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The *C. elegans* Sex-determining Gene *fem-2* Encodes a Putative Protein Phosphatase

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The genetic and molecular analysis of genes involved in the regulation of sex determination in *Caenorhabditis elegans* suggests that the gene *fem-2* plays an important role in regulating a pathway transducing a non-cell–autonomous signal to a nuclear transcription factor. The wild-type *fem-2* gene was cloned by identifying sequences from the *C. elegans* physical map that could restore normal Fem-2 function to homozygous mutant *fem-2* transgenic animals. cDNA sequences mapping to the minimal rescuing region correspond to an open reading frame with a sequence similar to protein phosphatase 2C enzymes from systems as diverse as yeast, humans, and plants, but the alignments suggest that FEM-2 falls into a separate class of proteins than the canonical homologues. Several *fem-2* mutant alleles were sequenced, and the mutations are predicted to cause protein changes consistent with their observed phenotypes, such as missense mutations in conditional alleles, and a nonsense mutation in a predicted null allele. This is the first evidence implicating phosphorylation and/or dephosphorylation as a control mechanism in *C. elegans* sex determination

INTRODUCTION

The primary sex-determining signal in the nematode *Caenorhabditis elegans* is the ratio of sex chromosomes (**X**) to autosomes (**A**) (Nigon, 1951). Males have a single **X** chromosome per diploid cell (**X/A** ratio of 0.5) while hermaphrodites have two (**X/A** ratio of 1.0). The latter may be thought of as females that express a transient male phase (spermatogenesis) in their germ line before switching to the exclusive production of oocytes. Mutations have been isolated in *C. elegans* that cause animals to ignore the **X/A** ratio. Loss-of-function mutations in the *fem-1*, -2, or -3 (feminization) genes cause XX and X0 animals to develop as females (the mutant hermaphrodites lose the ability to make sperm), while loss-of-function mutations in any of the three *tra* (transformer) genes cause XX animals to be masculinized. A genetic epistasis pathway has been proposed by Hodgkin using these, and other mutants, in which somatic sex determination in *C. elegans* is controlled by a hierarchy of negative regulatory interactions (Hodgkin, 1980, 1987), culminating in the gene *tra-1* (Figure 1). The genetic regulation of germline sex determination in *C. elegans* involves these, as well as germline specific genes (reviewed by Clifford *et al.*, 1994). Several genes linking the **X/A** ratio with both dosage compensation and sex determination have also been characterized (Villeneuve and Meyer, 1987; Miller *et al.*, 1988; Nusbaum and Meyer, 1989; DeLong *et al.*, 1993).

The *fem* genes have a central role in two decisions during development of *C. elegans*: whether to develop somatically as a male or female, and whether to make sperm or oocytes in the germ line (Kimble *et al.*, 1984; Hodgkin, 1986). Homozygous mutant alleles of the *fem* genes result in feminization of the animal, regardless of the karyotypic sex, implying that the wild-type function is to promote male development. Genetic evidence suggests that the *fems* act both as positive regulators of male germ cell development and as negative regulators of *tra-1*, a gene that promotes female somatic development. The *fem* gene products must be active in the male germ line and soma, and transiently active in the XX (hermaphrodite) germ line. The *fem* genes have been shown to be under different temporal control in the germ line and soma (Doniach and

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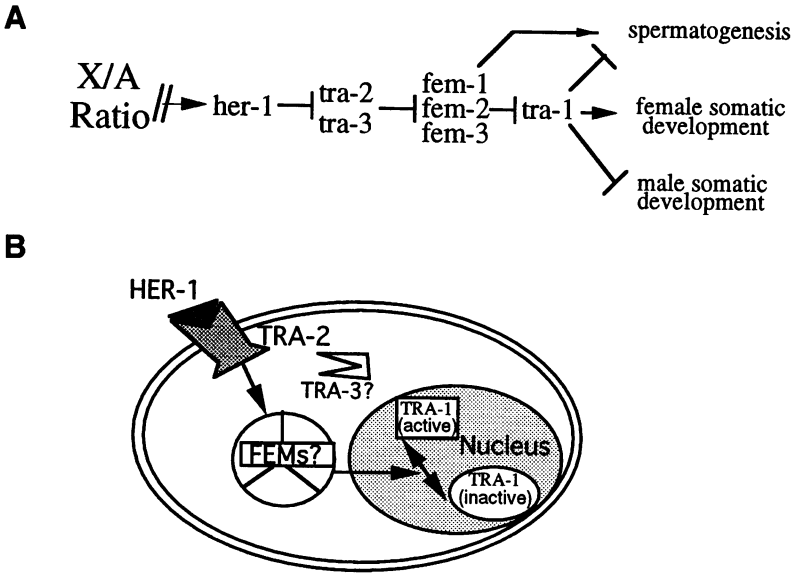

Figure 1. (A) Model of genetic interactions between the genes involved in somatic and germline sex determination in *C. elegans*. Only the genes downstream of *her-1* are illustrated, and genes affecting only the sex determination of the germ line (e.g. *fog* and *mog-1* genes [Schedl and Kimble, 1988; Graham and Kimble, 1993; Evans *et al.*, 1992]) have been omitted. Arrows indicate positive interactions, and bars indicate negative or repressive interactions. Adapted from Hodgkin, 1992. (B) Model for molecular interactions in the determination of somatic sex in *C. elegans*. Only gene products corresponding to the model in panel A are illustrated. See text for details. Adapted from Kuwabara and Kimble, 1992.

Hodgkin, 1984; Kimble *et al.*, 1984; Hodgkin, 1986), presumably due to negative regulation by the genes *tra-2* and *tra-3*. The activities of *tra-2* and *tra-3* are in turn repressed by *her-1*. Several of the genes in the pathway, including *fem-2*, demonstrate maternal rescue of the mutant phenotype (Doniach and Hodgkin, 1984; Kimble *et al.*, 1984), suggesting that either the mRNA or gene product is inherited through the female germ line. Because the *fem* genes act to promote male development, there must be control against inappropriate activity in XX animals. In contrast to *Drosophila*, where control of sex determination occurs at the level of mRNA splicing (reviewed in Hodgkin, 1990), evidence from *C. elegans* suggests control at both the transcriptional and translational levels (Doniach and Hodgkin, 1984; Rosenquist and Kimble, 1988; Evans *et al.*, 1992).

The genetic epistasis together with the molecular analyses of the genes have suggested that the cellular regulation of somatic sex determination in *C. elegans* involves a signal transduction pathway (Figure 1; reviewed by Kuwabara and Kimble, 1992) with control mechanisms that differ from other sex determination systems (Hodgkin, 1992). The *tra-1* and *-2*, *fem-1* and *-3*, and *her-1* genes have been cloned and characterized. HER-1 is predicted to be a secreted protein that acts non-cell autonomously (Hunter and Wood, 1992; Perry *et al.*, 1993). TRA-2 has the characteristics of a transmembrane receptor, possibly for HER-1 (Kuwabara *et al.*, 1992). The FEM-1 protein contains an ankyrin motif, suggesting that it is involved in protein-protein interaction (Spence *et al.*, 1990), whereas the predicted FEM-3 sequence is novel (Ahringer *et al.*, 1992). TRA-1 contains a zinc-finger DNA-binding domain and is probably a transcriptional regulator (Zarkower and Hodgkin, 1992). A complete molecular description of the pathway awaits characterization of the *fem-2* gene product.

Alignment of the physical and genetic maps of *C. elegans* on the left end of Linkage Group III (Pilgrim, 1993) predicted the physical position of the *fem-2* gene. In this work, we describe how this has permitted transformation rescue of the *fem-2* mutant phenotype using clones selected from this genomic region. The DNA sequence of the rescuing region predicts a single open reading frame (ORF). It is likely that this sequence encodes *fem-2*, because mutant *fem-2* alleles show single basepair (bp) changes consistent with a defect in the predicted FEM-2 protein. This protein shows sequence similarity to several proteins involved in the regulation of signal transduction pathways. The two proteins most similar to FEM-2 are ABI1, a protein phosphatase from plants involved in mediating hormone response, and a protein of unknown function from humans. The sequence similarities suggest that FEM-2 may have protein phosphatase activity and that *C. elegans* sex determination may be regulated by a kinase/phosphatase cascade.

MATERIALS AND METHODS

Strains and Genetics

Nematode stocks were maintained and handled as described by Wood (1988). The strains used (Table 1) were maintained at 20°C, except where indicated. The isolation and characterization of some of the *fem-2* alleles used in this work are described by Kimble *et al.* (1984) and Hodgkin (1986). XO animals were isolated from strains carrying the *him-8* mutation, which produces a high incidence of males due to X-chromosome nondisjunction (Hodgkin and Brenner, 1977; Broverman and Meneely, 1994). Two putative deficiencies (Df) for the *fem-2* region have been generously shared in advance of

Table 1. *C. elegans* strains used in this work

Strain genotype	Source
fem-2(b245ts)	J. Hodgkin
fem-2(q117ts)	J. Kimble
fem-2(b245ts); him-8(e1489)	J. Hodgkin
fem-2(b245e2005); tra-3(e1767)	J. Hodgkin[a]
unc-45(r450ts) sDf124(s2670) + sC1(s2073)[+ +dpy-1(s2171)]	H. Stewart and D. Baillie
wcDf1(wc5)+ +dpy-1(e1)	L. Venolia
+ daf-7(e1372) par-2(e2030) + unc-45(r450ts) sDf124(s2670) +; him-8(e1489)	This laboratory
sC1(s2073)[+ +dpy-1(s2171)] him-8(e1489) fem-2(e2105) + ; him-8(e1489)	This laboratory[a]
sC1(s2073)[+ dpy-1(s2171)] him-8(e1489) fem-2(e210n) +	n = 1 to 6
sC1(s2073)[+ dpy-1(s2171)]	This laboratory[a]

[a]The isolation of b245e2005 and the e210n series of alleles are described in Hodgkin, 1986.

publication by H. Stewart and D. Baillie (*sDf124*) and L. Venolia [*wcDf1*].

XO animals hemizygous for *fem-2* mutant alleles were generated as follows: *fem/+* males crossed to *fem/Df* females, or *Df/+* males crossed to *fem/fem* females. No difference was seen in the phenotype of the XO animals prepared by these two methods. Homozygous *fem/fem* XO animals were produced among the self progeny of *fem-2(e2105); him-8(e1489)* maternally rescued hermaphrodites. The progeny were raised at 15, 20, or 25°C.

Nematode Transformation

Adult hermaphrodites, homozygous for *fem-2(b245ts)*, were raised at 20°C and were transformed with cloned DNA, using microinjection as described by Mello *et al.* (1991). The animals were injected with a mixture of the pRF4 plasmid, containing a dominant allele of the *rol-6* gene, *su1006dm* (Mello *et al.*, 1991) and the test DNA. The pRF4 plasmid causes the animals to adopt a dominant characteristic rolling motion on the plates, and serves as a positive control for transformation. Following injection, the hermaphrodites were incubated at the restrictive temperature of 25°C, unless indicated. Progeny from the injected animals were raised at 25°C, and examined 3 to 4 days later, for the Rol-6 phenotype, as well as for self-fertility. Uninjected *fem-2(b245ts)* animals, or animals injected with pRF4 alone produce an F1 brood at 25°C consisting exclusively of self-sterile females. In some experiments, the injected hermaphrodite was kept at 20°C, and lines that transmit the transgene were selected by following the Rol-6 phenotype alone. The transgenic lines were subsequently tested for rescue of the *fem-2(b245)* XX phenotype at 25°C. The number of transgenic animals scored varied from injection to injection and clone to clone, but at least two independent transmitting lines were assayed for each of the rescuing clones described in the text. For the clones that are classed as "failure to rescue," three to ten F1 Rol-6 animals were examined at the restrictive temperature. Only representative clones are discussed in the text. In addition to these, other clones have also been tested for their ability to rescue the *fem-2* mutant phenotype. The only clones that have shown rescue are those that include at least the minimal rescuing region.

Once the minimum rescuing region had been defined by the suppression of the *b245ts* XX germline defect, the DP#DBP026 and DP#DBP141 plasmids were tested for their ability to rescue the XX germline and XO germline and soma defects at 20 and 25°C. Heterozygous animals of the strain *fem-2(e2105) +/sC1[+ dpy-1(s2171)]; him-8(e1489)* were injected with the test and *rol-6* plasmids as described above. Transmitting lines were established at 20°C. Homozygous *fem-2(e2105); him-8* animals were selected from the progeny, and these were shown to produce both self-fertile XX animals and somatically normal males (XO) at 25°C. XO mating ability was scored by placing several Rol-6 XO animals (which had been raised at 25°C) on a plate with four *unc-45(r450ts)* hermaphrodites, and screening the F1 brood for non-Unc rolling males. (Several males were used because the extrachromosomal arrays are not completely meiotically or mitotically stable, and can produce mosaic progeny, and also because of the poor mating efficiency of *rol-6* males). For each of the plasmids, two independent matings produced outcross progeny, indicating that at least some of the *fem-2(e2105)* XO animals were completely rescued by the transgenes at 25°C.

DNA Analysis

DNA manipulation was as described in Pilgrim (1993) except where otherwise stated. DNA fragments were subcloned from cosmid and lambda genomic clones into pBluescript (Stratagene, La Jolla, CA). All clones were sequenced using a Sequenase v2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH) as described by the manufacturer.

PCR Analysis

The presence of the test DNA in some, but not all, transgenic lines was assayed using DNA amplification by the polymerase chain reaction (PCR). Single worm PCR analysis was performed as described by Williams *et al.* (1992) and Pilgrim and Bell (1993). Confirmation of the 5′ end of the *fem-2* transcript used the rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988) with a Life Technologies (Gaithersburg, MD) 5′ RACE kit, following the directions of the manufacturer. The primer DHA9 (Table 2) was used for first strand synthesis, and AMC1 was used for PCR amplification. The RACE product was cloned into the vector pGEM-T (Promega, Madison, WI) before sequencing.

The deletion of at least part of the *fem-2* locus by the deficiency *sDf124* was noticed by D. Collins and H. Stewart (personal communication) and confirmed in our lab. Heterozygous *sDf124/+* hermaphrodites were placed on a plate and allowed to lay eggs for 24 h at 20°C. The adults were removed, and after a further 24 h, unhatched embryos were picked for PCR analysis. Embryos were picked from wild-type worms as a control. DNA from single embryos was prepared for PCR as described (Williams *et al.*, 1992). Primers DHA4, DHA17, DHA19, and SAD2 (Table 2) were used to test for DNA at the *fem-2* locus, while primers MMA1 and MMA2, which amplify DNA near the *unc-119* locus on the right end of Linkage group III (M. Maduro and D.P., unpublished data) were used as positive controls. A similar analysis was performed for the *wcDf1* deficiency.

DNA from the mutant alleles of *fem-2* was amplified as follows: for *b245ts* and *q117ts*, homozygous strains were grown at 20°C, and genomic DNA was isolated as described in Pilgrim (1993). For *e2103* and *e2105*, individual L4 hermaphrodites (either *fem/+* or *fem/fem*, which are indistinguishable) from a *fem-2/+* mother were set up on a plate at 20°C and allowed to produce an F1 brood. Six animals were selected from broods that consisted entirely of female adults (*fem-2/fem-2*), combined in one tube, and prepared as for the single worm PCR method described above. A portion of the DNA was amplified by PCR using the primers DHA3 and DHA4, and cloned into the pGEM-T (Promega) vector. For each mutant allele, clones from at least three independent PCR reactions were sequenced. For *e2101*, *e2102*, *e2104*, *e2106*, and *b245e2005*, genomic DNA was am-

Table 2. PCR primers used in this work

Primer	Sequence (5′ to 3′)	Position in fem-2 sequence[a]
AMC1	ACCTCGACGTCATAGCTAGT	464
DHA3	CAAAGATCTTGTCCCACCGAAGCCGGTAGTGG	278
DHA4	CGGTCTAGATGACGACGATATGGTGGATAG	2632
DHA9	CGTCTAGAAGCTCCAGCGAGCTTGCGAAGC	1524
DHA17	CCGAGCACTCGGAGATGTTC	1837
DHA19	CGATGGTCACGGTGGTCACGAG	1437
SAD2	AGAAATTCCATCACAAGCCAG	1912
MMA1	AGTCGGCCTTATTGTGCATTAC	n/a
MMA2	AAATTGCATGCCAGCACCGGTC	n/a

[a]Number refers to Figure 3, and most 3′ base in primer.

plified by PCR using the primers described above, and the PCR product was directly used as the template for sequencing.

cDNA Isolation and Analysis

A cDNA library, prepared from mixed stage *C. elegans* RNA and cloned into the λZap vector (Barstead and Waterston, 1989) was screened with a genomic DNA probe (15-kbp *Xba*I fragment containing sequences shown in Figure 2). From approximately 10^5 recombinant phage screened, 43 positive plaques were isolated. Subsequent characterization of the clones suggested that one-third came from within the 15-kb genomic region, but from outside the minimal rescuing region. Ten cDNA clones that fell entirely within the rescuing region were sequenced. One of the cDNA clones was chimeric, having been ligated to a second, unrelated cDNA. cDNA clones were excised in vivo to produce pBluescript SK(−) phagemid containing the cloned insert as described by the supplier (Stratagene).

Construction of Minigene

A partial minigene was constructed by ligating a 1.5-kb *Sal*I DNA fragment from a cDNA (containing part of exon 2 to the 3′ end of the cDNA, including the poly(A) tail and a portion of the polylinker) into a Bluescript plasmid containing 2.5-kb of upstream genomic sequence (*Xho*I–*Sal*I fragment) as well as exon 1, the first 56-bp intron, and part of exon 2. The cDNA fragment was cloned into both the sense and antisense orientations. The ability of the minigene to rescue the *fem-2(b245ts)* and *fem-2(e2105)* mutant phenotypes was tested as described above.

RNA Isolation and Characterization

Nematodes from N2 (XX) or *him-8(e1489)* (XX and XO) strains were grown in liquid cultures (mixed-stage or synchronized cultures) and purified according to Wood *et al.* (1988). Nematode pellets were then flash frozen in liquid nitrogen and stored at −80°C. RNA was isolated using glass bead homogenization as described by Hope (1994) and Johnson, Simpson, and Pilgrim (unpublished data). For embryos, RNA was prepared by grinding the frozen pellets with a mortar and pestle in liquid nitrogen. Northern analysis was performed using 1% agarose gels containing formaldehyde as described (Sambrook *et al.*, 1989). Radiolabelled antisense RNA probes were synthesized using T7 or T3 RNA polymerases as directed by the manufacturer (Promega), using a linearized cDNA clone as a template.

RESULTS

An alignment of the physical and genetic maps for the left end of *C. elegans* Linkage Group III (Pilgrim, 1993) suggested a molecular position for *fem-2*. The *fem-2* mutant alleles mapped between two RFLPs, *eP95* and *eP64*, which is a region of the physical map (approximately 0.2 map units or 50 kbp between the RFLPs; Pilgrim, 1993) that is completely covered by yeast artificial chromosome and cosmid clones from the *C. elegans* physical mapping project (Coulson *et al.*, 1986–

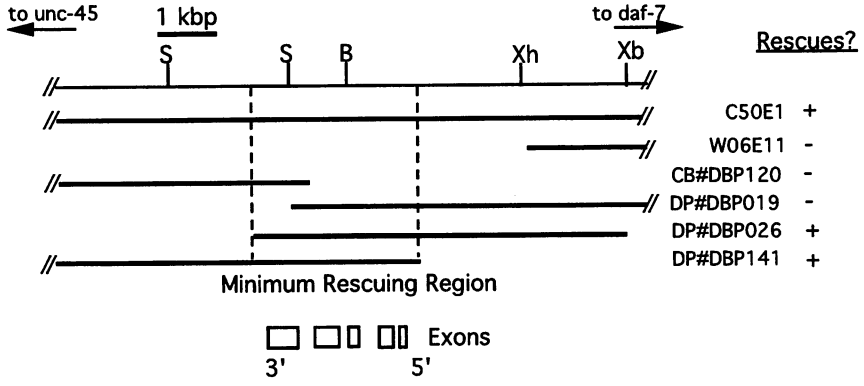

Figure 2. Restriction map of the *fem-2* region, aligned with the genetic map such that the left end of LGIII is to the left. Restriction enzyme recognition sites are shown above the line. Xb = *Xba*I; Xh = *Xho*I; B = *Bam*HI; S = *Sst*I restriction sites. The most informative subclones that have been tested for their ability to rescue the *fem-2* mutant phenotype are diagrammed, and indicated on the right. "+" indicates rescue, and "−" indicates failure to rescue by the criteria described (MATERIALS AND METHODS). The open boxes below the map indicate the positions of exons determined by sequencing the cDNA clones that mapped to the minimal rescuing region.

1991), as well as lambda clones identified by cross-hybridization to the yeast artificial chromosomes (Pilgrim, 1993). Cloned DNA fragments from this region of the physical map were tested for their ability to rescue the *fem-2* mutant phenotype, following germline transformation. The easiest *fem-2(b245ts)* mutant phenotype to score is the self-sterility of XX animals (females) at the restrictive temperature, due to their lack of spermatogenesis. Rescue was assayed by the restoration of self-fertility to homozygous *fem-2(b245ts)* transgenic animals at 25°C.

Three overlapping cosmid clones and one lambda clone were each able to restore spermatogenesis to transgenic animals raised at the nonpermissive temperature (Figure 2), while flanking cosmid and lambda clones failed to rescue. Transmitting lines were established with all of the rescuing clones, and several lines have been stably maintained for over 100 generations. The transgene is thought to be present as a meiotically unstable extrachromosomal array (Mello *et al.*, 1991) that is only passed to a fraction of the progeny. Animals that fail to inherit the array produce sterile progeny at the restrictive temperature. The rescuing region has subsequently been narrowed to 3.0 kbp, and its boundaries are defined by the ends of the subclones DP#DBP026 and DP#DBP141 (Figure 2). Clones containing less than this minimal region of genomic DNA fail to rescue. A subset of the rescuing clones have been successfully tested for XX germline rescue of a second temperature-sensitive allele *q117ts*, and a putative null allele *e2105*, both at 25°C.

The two minimal subclones DP#DBP026 and DP#DBP141 were then tested for their ability to rescue the *fem-2(e2105)* XO somatic and germline feminization at 25°C. At this temperature, *fem-2(e2105)* XO progeny of homozygous mothers develop as fertile females (Hodgkin, 1986). However, if they also carry either of the two plasmids as extrachromosomal arrays, the XO animals are somatically normal males, and are fertile when outcrossed (MATERIALS AND METHODS). Therefore, this confirms that the minimum rescuing region has been defined for both the somatic and germline phenotypes, in XX and XO animals, at all temperatures.

A 15-kbp genomic DNA fragment, which included the minimal rescuing region, was used to screen a mixed-stage cDNA library (Barstead and Waterston, 1989). Ten of the clones that mapped to the minimal rescuing region were characterized. Even though the small size and high A+T content of *C. elegans* introns (Wood *et al.*, 1988) allow the prediction of the position of exons within the genomic sequence; the intron/exon boundaries as well as the 5′ and 3′ ends of the gene were confirmed by sequencing the cDNAs. All clones were consistent with a single splicing pattern (see Figures 2 and 3), and were completely contained within the rescuing region. Both cDNA and genomic DNA sequences are given in Figure 3.

None of the cDNAs selected from the library were full length, although three extended into an apparent nematode spliced leader (SL1) sequence at the 5′ end and, therefore, contained all exons from the *fem-2* locus. The longest of the cDNA clones contained 9 bases at the 5′ end that matched the last bases of the SL1 sequence (Krause and Hirsh, 1987). The 5′ terminal segment was cloned using the PCR-based RACE procedure (Frohman *et al.*, 1988). Sequence analysis of the cloned RACE product confirmed that the SL1 leader is *trans*-spliced onto the *fem-2* mRNA. The 5′ end of the minimal rescuing region lies only 240 bp upstream of the spliced leader acceptor site. Because the transformation rescue assay for *fem-2* function produces multicopy transgenic arrays (Mello *et al.*, 1991), a basal level of transcription from each gene may prove sufficient to rescue, and therefore this small region may not contain all sequences responsible for wild-type *fem-2* transcriptional expression or regulation. The genomic and cDNA sequences are consistent with the presence of a single transcribed species, and a single transcript of the predicted size (1.8 kb) is seen on Northern blots (Figure 4). The developmental profile of the *fem-2* mRNA suggests that it is detectable at all stages of development, but is most abundant during adult development. When RNA prepared from cultures containing one-third XO animals is examined, no difference in the transcript patterns has been detected in embryos or adults (Figure 4 and our unpublished results). If there is a transcript from the *fem-2* locus that is specific to XO animals, it is either of low abundance, or co-migrates with the 1.8-kb transcript.

To further confirm that the cDNAs correspond to the *fem-2* locus, a minigene lacking all introns except the first was tested for its rescuing ability. The chimeric plasmids were constructed by fusing a genomic fragment containing 2.5 kbp of the upstream sequence, exon 1, intron 1, and part of exon 2 to a fragment from a cDNA clone containing the remainder of the exons and the poly(A) tail. In one chimera, the cDNA fragment was inserted in the "sense" orientation relative to the upstream region. In a second plasmid, the cDNA fragment was cloned in the "antisense" orientation. When transgenic lines were generated from the two chimeras, only the clone in the sense orientation was able to rescue the XX phenotype of *fem-2(b245ts)* at 25°C. The same sense clone is able to rescue the XO somatic and germline feminization of the *e2105* allele at 25°C, suggesting that no other transcript is necessary in XO animals. The second intron is almost completely composed of 12 repeats of a degenerate 30- to 35-bp sequence, itself an inverted repeat. Because this intron sequence can be removed with no apparent effect on the gene function, its role, if any, remains obscure.

Figure 3. DNA sequence of the minimal rescuing region of the *fem-2* gene. Exons are shown in capital letters, introns and flanking regions are shown in lower case. Numbers refer to nucleotides of genomic DNA from the beginning of the rescuing region. Predicted translation is given above the sequence, and the predicted initiation codon is underlined. The DNA changes in the *b245*, *q117*, *e2102*, and predicted *e2005* mutant alleles are indicated in boldface above the protein sequence. The *e2005* mutation is inferred from the sequencing of the doubly mutant *b245e2005* allele as described in the text. GenBank accession number for this sequence is U29515.

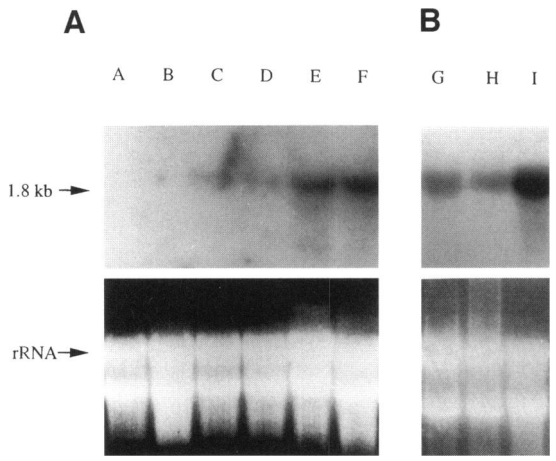

Figure 4. Northern blot of total *C. elegans* RNA. Fifteen micrograms of total RNA, prepared from wild-type hermaphrodite (lanes A-G and I) or *him-8(e1489)* XX and XO (lane H) worms synchronized at each developmental stage (embryo, first to fourth larval [L] stages, and adult), was loaded in each lane. Following electrophoresis and capillary transfer, the blot was hybridized to an antisense riboprobe prepared from a *fem-2* cDNA clone. The top panel shows the resulting autoradiogram. The bottom panel shows the ethidium bromide-stained gel before Northern transfer to show relative loading. Lanes A, G, and H are embryo RNA. Lane B is L1, C is L2, D is L3, E is L4, and F and I are adult RNA. The blot in panel B was hybridized with a probe of higher specific activity than the blot in panel A to allow more sensitive detection of the embryonic mRNA.

The sequence of the cDNAs representing the *fem-2* transcript is shown in Figure 3. Assuming that the first in-frame ATG sequence corresponds to the initiator methionine, the single open reading frame encodes a protein of 449 amino acids. In a BLAST search of the protein sequence databases (Altschul *et al.*, 1990), six separate regions of the potential FEM-2 protein (conserved motifs I-VI in Figure 5) show significant similarity to an uncharacterized ORF from humans, as well as several mammalian and yeast protein phosphatase 2C (PP2C) genes (Figure 5). In addition, the ABI1 gene from the plant *Arabidopsis thaliana*, which is involved in regulation of the plant cell's response to abscisic acid (Leung *et al.*, 1994; Meyer *et al.*, 1994), also shows similarity over the same regions. A second *Arabidopsis* phosphatase, KAPP (Stone *et al.*, 1994), also shows similarity to FEM-2, but at a lower level. When comparing the predicted amino acid sequence of FEM-2 with that of the other proteins over the six motifs, the human ORF is 37% identical and 56% similar over 190 amino acids, rat PP2C is 37% identical and 49% similar over 181 amino acids, and ABI1 is 31% identical and 46% similar over 189 amino acids.

The amino terminal third of the predicted FEM-2 protein showed no significant similarity to any sequence in the database, as judged by the BLAST program. Most of the "classical" PP2C proteins have short (30–40 amino acids) amino terminal regions proximal to motif I. In contrast, FEM-2, ABI1, and the human ORF have much larger amino terminal regions, extending 135–170 residues amino terminal to motif I (Figure 5). The amino terminal domains of the three proteins have no significant sequence alignment with one another, although all are rich in charged amino acids and proline. FEM-2 also shows protein sequence similarity to another predicted *C. elegans* protein, F42G9.1, which corresponds to the first ORF from cosmid clone F42G9 sequenced by the *C. elegans* genome sequencing project (Sulston *et al.*, 1992; Wilson *et al.*, 1994). Cosmid F42G9 has been placed on the physical map at the left end of Linkage Group III, close to *fem-2* on the *eP64* contig, about 100 kbp toward *daf-7* (Pilgrim, 1993). (The F42G9 cosmid is colinear with cosmid F10H6 in Figure 5 of Pilgrim, 1993). The sequence identity between FEM-2 and F42G9.1 is restricted to the conserved motifs, suggesting that the two proteins are distantly related (Figure 5). The predicted F42G9.1 product appears to contain a large in-frame insertion between motifs II and III, relative to all the other homologues. Because the transcript from this region has not been examined, it is possible that this represents a cryptic intron.

Figure 5 shows a cartoon of the alignment of the protein homologues. The proteins fall into three structural classes, based on the following criteria: 1) the presence of an extended amino-terminal domain (Class A); 2) the presence of an "Asp-Trp" dipeptide at the end of motif I (Class B and C); and 3) the presence of an extended carboxy terminus and a conserved acidic 9- or 10-amino acid peptide between motifs IV and V (Class C: ILRAEEDEF in the rat PP2C β isoform, IERSEEDDQF in the human α isoform, and EALTPEDEF in F42G9.1).

Twelve *fem-2* mutant alleles have been isolated and genetically characterized (Kimble *et al.*, 1984; Hodgkin, 1986). Two of the existing alleles are temperature sensitive, and at least four others are thought to have two mutations in the *fem-2* gene (Hodgkin, 1986). All of the putative *e2101* to *e2106* single mutants were isolated *in trans* to a doubly mutated allele *b245e2005* (Hodgkin, 1986). Of these alleles, *e2105* has been proposed as a candidate for a null allele (Hodgkin, 1986). However, because homozygous *fem-2(e2105)* XO animals are completely feminized only at 25°C, the possibility remains that the *e2105* allele retains some low level of Fem-2 activity. The recent isolation of genetic deficiencies for portions of the left end of Linkage Group III (H. Stewart and D. Baillie, personal communication; L. Venolia, personal communication) allowed a more definitive test for a null allele. One of the predicted characteristics of a null allele is that the phenotype of the allele *in trans* to a deficiency for the locus is no more severe than the phenotype of the homozygous

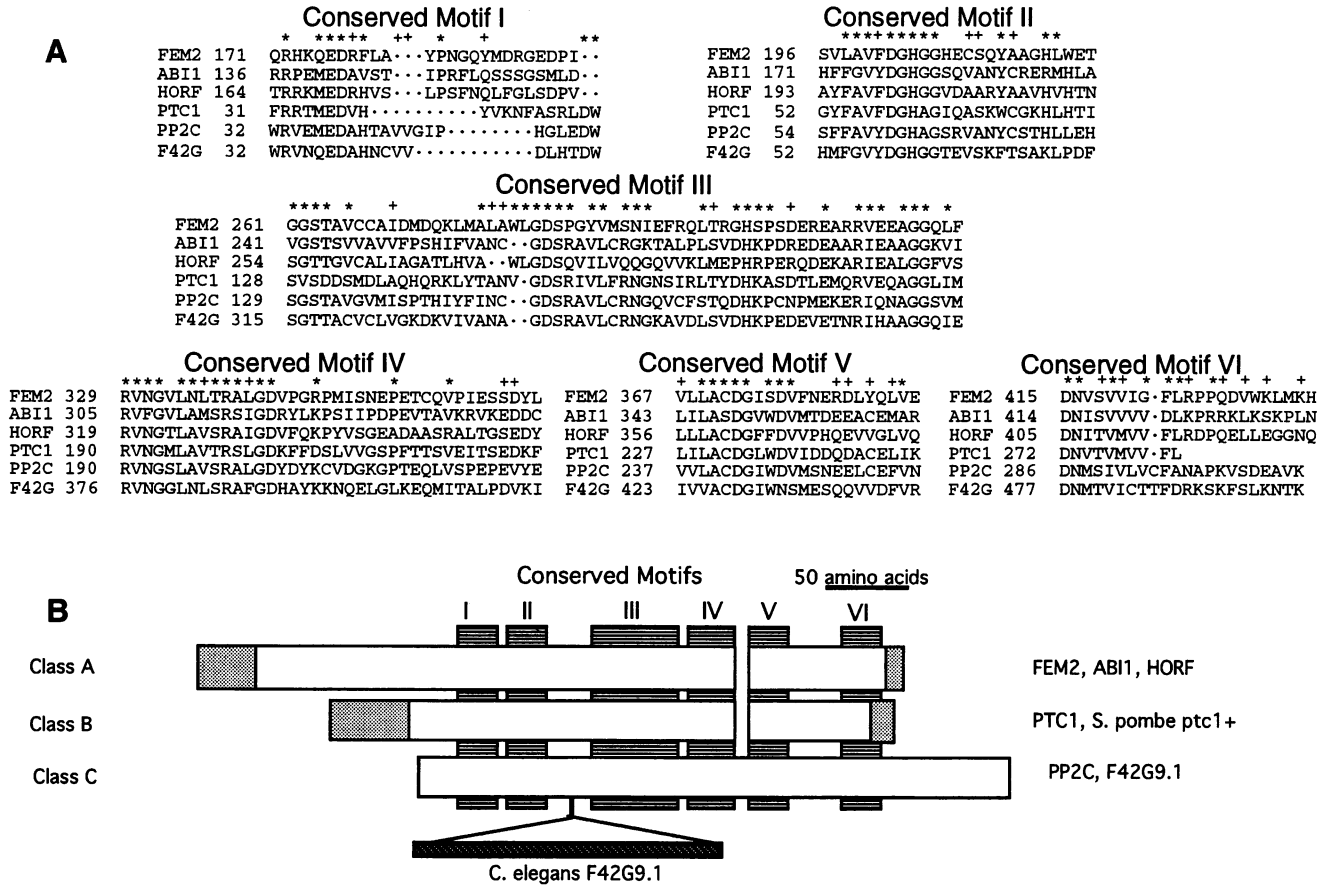

Figure 5. FEM-2 alignment with putative homologues. (A) Alignment of putative homologues to illustrate conserved motifs. Sequences are as follows: FEM2, predicted protein from *fem-2* locus; ABI1, *ABI1* protein from *Arabidopsis thaliana* (Leung *et al.*, 1994; Meyer *et al.*, 1994); HORF, predicted human protein from cDNA sequence (Nomura *et al.*, 1993); PTC1, *Saccharomyces cerevisiae* product of *PTC1* gene (Maeda *et al.*, 1993); PP2C, rat protein phosphatase 2C β isoform (Wenk *et al.*, 1992); F42G, F42G9.1 ORF from *C. elegans* sequencing project (Wilson *et al.*, 1994). * = >60% of proteins have same amino acid; + = >80% of proteins have one of two amino acids; • = gap introduced to maximize alignment. Numbers refer to the residue number at the beginning of the motif. Other mammalian PP2C homologues are similar to the P2CB sequence. Motifs IV and V are contiguous in FEM-2, *ABI1*, HORF, and *PTC1*, but are separated by a 9- to 10-amino acid peptide in the mammalian PP2C homologues and F42G9.1 (ILRAEEDEF in the rat PP2C β isoform, IERSEEDDQF in the human α isoform, and EALTPEDEF in F42G9.1). Sequence accession numbers: *ABI1*, gp X77116; HORF, gp D13640; *PTC1*, sp P35182; PP2C, sp P35815; and F42G9.1, gp U00051. (B) Alignment of proteins to show relative positions of conserved motifs I-VI. Stippled boxes represent position and extent of the conserved motifs. Open boxes represent protein sequences, shaded boxes represent extensions that are not present in all members of a class. The dark hashed box is the predicted extra sequence in the *C. elegans* F42G9.1 protein that lies between motifs II and III. Motif IV and V are colinear in Class A and B proteins, but separated by a 9- to 10-amino acid peptide in Class C proteins. The spacing between motif V and VI varies between the proteins.

null allele. Embryos homozygous for either the *sDf124* or *wcDf1* deficiencies fail to show a PCR product with two different primers sets from the *fem-2* coding region (Figure 6), although primers corresponding to sequences elsewhere in the genome are able to amplify products. Therefore, both deficiencies appear to delete much if not all of the *fem-2* gene.

The *e2105* allele was placed *in trans* with a deficiency, and the phenotype of the resulting animals was examined. In the XO, the requirement for Fem-2 activity is least stringent at lower temperatures (Hodgkin, 1986). At 20°C, many *e2105* XO homozygotes die during development (the animals most often break open due to a weakening in the ventral hypodermis), but a few survive, and they have abnormal gonads, containing oocyte-like cells. These animals also have rudiments of the male tail, with a small fan. Rays are often present (Figure 7; Hodgkin, 1986) and the animals are sterile. At 15°C, the feminization of XO animals is weaker (Figure 7). The gonad has a typically female shape, but only rarely are oocyte-like cells seen. These animals also have projections or bulges in the ventral midbody region, where the vulva would

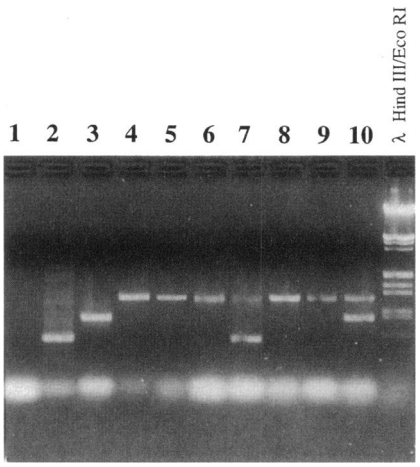

Figure 6. PCR analysis of putative deficiencies for the *fem-2* region. DNA was prepared from embryos that were wild type for *fem-2* (lanes 1–4, 7, and 10), homozygous for *wcDf1* (lanes 5 and 8), or homozygous for *sDf124* (lanes 6 and 9). Three primer sets were used to PCR amplify the DNA. Two sets correspond to *fem-2* (SAD2-DHA19 and DHA4-DHA17) whereas the third set amplifies a region of the unlinked *unc-119* gene (MMA1-MMA2). Lane 1 contains one primer from each of the three primer sets (DHA4, MMA1, and SAD2). Lanes 2–4 show the bands amplified for each of the three primer sets alone on wild-type DNA (SAD2-DHA19, DHA4-DHA17, and MMA1-MMA2, respectively). Lanes 5–7 show amplified products of two primer sets (SAD2-DHA19 and MMA1-MMA2) with *wcDf1*, wild-type, and *sDf124* embryos. Lanes 8–10 show amplified products of the primer sets DHA4-DHA17 and MMA1-MMA2, again with *wcDf1*, wild-type, and *sDf124* embryos. Each lane represents the PCR results from a single embryo.

normally form in a female. The fan and rays in the tail are more wild type. Although the XO phenotype can vary from animal to animal, the range of feminization seen in hemizygous *e2105/Df* XO animals is indistinguishable from *e2105* homozygotes at either temperature (Figure 7). Identical results were seen with *e2105 in trans* to either *sDf124* or *wcDf1*. Because one of the tests for a null allele is that the homozygous and hemizygous phenotypes are identical, this suggests that *e2105* is a putative null mutation for the *fem-2* gene.

The DNA encoding the *fem-2* locus (between nucleotide positions 250 and 2650; Figure 3) was sequenced from eight of the mutant *fem-2* alleles (*b245ts*, *q117ts*, and *e2101–6*), and from two different strains containing wild-type *fem-2* genes. All alleles were reportedly induced using ethylmethane sulfonate (EMS), which causes primarily transition mutations of G-C to A-T bp. Both PCR amplified wild-type alleles showed an identical sequence to that determined from the clones isolated from the genomic and cDNA libraries. All mutant alleles showed base changes when compared with the wild-type sequence. The positions of the mutations in these alleles are shown in Figure 3. The *q117ts* allele contains a single G-C to A-T base change at position 1242, which is predicted to cause the substitution of glutamic acid for glycine. The *b245ts* allele contains a single G-C to A-T change at position 1828, predicted to cause a substitution of arginine for glycine at amino acid 341 in motif IV. This position is absolutely conserved among all homologues shown. The *e2102* allele contains a single G-C to A-T change at position 1262, creating an in-frame stop codon.

The *e2101*, *e2103*, *e2104*, *e2105*, and *e2106* sequences were identical to one another, and unexpectedly showed two base changes from wild type. One change was identical to the *b245* mutation at position 1828. The other was a G-C to A-T transition at position 1466, which creates an in-frame stop codon (Figure 3). These alleles (along with *e2102*) were isolated in a non-complementation screen in a strain containing the *b245e2005* double mutation (Hodgkin, 1986). Although the entire DNA sequence from this allele has not been determined, *b245e2005* also shows the *b245'* change at position 1828, and the "stop" at position 1466. It therefore appears that the *e2101*, *-3*, *-4*, *-5*, and *-6* alleles were not "new" *fem-2* alleles, but recombinational reisolates of *b245e2005*. Regardless of their origin, both the presence of a translational stop codon, and the hemizygous phenotype support the contention that these represent the null phenotype of *fem-2*.

DISCUSSION

The *fem-2* gene product acts in the sex determination hierarchy, as a positive regulator of the male fate (spermatogenesis) in the germ line, and as a negative regulator of the female fate in the soma. If all genes downstream of *tra-2* in the epistatic pathway act in a cell autonomous manner, the interactions in the soma can be modeled as a signal transduction pathway, culminating in the TRA-1 transcriptional regulator protein (Figure 1). The genetic epistasis suggests the following: 1) that the *tra-2* and *tra-3* genes negatively regulate the *fem* genes, and 2) that the *fem* genes negatively regulate the activity of *tra-1*. The activity of all three of the *fem* genes seems to be required for normal male development, but it is not known how, or if, they interact. FEM-2 may play different regulatory roles in the germ line and in the soma. Loss-of-function (lf) mutations in *tra-1* suppress the somatic feminization caused by *fem-2* mutations (Hodgkin, 1986), but Fem-2 activity is still required for spermatogenesis at 25°C (Hodgkin, 1986). *fem-3* gain-of-function (gf) mutations suppress the germline feminization caused by a temperature-sensitive *fem-2* mutation, but do not affect the soma (Barton *et al.*, 1987).

The *fem-2* locus has been cloned based upon two lines of evidence. First, transgenes that contain the intact coding region for the putative FEM-2 protein are necessary and sufficient to rescue the mutant phenotype in both germline and somatic tissues, in both XX

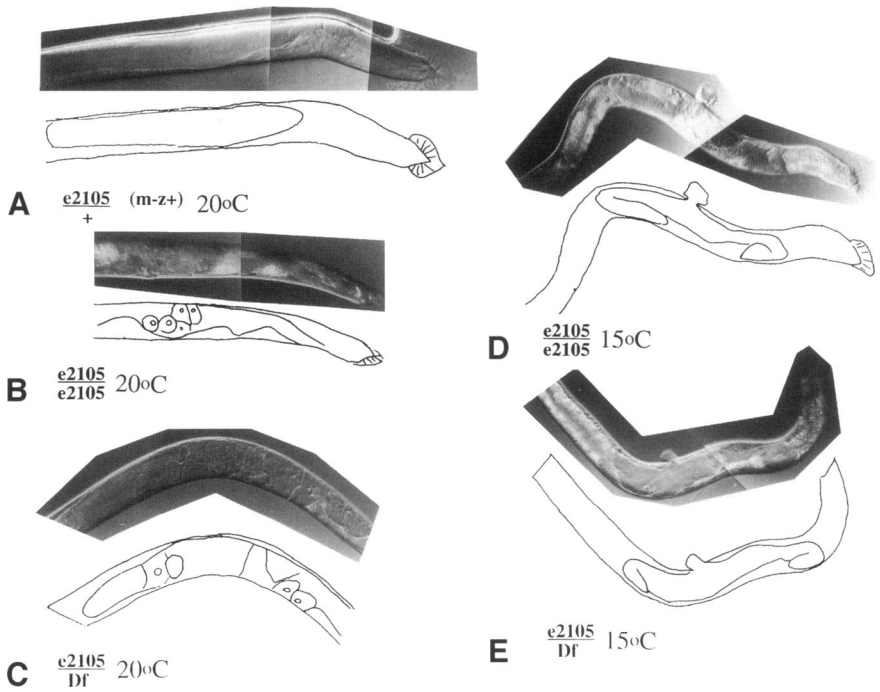

Figure 7. Photographs using differential interference contrast microscopy (top) and cartoons of the photographs (bottom) of the *fem-2(e2105)* heterozygous, homozygous, and hemizygous XO phenotypes at different temperatures. All animals were progeny from *fem/fem* mothers. In panels B and C, there are oocyte-like cells apparent in the gonad. The deficiency used in these examples was *sDf124*, but identical results were seen when *wcDf1* was used instead. The XO phenotype can be quite variable, so the examples shown do not portray all the defects seen in animals of the same genotype. Although the tail phenotype is not very clear from these examples, there were no apparent differences in the ranges of defects seen in the homozygous vs. hemizygous animals.

and XO animals. The minimal rescuing region includes 300 bp 5′ to the predicted translation start codon, and 600 bp 3′ to the predicted translation termination codon. Second, all mutant alleles of *fem-2* that were examined contain base changes within the predicted coding region, compared with two different wild-type alleles as well as to cDNA and genomic DNA clones from the physical mapping project. The base changes are predicted to result in alterations of amino acid sequence in all cases, and are consistent with the mutations resulting from EMS mutagenesis. Two different chromosomal deletions that remove at least part of the predicted *fem-2* coding region fail to complement the *fem-2(e2105)* allele.

The transgenic rescue results with genomic and cDNA clones suggest that a single transcription unit is sufficient to account for all the functions of *fem-2* in XX and XO animals. There is a single transcript from the locus in XX animals, which is detected throughout development, but is most abundant in adults. Although this may seem unusual given the masculinizing role of *fem-2*, and the observation that Fem-2 activity is completely dispensable for adult female development, the *fem-2* mutant phenotype can be maternally rescued (only *fem-2/fem-2* progeny of *fem-2/fem-2* mothers are completely feminized), suggesting that the FEM-2 protein or its mRNA can be inherited through the female germ line. The high expression of *fem-2* mRNA in the adult hermaphrodite may represent transcription that is restricted to the germ line. Somatic expression of *fem-2* is predicted in males throughout their life (Kimble *et al.*, 1984). In mixed male/hermaphrodite cultures, no other transcript has yet been detected.

The sequence similarity found in this work between the predicted FEM-2 protein and protein phosphatase 2C (PP2C) homologues suggests that FEM-2 might have phosphatase activity. PP2C enzymes have been characterized as serine/threonine specific phosphatases that are Mg^{+2} dependent, and resistant to okadaic acid (Cohen, 1989), but their regulatory role is poorly understood. Maeda *et al.* (1993 and 1994) showed that the yeast homologue *PTC1* is an essential gene in yeast under certain conditions, and suggested that *PTC1* is involved in the regulation of a two-component signal transduction system, possibly via a mitogen-activated protein kinase homologue involved in osmosensing. *S. cerevisiae* has at least three separate mitogen-activated protein kinase cascades (reviewed in Nieman, 1993). The mutant phenotypes of genes involved in each separate yeast pathway do not show obvious pleiotropic defects, suggesting that there is little or no interaction between the pathways under normal circumstances. None of the other proteins in these pathways show similarity to any of the gene products in the *C. elegans* sex determination pathway.

Another apparent homologue of FEM-2 is the *ABI1* gene of *Arabidopsis thaliana*. The *abi1* mutant is insensitive to the phytohormone abscisic acid (ABA) (Koornneef *et al.*, 1984). ABA is involved in many aspects of plant growth and development. In at least one fern species, ABA-resistant mutations affect sex

determination (Banks, 1994). The *abi1* mutant phenotype in *Arabidopsis* has been proposed to result from a lack of negative feedback regulation in an intracellular step of hormone signaling (Leung *et al.*, 1994; Meyer *et al.*, 1994). The mutation in the *abi1–1* allele affects a conserved glycine in motif II, suggesting a role for this motif in the negative regulation of the phosphatase activity (Leung *et al.*, 1994; Meyer *et al.*, 1994). *ABI1* has been shown to have protein phosphatase function in vitro (Leung *et al.*, 1994; Meyer *et al.*, 1994). Again, although FEM-2, *PTC1*, and *ABI1* appear to have roles in the regulation of signal transduction pathways, there is no evidence that any other components in the signaling pathways are common to the systems.

Despite the similarity between FEM-2 and canonical mammalian PP2C enzymes, FEM-2 is most similar in sequence to an unidentified human ORF. With the long amino-terminal domain, short carboxy-terminal domain, and lack of a conserved decapeptide between domains IV and V, FEM-2 (along with *ABI1* and the human ORF) appears to fall into a distinct class of PP2C homologues. The presence of the long amino terminal domain suggests that the three proteins may regulate their protein activity in a manner different from the classical PP2C proteins. The Arabidopsis KAPP phosphatase also appears to be a Class A PP2C enzyme, based upon its long amino-terminal domain (320 amino acids; Stone *et al.*, 1994). In this case the amino-terminal domain appears to directly interact with the receptor serine/threonine kinase RLK5. The F42G9.1 protein from *C. elegans* is more similar in structure to the canonical mammalian PP2C proteins. F42G9.1 does not have the long amino terminus found in FEM-2 and ABI1, but does have the decapeptide between domains IV and V, which makes it most similar to the mammalian PP2C isoforms. No genetic loci have yet been identified that map to the predicted position of F42G9.1, and the existence of a protein product has not been confirmed.

Could the sex determination pathway be regulated by phosphorylation? There are several well studied paradigms for regulation of transcription by phosphorylation (Hunter and Karin, 1992). For example, the nuclear localization of the transcription factor NF-[κ]B is controlled by the phosphorylation of its regulatory subunit I[κ]B (Ghosh and Baltimore, 1990). I[κ]B contains ankyrin repeats (Haskill *et al.*, 1991), like FEM-1, therefore the localization of TRA-1 may be controlled by interactions with FEM-1 to control somatic sex. FEM-2 may regulate such an interaction by dephosphorylating FEM-1. However, there is no evidence to suggest that FEM-1 and TRA-1 interact, and limited evidence that TRA-1 is always nuclearly localized (D. Zarkower, personal communication). An alternative model is that *fem-3* product may be the target. The predicted FEM-3 protein contains several putative casein kinase phosphorylation sites (Ahringer *et al.*, 1992) but it is not known whether any of these are used in vivo or in vitro. In addition, *fem-3* gain-of-function mutations have been shown to suppress the *fem-2* phenotype of the conditional allele *b245* in the germ line, but not the soma (Barton *et al.*, 1987). These gain-of-function mutations alter a control sequence in the *fem-3* 3′-untranslated region (UTR) of the mRNA (Ahringer and Kimble, 1991; Evans *et al.*, 1992). Perhaps FEM-2 is a molecule that normally controls *fem-3* translation in the germ line, possibly by de-phosphorylating an unidentified untranslated region binding factor. The suppression has only been seen for one allele of *fem-2*, and is limited to the germ line and therefore, this model does not explain all the observations. A third possibility is that the TRA-1 protein, which has several putative phosphorylation sites, is the target (Zarkower and Hodgkin, 1992). In this model, there must be a second target in the germ cells, where *fem-2* activity is normally required and is independent of *tra-1*. Because *fem-2* has different genetic roles in the germ line and soma, neither of these speculations can be ruled out.

A further complication to these models is the absence of a characterized kinase in the sex determination pathway. Because *fem-2* mutants show defects in sex determination in both the germ line and soma, one might expect that an associated kinase must exist in the two tissues. If the activity of FEM-2 is to remove phosphate groups from a protein target to produce the "male" phenotype, a protein kinase must initially phosphorylate the target. No kinase has been found among the genes of the pathway; if an associated kinase exists, loss-of-function mutations in the gene are either rare, or do not lead to a simple sexual transformation phenotype (the predicted phenotype of such a mutation would be "Tra" phenotype of masculinization). Perhaps the kinase is constitutive, and essential, or redundant. Alternatively, one of the characterized genes in the pathway may have a kinase activity that cannot be predicted from the primary sequence.

Finally, it is clear that under some circumstances, the FEM-2 product is dispensable for normal male development. There is evidence from the current work that the strongest alleles of *fem-2* completely eliminate FEM-2 activity. The sequence of the *e2102* and *e2105* alleles predicts a truncated protein, missing much of the PP2C conserved region. The phenotype of the *e2105* allele gets no stronger when *in trans* to a deficiency, suggesting that there is no partial function supplied by one copy of *e2105*. Yet in *fem-2(e2105)* animals, male somatic structures as well as sperm can still be formed in certain conditions (this work; Hodgkin, 1986). Therefore, *C. elegans* can bypass the requirement for *fem-2* activity. If FEM-2 is a phosphatase whose activity leads to

the activation of spermatogenesis and the inactivation of TRA-1 in the soma, such dephosphorylation is not an essential part of the regulation. Identification of the cellular targets of FEM-2 activity will help to clarify its apparent dual role in somatic and germline sex determination.

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