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 XXs, but can rescue X0-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 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; Parhurst 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 Xs 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 Xs (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 X0-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, feminizing on 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.
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 animals and its main function is to specify male development. Therefore a high X:A ratio is predicted to downregulate xol-1 in XXs, while a low X:A ratio permits its expression in XOs. The X0-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°C (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(e114), foa-1(h1c15); LG II, bli-2(e268), tra-2(e095, q276); LG III, unc-32(e189), tra-1(e1099, e076); LG IV, him-8(e489), unc-5(e363), dpy-9(e2), tra-3(e1767), fem-1(h1c17s); LG V, dpy-11(e224), unc-51(e369), her-1(y101s); LG X, lon-2(e768), dhd-1(y9), dpy-3(e27), unc-1(e358dm, e19), unc-2(e55), dpy-18(e11). Duplications, dP26 (X;X), dP313 (X;X); deficiencies, dM66 X.

Detection of Tc1 insertions: All procedures were performed as described in Zwaal et al. (1993), except that the first and second PCR series were increased from 30 to 35 cycles. The following primers were used: marker, A and B (et al. 1993), and the second PCR series were increased from 30 to 35 cycles. F1 primers used were gene specific and flanked by 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 (5W) buffer (50 mm KCl, 10 mm Tris-HCl pH 8.3, 2.5 mm MgCl2, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin) with proteinase K 100 μg/ml, at 65°C. 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: right 1 (GCTGATCGTCTGATCGCGTC) right 2 (GATTTTGTGAACACTGTGGTGAAG) left 1 (GTGTTCCAGCCAGCTCAATAGGC) left 2 (TCAAGTGCGAGTTGATCAGC).

Once the positive address was identified, a corresponding pool of worms was thawed and worms were single 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 MgCl2, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin) with proteinase K 100 μg/ml, at 65°C. 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:

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 vec-
XX males were mated with fox-1(Δ) unc-2; tra-2(q776)/+ hermaphrodites. F1 dpy-3/fox-1(Δ) unc-2; tra-2(q776) XX males were mated with dpy-3 unc-2 hermaphrodites. Rare non-Dpy and non-unc F1 hermaphrodites were isolated. They must have been of one of the following genotypes: fox-1(Δ)/dpy-3 unc-2; tra-2(q776)/+ or /+dpy-3 unc-2; tra-2(q776)/+. 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°C with four young fem-1(ncl-175) females (raised at 25°C) 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 EcoRI-PstI 5.5-kb fragment into the pPD89.20 lacZ vector. CB#1504 was made by subcloning a XhoI-PstI 4.5-kb fragment into the pPD89.20 lacZ vector. The plasmids were co-injected with a RF4, 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(Δ); 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 Flaming 1995). 32P-labeled RNA probes for RPA were made using a MaxiScript kit (Ambion, 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 described by Anderson (1989). Amplification of the region corresponding to the 287-bp probe was achieved by PCR using the overlap extension procedure (Higuchi et al. 1988). Amplification of the region downstream of the 287-bp probe was achieved with primer C 5′-CCACCTGGAGACAGCGACT3′ (NC6 site underlined) and primer D 5′-GTGTTGCAAATATAGAGCCTTACGAGCTAGTTAC3′ (PstI site underlined and amplified). The plasmids were co-injected with a PstI and subcloned into pBlueScript SK+ (Stratagene). The following PCR primers were used: 22MS1 (TAGCTATTGCTACTGAAT with primer A 5′-CAAGG) 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 (Ambion). Hybridization was performed at 45°C for 14 hr. In each reaction 20 µg of total RNA, 1 × 105 cpm of act-1 probe, and 1 × 105 cpm of xol-1-specific probe were used. Results were visualized on a polycrylamide gel, which was subsequently exposed to film over several days. RPA results were scanned using a Densitometer and analyzed using ImageQuant (Aladdin Systems).

**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 described in Zarkower and Hodgkin (1992).

**Construction of the fox-1 genomic construct expressing only the second exon start:** A 16-kb KpnI-Stul genomic fragment containing fox-1 and its 5′ and 3′ flanking regions was subcloned from cosmid R0483 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 et al. (1990). Amplification of a region upstream of the first exon was performed with primer A 5′-GCGCATGCCGACCTTACGCCCTCC-3′ (NcoI site underlined) and primer B 5′-TGAATTCCTCAATTTGGGTCTTCATTTTTTTCTTTTGAATTTTGGAACAGCTGATTTCTTTTTTTTTT-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 NcoI and PstI, and subcloned into PRS314 (Sikorski and Hieter 1989). After sequencing confirmed the correct introduction of the deletion, the NcoI-PstI 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
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). 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 of 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
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. 

### Figure 3.

In vitro RNA-binding analysis of fox-1. 

**A** 35S 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 protein analyzed by 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.

### 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 (ds26) 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 transcription activity between the two regions.

No phenotype was detected in the XX or X0 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 XXs is distinct from that in XOs.
is a biological null, then males carrying fox-1(eDp26) expression of lethal and feminizing duplication that increases the XO ratio. A null mutation, we tested the ability of no phenotype. To establish if the deletion represented no phenotype at the sequence level, and yet fox-1(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 Xs 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 material and methods) and 50 F2 populations were examined for each strain. In all strains tested fox-1(Δ) was compatible with normal XX hermaphrodite development.
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 incompletely nonmating males (Hodgkin and Brenner 1977). A complete transformation toward a male fate can be achieved when the wild-type function of fox-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) and 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 X0 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 1**

<table>
<thead>
<tr>
<th>Genotype of mother</th>
<th>No. of broods scored</th>
<th>No. of individuals counted</th>
<th>No. of wt F1 males/ no. of wt F1 hermaphrodites × 100</th>
<th>No. of Dpy F1 males/ no. of wt F1 hermaphrodites × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpy-3; yDp13</td>
<td>3</td>
<td>199</td>
<td>4% (2-5%)</td>
<td>NA</td>
</tr>
<tr>
<td>dpy-3</td>
<td>3</td>
<td>1375</td>
<td>NA</td>
<td>101% (96-104%)</td>
</tr>
<tr>
<td>fox-1(Δ) dpy-3; yDp13</td>
<td>12</td>
<td>827</td>
<td>100% (78-177%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable.

**Table 2**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>tra-2(e1095) XX</td>
<td>Nonmating pseudomales</td>
</tr>
<tr>
<td>tra-2(q76) XX</td>
<td>Mating males</td>
</tr>
<tr>
<td>tra-3(e1767) XX</td>
<td>Nonmating pseudomales</td>
</tr>
<tr>
<td>tra-1(e099) XX</td>
<td>Mating males (gonad often defective)</td>
</tr>
<tr>
<td>tra-1(e076) XX</td>
<td>Weak masculinization</td>
</tr>
<tr>
<td>tra-2(e1095); xol-1(y9) XX</td>
<td>Many mating males</td>
</tr>
<tr>
<td>tra-2(q76); xol-1(y9) XX</td>
<td>Many mating males</td>
</tr>
<tr>
<td>tra-3(e1767); xol-1(y9) XX</td>
<td>Many mating males</td>
</tr>
<tr>
<td>tra-1(e099); xol-1(y9) XX</td>
<td>Mating males (gonad often defective)</td>
</tr>
<tr>
<td>tra-1(e076); xol-1(y9) XX</td>
<td>Weak masculinization</td>
</tr>
</tbody>
</table>

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.
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 XXs. Despite their almost wild-type morphology, they do not show mating behavior. The alteration in brood profile in fox-1(gf); her-1 XXs (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 strong 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 X0-specific lethality, but not sufficient to remove feminizing effects of xol-1 in XX animals. It is possible that X0 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, 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>X chromosome complement</th>
<th>Progeny sired</th>
</tr>
</thead>
<tbody>
<tr>
<td>fox-1(Δ)</td>
<td>X0</td>
<td>437.3 ± 256.4 (99-947), N = 11</td>
</tr>
<tr>
<td>fox-1(Δ) outcrossed</td>
<td>X0</td>
<td>455.45 ± 205.9 (238-949), N = 11</td>
</tr>
<tr>
<td>N2</td>
<td>X0</td>
<td>1046.6 ± 153.24 (820-1294), N = 5</td>
</tr>
<tr>
<td>fox-1(Δ); tra-2(e1095) xol-1(y9)</td>
<td>XX</td>
<td>327.2 ± 146.7 (122-761), N = 19</td>
</tr>
<tr>
<td>xol-1(y9); tra-2(e1095)</td>
<td>XX</td>
<td>761 ± 124.2 (557-975), N = 10</td>
</tr>
<tr>
<td>fox-1(Δ); tra-2(q276)</td>
<td>XX</td>
<td>2 ± 0.6 (0-2); N = 10</td>
</tr>
<tr>
<td>tra-2(q276)</td>
<td>XX</td>
<td>181 ± 254 (0-728); N = 9</td>
</tr>
</tbody>
</table>

In the 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 strains than in XX; 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 X0-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) X0 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>fox-1(Δ)</td>
<td>66% Egl hermaphrodites, 34% intersexes and pseudomales female soma, male gonad</td>
</tr>
<tr>
<td>tra-2(e1095); xol-1(y9) XX</td>
<td>92% intersexes and pseudomales, 8% mating males</td>
</tr>
</tbody>
</table>

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 X0-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 X0-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 X0 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 X0 animals, such that overexpression in X0 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 X0s, 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 XXs, 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 X0s (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 posttranscriptional 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 XXs, it is important that XOL-1 levels are high in the X0s, 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(If ) in some of the genetic experiments. xol-1 is an X0-specific gene, responsible for the male mode of development. fox-1(gf ) effectively mimics the xol-1 X0 lethal and feminizing phenotype in X0 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 XXs, tra-2 XXs develop into nonmating pseudomales, but tra-2; xol-1 XXs 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 sdcs in XOs, 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 X0 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) X0 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 (Whiteleld 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 X0 animals disturbs the balance beyond compensation. fox-1 overexpression is capable of downregulating xol-1 sufficiently in X0 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 X0 lethality and feminization, but is insufficient to mimic the effects of xol-1 loss of function in XXs.

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LITERATURE CITED


Cline, T. W., 1988 Evidence that sisters a and sisters b are two of several discrete “enumerator elements” of the X/A sex determination signal in Drosophila that switch Sxl between two alternative stable expression states. Genetics 119: 829–862.


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