
tra-2(q276); xol-1(y9) XX	Many mating males	ND
tra-3(e1767); xol-1(y9) XX	Many mating males	ND
tra-1(e1099); xol-1(y9) XX	Mating males (gonad often defective)	ND
tra-1(e1076); xol-1(y9) XX	Weak masculinization	ND
her-1(y101sd) XX	66% Egl hermaphrodites, 34% intersexes and pseudomales female soma, male gonad	57% Egl hermaphrodites and 43% intersexes and pseudomales
her-1(y101sd); xol-1(y9) XX	92% intersexes and pseudomales, 8% mating males	ND

Phenotypes described in this table are highly penetrant (a majority of animals of a given genotype exhibit the phenotype described). See Figure 7 for representative examples. ND, not determined.

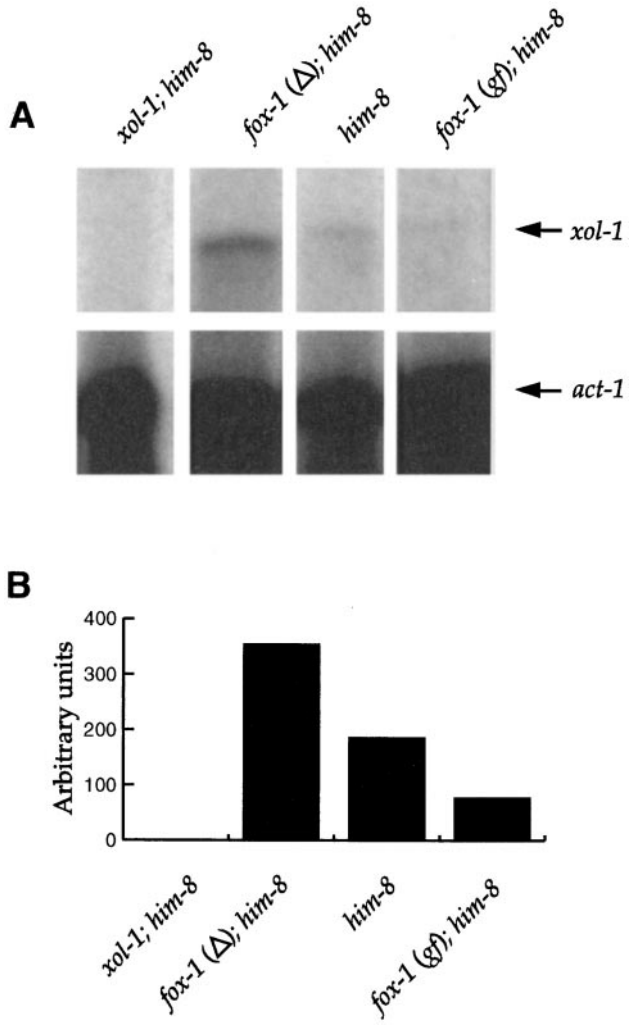


Figure 8.—*fox-1* negatively regulates *xol-1* post-transcriptionally. The 2.2-kb *xol-1* transcript levels are increased in the absence of *fox-1* and reduced, but not completely eliminated, when *fox-1* is overexpressed. (A) For all four strains RNA was isolated from embryos. RNase protection assay (RPA) was performed using a *xol-1* probe specific to the unique portion of the 2.2-kb transcript and an *act-1* probe, which served as a control. A *xol-1*; *him-8* strain was used as a negative control for the *xol-1*-specific probe, and a *him-8* strain was a positive control, equivalent to wild type. Note that the apparent size difference of the *xol-1*-specific bands across the four lanes is an artifact. (B) The relative levels of the normalized *xol-1*-specific signal. (Normalization was done as follows: the levels of *act-1* signal were brought to the highest common denominator; for each lane, the actual *xol-1* signal was multiplied by the factor by which the actual *act-1* signal differed from the highest common denominator.)

age number of progeny sired by a single *fox-1(Δ) XO* male is approximately one-half of the wild-type value. To test whether *fox-1* effects on male mating efficiency are *xol-1* dependent, *xol-1*; *tra-2(e1095) XX* males were compared with *fox-1(Δ) xol-1*; *tra-2(e1095) XX* males for their mating efficiency. If *fox-1* acts through *xol-1* in a relationship similar to that seen during primary sex determination, then *xol-1* should be epistatic to *fox-1*, and no difference between *xol-1*; *tra-2(e1095)* and *fox-1(Δ) xol-1*; *tra-2 XX* males should be observed. It is evident from Table 3 that *xol-1* is not epistatic to *fox-1(Δ)* in this interaction, because *fox-1(Δ) xol-1*; *tra-2(e1095) XX* males sire significantly fewer progeny than *xol-1*; *tra-2(e1095) XX* males. These results suggest that *fox-1* effects on male mating are not mediated via *xol-1*. The effects of *fox-1(Δ)* on male mating efficiency in the *tra-2(e1095)* background are similar to those observed in *tra-2(q276)* background (see above and Tables 2 and 3). To exclude the possibility that this reduction in male mating efficiency was due to genetic factors other than *fox-1(Δ)*, a series of crosses was designed to outcross the deletion from any closely linked factors. Because the deletion was isolated from a strain with a high Tc1 transposon copy number, a possibility existed that the observed mating effects were due to a high genetic load caused by a high transposition rate. The strategy adopted to outcross *fox-1(Δ)* is described in detail in materials and methods. The males from the outcrossed strain were tested for their mating efficiency. As can be seen in Table 3, the number of progeny sired by single outcrossed males is on average the same as for the previous *fox-1(Δ)* strain. Therefore we conclude that *fox-1(Δ)* is responsible for the observed effects on mating efficiency.

DISCUSSION

FOX-1 is an RNA-binding protein involved in the assessment of the *X*:*A* ratio in the initial stages of sex determination and dosage compensation in *C. elegans*. We describe construction and analysis of a Tc1-derived deletion within the *fox-1* locus and present genetic and molecular evidence to establish the role of *fox-1* as a numerator element. We show that *fox-1* influences the sex determination and dosage compensation pathway probably by regulating the levels of the 2.2-kb transcript of *xol-1*. Furthermore, we describe a postembryonic function for *fox-1*, which is distinct from its role as a numerator element.

Duplication of the left end of the *X* chromosome results in *XO*-specific feminization and lethality due to an increase in the number of numerator elements that act to downregulate *xol-1* activity. We have generated a deletion that removes 1.2 kb of *fox-1* genomic sequence, including the RRM domain. Its ability to rescue *XO*-specific feminization and lethality caused by duplications of the left end of the *X* demonstrates that it is a *bona fide* loss-of-function allele. The duplication used in our analysis, *yDp13*, duplicates two distinct regions with putative numerator function, as well as *fox-1*. The ability of *fox-1(Δ)* to almost completely rescue the *XO* effects of *yDp13* argues that *fox-1* is by far the strongest element in this part of the chromosome.

A strong numerator element might be expected to have reciprocal effects in *XX* and *XO* animals, such that overexpression in *XO* should result in a strong feminiza-

tion and lethality, while loss of function should have reciprocal effects in *XX*. This is not the case for *fox-1*. Although increasing the dose of *fox-1* is almost completely lethal to *XO*s, *fox-1(Δ) XX* animals develop as apparently normal hermaphrodites. Comparable genetic behavior, revealing that numerator elements are not equipotent and can be redundant, has been demonstrated in Drosophila (Cline 1988). Synergistic relationships between areas of the *X* chromosomes with numerator activity was shown by Akerib and Meyer (1994), and synergism between mutations in *fox-1* and *sex-1*, another putative numerator element, was shown by Nicoll *et al.* (1997). Although neither *fox-1(Δ)* nor *meDf6*, which removes two putative numerator elements, has a phenotype on its own in *XX*s, both synergize *in trans* to result in strong dumpy and partially masculinized hermaphrodites. *fox-1* therefore has both reciprocal functions expected from a numerator element, but its loss in *XX* animals is masked by contributions from other numerators.

It is conceivable that in different biological contexts the relative importance of the same signal will vary. This phenomenon was reported in Drosophila (Cline 1988), where natural differences in the *X*:*A* ratio bias exist among wild-type strains. In some strains, an increase of numerator elements in males has a stronger effect than their decrease in females, whereas in other strains the reverse is true. We investigated the role of *fox-1(Δ)* in 11 different wild-type genetic backgrounds in an attempt to find an *XX*-specific *fox-1(Δ)* phenotype. In contrast with the fly, the partial numerator redundancy is robust in the worm (at least in the case of *fox-1*), because no abnormal phenotypes were ever observed in any of the hybrid strains tested.

fox-1 may be dispensable for hermaphrodite development, but its overexpression has strong lethal and feminizing effects in *XO*s (Hodgkin *et al.* 1994). In *C. elegans*, numerator elements work together to negatively regulate their downstream target, *xol-1*. Recent evidence suggests that although they all affect the same downstream target, their modes of action differ. Among numerators discovered to date, two of them influence *xol-1* at transcriptional level (*sex-1* and region 1) and two at post-transcriptional level (region 2 and *fox-1*) (Nicoll *et al.* 1997). We show, using *in vitro* RNA-binding assays, that FOX-1 is capable of binding RNA with some sequence preference for poly(A). This ability to bind RNA is consistent with the presence of the RRM-type RNA-binding motif and supports the idea of a post-transcriptional role for FOX-1. Decreasing the dose of numerators should derepress *xol-1* in *XX* animals, thus leading to masculinization and inappropriate dosage compensation. Such effects are not observed when *fox-1* levels are decreased. The following has to be appreciated to account for this observation. In *fox-1(Δ) XX* animals the expression of numerators that control *xol-1* at transcriptional levels is wild type. Transcriptional control is the primary stage in *xol-1* regulation and accounts for about a 10-fold difference in *xol-1* levels between the sexes (Rhind *et al.* 1995). In wild-type *XX* animals *xol-1* is repressed. In *fox-1(Δ) XX* animals transcriptional repression is administered correctly by other numerator elements. It seems that in this case a further post-transcriptional repression is not critical; therefore its absence in *fox-1(Δ)* animals is of no great consequence. In other words, the absence of a repressor when its target is already repressed will go unnoticed.

In contrast to the *XX*s, it is important that XOL-1 levels are high in the *XO*s, because XOL-1 activity directs the male mode of development. When *fox-1* is overexpressed, the levels of transcriptional regulators of *xol-1* remain low, allowing for a higher transcription of *xol-1*. However, *xol-1* transcripts have to be processed correctly to achieve high levels of a 2.2-kb message, which is both necessary and sufficient for all known *xol-1* functions (Rhind *et al.* 1995). It is at this step that *fox-1* levels are critical. We postulated that *fox-1* overexpression might reduce the level of the 2.2-kb *xol-1* message and perhaps eliminate it altogether. RNase protection assays were performed to test this prediction. We looked at the *xol-1* 2.2-kb message levels in strains with *fox-1* deletion and overexpression. The results show clearly that eliminating *fox-1* leads to increased levels of this transcript, while overexpressing *fox-1* reduces its levels below wild type. While *fox-1(gf)* leads to a substantial reduction in *xol-1* message levels, it does not completely eliminate the 2.2-kb splice form. The exact mechanism by which *fox-1* regulates the levels of *xol-1* 2.2-kb transcript is not known at present, although Nicoll *et al.* (1997), using *xol-1::GFP* reporter transgenes, have shown that the sixth intron of *xol-1* is required for *fox-1* action. The three transcripts of *xol-1* share a common 5′ end up to the sixth intron, where there is a choice of three different splice acceptors (Rhind *et al.* 1995). The most likely role of *fox-1* is to hinder the formation of the 2.2-kb splice form by blocking the splice acceptor specific for this message. *fox-1* does not prevent the formation of the 2.2-kb message completely, because even when *fox-1* is overexpressed, low levels of this splice form are detectable.

The observation that *fox-1* overexpression does not completely eliminate the 2.2-kb *xol-1* message probably accounts for the fact that the effects of *fox-1(gf)* are less strong than *xol-1(lf)* in some of the genetic experiments. *xol-1* is an *XO*-specific gene, responsible for the male mode of development. *fox-1(gf)* effectively mimics the *xol-1 XO* lethal and feminizing phenotype in *XO* animals, suggesting it sufficiently reduces the levels of *xol-1* function for sex determination and dosage compensation. However, *xol-1* also has a minor *XX*-specific role. A weak feminizing effect can be seen in sexually transformed *XX* animals, *e.g.*, *tra-2 XX*s. *tra-2 XX*s develop into nonmating pseudomales, but *tra-2*; *xol-1 XX*s develop into complete males capable of mating (Miller *et al.* 1988). This *XX*-specific effect was exploited in our genetic analysis to investigate the effects of varying the dose of *fox-1* on the

extent of the transformation. Because *fox-1* is predicted to lie upstream of *xol-1* and negatively regulate it, increasing *fox-1* dose is predicted to mimic the effects of *xol-1* loss of function, while removing *fox-1* is predicted to mimic the effects of *xol-1* overexpression. The *xol-1(y9)* allele is a 40-kb deletion that completely removes *xol-1*. *fox-1(gf)* greatly reduces the level of *xol-1*, but the RNase protection results show that a low level of *xol-1* activity remains in *fox-1(gf)* animals. In *XX*s *fox-1(gf)* is able to mimic *xol-1(lf)* only partially. It may be that high levels of *xol-1* are necessary to adequately repress its target, the *sdc*s in *XO*s, and that *fox-1(gf)* reduces those levels enough to bring them below these critical levels. In contrast, the *xol-1 XX*-specific function may require very little *xol-1* and is therefore less sensitive to variations in *xol-1* levels.

Interestingly, *fox-1(gf)*; *tra-2 XX* animals resemble *xol-1*; *tra-2 XX* animals morphologically, but not behaviorally. It seems that *xol-1* influences morphology and behavior at two distinct times in development, L3 and L4, respectively (Rhind *et al.* 1995). The ability of *fox-1(gf)* to phenocopy loss of the L3 function of *xol-1* suggests that perhaps the morphology is less sensitive to low levels of *xol-1* than behavior, so that even small amounts of *xol-1* can prevent *fox-1(gf)*; *tra-2 XX* males from developing mating behavior.

An indication of an additional late *fox-1* function came from a comparison of male mating efficiencies between *fox-1(Δ)* and wild type. *fox-1(Δ)* males consistently sired fewer progeny. This function of *fox-1* appears to be *xol-1* independent, because *fox-1(Δ) xol-1*; *tra-2* males sire significantly fewer progeny than *xol-1*; *tra-2*. Therefore *fox-1* must have another target or targets, whose nature is yet unknown. The existence of a late function is further supported by the results of our expression studies. We found a very restricted expression pattern of *fox-1::lacZ* transgenes, which we suggest is neuronal. Western analysis of staged animals also shows clearly that FOX-1 protein is present throughout the life of the animal. Multiple forms of the protein are observed throughout development. The two different transcripts of *fox-1* could account for a subset of the protein products observed. In fact, animals that express only the shorter SL1-spliced transcript produce only the 44- and 45-kD forms of FOX-1 as detected on Western blots. The presence of two different-sized products from one transcript suggests there may be some form of post-translational modification of the protein that may be involved in its activity. The significance of the two different starts is unclear as the additional 39 amino acids found in the longer ORF have no obviously remarkable features. The multiple forms of the protein raise the possibility of division of labor, with some forms being involved in early function and others responsible for the late effects. All four forms are observed in L4/adult populations of males so none of the forms can be ruled out for providing the late activity. The predominance of the 45-kD form of FOX-1 in the embryo may indicate that it is this form that is responsible for the early functions in sex determination or it may simply reflect an increased stability or more efficient processing of the shorter SL1-spliced transcript. Evidence suggests that both the long and short transcripts of *fox-1* are sufficient to cause *XO* lethality when overexpressed in males (data not shown).

Defects in mating efficiency can result from a lack of interest in hermaphrodites or from a reduced sperm count. We suggest that *fox-1(Δ) XO* male mating deficiency has a behavioral cause, because rare males manage to sire almost-wild-type numbers of progeny. We suggest that the reduced male mating efficiency and the smaller brood sizes, which reveal the late function of *fox-1*, represent its ancestral role. The finding of a surprisingly conserved human homologue (68% identical within the RRM domain; J. Collins, unpublished results, EMBL accession number AL009266) further suggests the involvement of *fox-1* in a process other than sex determination. Such high homology is unlikely to be fortuitous, especially in view of well-documented rapid divergence of sex-determining genes (Whitfield *et al.* 1993; De Bono and Hodgkin 1996; Kuwabara 1996).

The uncovering of *fox-1* involvement in male mating behavior forms an interesting parallel with the Drosophila system in which many numerator elements are also involved in aspects of neural development.

The genetic and molecular data presented here strongly argue in favor of a model in which *fox-1* is one of a small number of numerator elements whose function is to downregulate *xol-1* at the post-transcriptional level. Due to a redundancy among the elements, removing *fox-1* in *XX* animals results in normal development. The loss of one numerator is compensated for by the others. However, adding multiple copies of *fox-1* in the *XO* animals disturbs the balance beyond compensation. *fox-1* overexpression is capable of downregulating *xol-1* sufficiently in *XO* animals to result in their death and feminization. However, it is not able to eliminate completely the *XX*-specific *xol-1*-dependent feminization. We demonstrate that altering the levels of *fox-1* results in reciprocal changes in the levels of the functional *xol-1* message, so that removing *fox-1* leads to increased levels of *xol-1*, while *fox-1* overexpression leads to a reduction, but not complete elimination, of *xol-1*. We suggest that this reduction in *xol-1* levels is sufficient to cause strong *XO* lethality and feminization, but is insufficient to mimic the effects of *xol-1* loss of function in *XX*s.

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