Molecular Evolution of a Sex Determination Protein: FEM-2 (PP2C) in Caenorhabditis

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ABSTRACT

Somatic sex determination in *Caenorhabditis elegans* involves a signal transduction pathway linking a membrane receptor to a transcription factor. The *fem-2* gene is central to this pathway, producing a protein phosphatase (FEM-2) of the type 2C (PP2C). FEM-2 contains a long amino terminus that is absent in canonical PP2C enzymes. The function of this domain is difficult to predict, since it shows no sequence similarity to any other known proteins or motifs. Here we report the cloning of the *fem-2* homologue from *Caenorhabditis briggsae* (*Cb-fem-2*). The sequence identity is much higher than that observed for other *C. briggsae* homologues of *C. elegans* sex determination proteins. However, this level is not uniform across the entire lengths of the proteins; it is much lower in the amino termini. Thus, the two domains of the same protein are evolving at different rates, suggesting that they have different functional constraints. Consistent with this, Cb-FEM-2 is able to replace some, but not all, of the Ce-FEM-2 *in vivo* function. We show that removal of the amino terminus from Ce-FEM-2 has no effect on its *in vitro* phosphatase activity, or its ability to replace the *in vivo* function of a yeast PP2C enzyme, but that it is necessary for proper FEM-2 function in worms. This demonstrates that the amino terminus is not an extended catalytic domain or a direct negative regulator of phosphatase activity.

THE primary sex determination signal in many animals, including the nematode *Caenorhabditis elegans*, is the ratio of sex chromosomes to sets of autosomes (Madl and Herman 1979). In *C. elegans*, somatic and germline sex (exclusive of dosage compensation) is regulated by a set of gene products whose order and function have been predicted through genetic analysis (Hodgkin 1986). Most of the genes identified through genetic means as being involved in sex determination have been cloned, and their predicted protein products are known (Kimble 1988; Spence *et al.* 1990; Kuwabara *et al.* 1992; Zarkower and Hodgkin 1992; Perry *et al.* 1993; Pilgrim *et al.* 1995; Barnes and Hodgkin 1996). This has allowed a molecular model to be developed for the biochemical activities involved in transducing the sex determination signal (Kuwabara and Kimble 1992). *fem-2* is required for normal male somatic development and spermatogenesis (Kimble *et al.* 1984). Its sequence shows similarity to protein phosphatases type 2C (PP2C), and is most similar to PP2C homologues from plants and humans (Pilgrim *et al.* 1995). FEM-2 is able to dephosphorylate targets *in vitro*, and this activity is necessary for FEM-2's masculinizing function (Chin-Sang and Spence 1996).

The areas of similarity among all PP2C enzymes span a region of approximately 260 amino acids (Pilgrim *et al.* 1995), which is almost the entire length of many PP2C enzymes, including the PP2C enzyme from yeast, Ptc1p (Maeda *et al.* 1993; Robinson *et al.* 1994; Huang and Symington 1995; Jiang *et al.* 1995). FEM-2 is a member of Class A PP2C enzymes (Pilgrim *et al.* 1995), which also includes the Arabidopsis proteins ABI1, ABI2 and KAPP (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Stone *et al.* 1994), and a predicted protein of unknown function from humans (Nomura *et al.* 1994). They are much larger than Ptc1p due to an additional domain at the amino terminus. With the exception of ABI1 and ABI2, none of the Class A proteins show sequence similarity to each other in their amino termini, suggesting that their functions differ. The amino terminus of KAPP has been shown to interact with the protein kinase RLK5 (Stone *et al.* 1994), and this binding is thought to regulate KAPP's phosphatase activity. FEM-2's amino terminus could also be involved in regulating PP2C activity. This could be accomplished in a number of ways including binding to a positive or negative regulator, providing target specificity, or determining subcellular localization. It is also possible that it is an integral part of an extended catalytic domain needed for phosphatase activity.

FEM-2's amino terminus does not show similarity to any domains of known biochemical function making it difficult to predict its role in sex determination. One approach to understand its role is to identify and characterize functional homologues of *fem-2*. The *Caenorhabditis briggsae* homologues of two other *C. elegans* sex determining genes have been reported (De Bono and

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Hodgkin 1996; Kuwabara 1996). Cb-TRA-1 and Cb-TRA-2 both show surprisingly low amino acid identity to their *C. elegans* counterpart (44% and 43%, respectively). This level of conservation is much lower than that observed for other, nonsex-determining, *C. briggsae* homologues that have been identified (*e.g.*, Table 1; De Bono and Hodgkin 1996), suggesting that the sex-determining genes are diverging much faster than those involved in other developmental processes. It is tempting to speculate that this rapid divergence is due to strong evolutionary pressure on a set of interacting proteins, which must coevolve. Since there is evidence that FEM-2 interacts with other members of the sex determination cascade, at least *in vitro* (Chin-Sang and Spence 1996), we expect that at least a portion of the FEM-2 sequence should be diverging at the same accelerated rate. It is unlikely that the carboxy terminus is diverging at this high rate due to constraints needed to maintain the PP2C activity; however, we do expect the amino terminus to be diverging at a rate similar to the TRA-1 and TRA-2 homologues from *C. elegans* and *C. briggsae*.

Herein we report the cloning of the *C. briggsae* homologue of *fem-2* (*Cb-fem-2*). The degree of identity between the two predicted proteins is much higher than has previously been reported for other *C. elegans* sex-determining proteins and their *C. briggsae* homologues (De Bono and Hodgkin 1996; Kuwabara 1996), although a higher level of conservation is seen over the PP2C domain than the amino terminus. We show that the amino terminal domain of Ce-FEM-2 is not necessary for *in vitro* phosphatase activity. Also, Ce-FEM-2 is able to functionally replace the yeast PP2C enzyme, Ptc1p, independent of the presence or absence of this domain. These results suggest that the function of the amino terminal domain is distinct from that of the phosphatase domain and their functions may impose different stringencies of sequence constraints. These different stringencies could cause the amino termini of the *C. elegans* and *C. briggsae* FEM-2 proteins to diverge from each other at a much faster rate than the phosphatase domains.

MATERIALS AND METHODS

DNA primers used in this work: DHA17, 5′ CCGAGCACT CAGAGATGTTCC 3′; DHA22, 5′ CGACAAGATCTTCCAG CAAAACGC 3′; DHA23, 5′ GGATCAGATCTGCGATCGCC GTC 3′; DHB.2, 5′ CGGATGGGAATGTATGACTGTGG 3′; DHB.3, 5′ GCACTCGTGGCCTCCATGACCATCG 3′; DHB.4, 5′ GGTGGACGGAGGAATCCAATGACG 3′; DHB.5, 5′ CCAC AGTCATACATTCCCATCCG 3′; PJA1, 5′ AATTCGTCGA CG 3′; PJA2, 5′ TCTAGATCTCGAGAGAGAAAATTGGAA TACCC 3′; PJA3, 5′ TCTAGATCTCGAGATGGAAAAAGTA AACGAGGAG 3′.

Cloning of the *C. briggsae* homologue: Southern blotting was performed using *C. briggsae* genomic DNA digested with various restriction endonucleases and probing with a full-length *C. elegans fem-2* cDNA. Hybridization was performed at 50° with three subsequent 15-min 50° washes of 2× SSC, 0.1% sodium dodecyl sulfate (SDS). Autoradiography revealed a single 3.7-kbp band when the *C. briggsae* DNA was digested with *Xba*I. A genomic minilibrary was made by gel purifying *Xba*I-digested *C. briggsae* DNA that was 3.2–4.0 kbp in size. These fragments were ligated into *Xba*I-digested pBluescript II SK−, transformed into bacteria, and screened using colony hybridization as described (Sambrook *et al.* 1989). Hybridization and washing conditions for colony hybridization were the same as those used for the Southern blot mentioned above. Analysis of the sequence of a positive clone (pDP#DH42) showed that all of the predicted coding region was contained in the 3.7-kbp *Xba*I fragment; however not all of the predicted 3′ untranslated region (3′ UTR) and only 414 bp of upstream sequence was contained in the clone (Figure 1). To get a larger clone, another minilibrary was constructed by digesting *Hin*dIII-digested genomic DNA and isolating fragments between 10 and 20 kbp. These were cloned into pBluescript II SK− and transformed into bacteria. pDP#DH42 was used to probe colony lifts. The blots were hybridized at 65° and then, at the same temperature, washed twice with 2× SSC (10 min), once with 2× SSC, 0.1% SDS (15 min) and once with 0.1× SSC (5 min). One positive clone was isolated (pDP#DH53) and confirmed to completely contain pDP#DH42 region by restriction mapping, PCR, and partial sequencing (Figure 1). The sequence of *Cb-fem-2* can be obtained from GenBank (accession no. AF054982).

***C. briggsae* mRNA transcript analysis:** Total RNA was isolated from hermaphrodite (*XX*) worms which were grown in liquid culture as described (Sulston and Hodgkin 1988). Four volumes of TRIZOL (Life Technologies) were added to packed worms and resuspended by vortexing, then incubated at 20° for 5 min. The solution was centrifuged at 16,000 × *g* (4°) for 10 min and liquid was transferred to a new tube. One-fifth volume of chloroform was added to the solution and vortexed for 15 sec. Centrifugation was performed as described above for 15 min and the aqueous phase was transferred to a new tube containing one volume of isopropanol and incubated for 10 min at 20°. The RNA was recovered by centrifuging at 16,000 × *g* for 10 min (4°) and the pellet was washed with 75% ethanol and suspended in DEPC-treated water.

A DNA copy of the 5′ end of the *Cb-fem-2* transcript was obtained by Rapid Amplification of cDNA Ends (RACE) using a 5′ RACE Kit (Life Technologies), following the manufacturer's instructions. Primer DHB.4 was used for first strand synthesis and primer DHB.3 was used for PCR amplification (Figure 1). The PCR product was cloned into pGEM-T (Promega, Madison, WI). Sequencing revealed the presence of a *trans*-spliced leader with the sequence 5′GGTTTAATTACCCAAGT TGAG3′, although the number of G's at the 5′ end cannot be precisely determined because the RACE protocol used incorporates a string of G's at this location. The leader splices into the sequence UUUCAG***AUG**, with * being the site of splicing. The AUG shown (bold) is predicted to encode the initiator methionine. Therefore, as seen in other *C. elegans* transcripts (Blumenthal and Steward 1997), there is no sequence between the *trans*-spliced leader and the coding region. Since the RACE product contained only one intron splice site, reverse transcription (RT)-PCR was used to obtain a cDNA containing all putative intron splice sites. Primer DHB.4 was used for first strand synthesis and primers DHB.4 and DHB.5 were used for amplification (Figure 1). The product was cloned into pGEM-T (pDP#DH113) and sequenced.

RNA gel electrophoresis and Northern blotting were performed as described (Sambrook *et al.* 1989). Ten micrograms of the total RNA described above was electrophoresed on a 1.3% agarose gel and transferred to GeneScreen Plus nylon membrane (New England Biolabs, Beverly, MA). The RNA

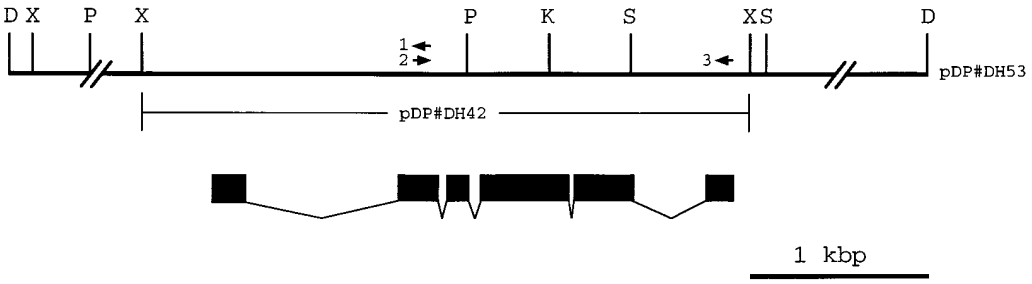


Figure 1.—Restriction map of genomic region containing *Cb-fem-2* and location of intron and exon boundaries. The horizontal line represents the *C. briggsae* genomic DNA insert of clone pDP#DH53. Vertical lines represent restriction enzymes (D, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sst*I; and X, *Xba*I). The hash marks represent portions of DNA not included on this figure. Clone pDP#DH42 contains the region flanked by the two *Xba*I sites within the hash marks. Clone pDP#DH53 contains the entire region shown on the restriction map. The distance between the first *Hin*dIII site and the second *Xba*I site is ~4.5 kbp while the distance between the third *Xba*I site and the final *Hin*dIII site is ~7.0 kbp. The numbered arrows show the location of the primers used for RT-PCR and 5′ RACE (1, DHB.2; 2, DHB.5; and 3, DHB.4). The black boxes below the line represent coding regions of the six exons.

was hybridized to a ^{32}P-labeled DNA probe made from the insert of clone pDP#DH113. Hybridization was performed at 65° overnight using Hybridsol II hybridization solution (Oncor). The blot was washed at 65° twice with 2× SSC for 15 min, once with 2× SSC, 0.1% SDS for 30 min and once with 0.1× SSC, 0.1% SDS for 10 min.

Partial rescue of *fem-2* animals with *C. briggsae* clone: pDP#DH53 was coinjected with pRF4 (*rol-6(su1006dm)*), into the gonads of hermaphrodites of strain DP51 (*fem-2(e2105) unc-45(r450ts)/sC1* [*dpy-1(s2171)*];*him-8(e1489)*). pDP#DH53 was injected at concentrations of 50 ng/μl and 100 ng/μl as described in Mello *et al.* (1991). F_1 progeny with the rolling phenotype were confirmed to also be transmitting pDP#DH53 by single worm PCR using primers DHB.3 and DHB.5 as described in Pilgrim *et al.* (1995) and Williams *et al.* (1992). Single rolling Unc hermaphrodites were transferred onto plates and allowed to self-fertilize at 21° and 25°. F_2 hermaphrodite (*XX*) and male (*X*O) rolling animals were scored for their ability to self-fertilize, as well as for somatic sexual phenotype, compared to control DP51 animals grown at the same temperature.

Attempted rescue of *fem-2* animals with truncated FEM-2: pDP#DH28 was coinjected with pRF4 into strains DP51 and DP53. DP53 is similar to DP51 except that it carries a lethal allele (*s2683*) on the balancer *sC1* chromosome (gift of H. Stewart and D. Baillie). Construction of pDP#DH28 is described below. Analysis of the transformed worms was performed at 21° as described above.

Yeast strains, media, and transformation: *Saccharomyces cerevisiae* strain TM126 (*MAT***a** *leu2 ura3 his3 ptc1::URA3*) was obtained from H. Saito (Maeda *et al.* 1994). Transformation of plasmids into strain TM126 was carried out as described (Schiestl *et al.* 1993) following protocol number 3. Transformation of pDP#DH1 into TM126 yielded strain sDH2, pDBL yielded sDH3, and pDP#DH29 yielded sDH5 (DNA constructs described below). For growth curves, cells were grown overnight at 30° with constant shaking in selective (SC-leu) liquid media. The next day, 5 μl of each overnight culture was added to 5 ml of fresh media. These samples were grown with constant shaking at 30° or 37° at 300 rpm and the OD_{550} was measured at specified intervals in a Bausch and Lomb Spectronic 20 spectrophotometer. Growth was assayed for five independent cultures of each strain at both temperatures.

Detection of Ce-FEM-2 in yeast and worms: Protein extracts of the yeast strains sDH2, sDH3, and sDH5 were obtained following overnight growth at 30° in liquid SC (minus leucine and uracil) media. The cells were pelleted and resuspended in 1.5 ml of water. 0.24 ml of 1.85 m NaOH, 7.4% β-mercaptoethanol was added to the cells and incubated at 4° for 10 min. 0.24 ml of 50% trichloroacetic acid was added to the solution and incubated again at 4° for 10 min. The solution was centrifuged and the pellet was resuspended in 1.5 ml of acetone. The acetone was removed after centrifugation and the dried pellet was resuspended in 0.1 ml of 2× sample buffer (Sambrook *et al.* 1989). Protein extracts of worm strains DP51 and DP151 were isolated as described (Goetinck and Waterston 1994).

SDS-PAGE was performed as described in Sambrook *et al.* (1989) using a 12% resolving gel. Western blotting and detection were performed using the ECL kit (Amersham, Arlington Heights, IL) following the supplier's instructions using a 1:2500 dilution of a polyclonal antibody made against Ce-FEM-2 (Jackle-Baldwin 1996).

DNA manipulation: pDBL, containing the 2 μm origin of replication and ADH1 promoter, has been described previously (Milne and Weaver 1993) and was obtained from T. Milne (Harvard Medical School, Cambridge, MA). This plasmid was used as the backbone for the construction of pDP#DH1 (directs the synthesis of FEM-2 under the control of the ADH1 promoter in yeast) and pDP#DH29 (same as pDP#DH1 but contains a deletion in the region encoding the amino terminal domain of FEM-2). For pDP#DH1, a cDNA, previously shown to contain the entire *Ce-fem-2* open reading frame and upstream sequences including the first few bases of the SL1 spliced leader cloned into the *Eco*RI site of pBluescript (Pilgrim *et al.* 1995), was liberated from the plasmid by digesting with *Not*I and *Eco*RV. *Not*I linkers were added to the blunt end, the insert was redigested with *Not*I and then cloned into the *Not*I site of pDBL. For pDP#DH29, the insert from pDP#DH1 was cloned into the *Not*I site of pBluescript II SK− and subjected to *in vitro* mutagenesis (Leatherbarrow and Fersht 1986), using mutagenic primers DHA22 and DHA23. These created in-frame *Bgl*II restriction sites at positions 396 and 1187 of the *fem-2* sequence (Pilgrim *et al.* 1995), which were digested and then religated, removing the sequence between the two sites (Figure 4A). This resulted in a truncated open reading frame and the resulting protein is referred to as FEM-2ΔN2 (Figure 4B). The engineered insert was then removed by digesting with *Not*I and ligated into the *Not*I site of pDBL. pDP#DH28 was made by *in vitro* mutagenesis (Leatherbarrow and Fersht 1986) of a wild-type genomic *Ce-fem-2* clone (pDP#DH11) that can completely rescue *fem-2* mutant defects [data not shown; insert includes the minimal rescuing region shown in Pilgrim *et al.* (1995)]. Primers DHA22 and DHA23 introduced in-frame *Bgl*II restriction sites as described above (Figure 4A). The plasmid was digested with *Bgl*II and religated after removal of the intervening sequence. The resulting protein is the same as FEM-2ΔN2, which was expressed in yeast.

For the production of glutathione *S*-transferase (GST)-FEM-

2 fusion proteins in bacteria, the following plasmids were constructed: pDP#DH14: pGEX-1λT (Pharmacia, Piscataway, NJ) vector was digested with *Eco*RI and ligated with primer PJA1, which introduces a *Sal*I site at the location of the original *Eco*RI site to make pGEX-Sal. Full-length *fem-2* cDNA was amplified by PCR using primers PJA2 and PJA3 and a 20:1 mixture of Taq polymerase to Pfu DNA Polymerase (Stratagene, La Jolla, CA). The amplified product was gel purified and digested with *Xho*I, which cuts at the sites introduced by the two primers. The product was then cloned into the introduced *Sal*I site of the pGEX vector and orientation was confirmed by restriction digestion. pDP#DH19: The pDP#AMc001 "minigene" described previously (Pilgrim *et al.* 1995), was mutagenized *in vitro* using primer DHA17 to introduce a mutation into the FEM-2 coding region identical to that found in the temperature-sensitive *b245* mutation (Pilgrim *et al.* 1995). The clone was then digested with *Sal*I and *Sst*I, and the insert containing the *b245* mutation was gel purified and cloned into pDP#DH14 that had been digested with the same enzymes and separated from the corresponding insert. pDP#PJB1 was a gift of Petra Jäckle-Baldwin and consists of a fragment of the *fem-2* cDNA from the *Sal*I site to the 3′ end (Figure 4A) cloned into the pGEX-*Sal*I plasmid. This results in a GST-FEM-2 fusion protein which is missing 87 amino acids at the amino terminus of FEM-2. This truncated form of FEM-2 is referred to as FEM-2ΔN1 (Figure 4B).

Isolation of the FEM-2 protein produced in *Escherichia coli*: GST fusion vectors pDP#DH14, pDP#DH19 and pDP#PJB1 were transformed into *E. coli* strain BL21 DE3, then grown in LB-ampicillin to an OD_{550} of ~0.9 at 30°. Induction of the fusion protein was achieved by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 400 μm as described by the supplier (Pharmacia) for an additional 2–3 hr. The cells were pelleted and resuspended in lysis buffer (100 mm NaCl, 2.5 mm EDTA, 0.1% Tween-20, 50 mm Tris-HCl, pH 8.0). Cells were lysed using a Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) by sonication on ice with four bursts of 60 sec each on power setting 3. The debris was removed by centrifugation at 20,000 × *g* for 15 min in a Sorvall SS-34 rotor and the supernatant was added to 0.5 ml bed volume of washed Glutathione Sepharose 4B (Pharmacia) and allowed to bind at 4° for 2 hr with inversion. Beads were washed three times with phosphate buffered saline (PBS) and protein was eluted with two washes each of 10 and 15 mm glutathione in washing buffer (100 mm NaCl, 50 mm Tris-HCl, pH 8.0). The eluant was dialyzed against washing buffer for 2 hr, then overnight against 50 mm NaCl, 20 mm HEPES-KOH pH 7.2, 20% glycerol, 10% polyethylene glycol 20,000. Isolated proteins were quantified using the method of Bradford (1976) and by comparing against known standards on Coomassie Brilliant Blue-stained SDS-PAGE gels.

Phosphatase assays: Bovine milk casein (C-4765; Sigma, St. Louis) was radioactively labeled using 7000 Ci/mmol [γ-^{32}P]ATP (ICN) with the catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma P-2645) as described by the supplier. The labeled protein was then precipitated with 20% trichloroacetic acid/20 mm NaH_2PO_4 and washed four times with the same solution. The pellet was dried and resuspended in 0.2 m Tris-HCl (pH 8.0) to a final concentration of ~10^5 cpm/μl. The phosphatase assay was performed as described (Maeda *et al.* 1993) at 22° and similar results were obtained in at least two separate reactions. All reactions contain 50 mm $MgCl_2$ unless otherwise noted.

RESULTS

***C. briggsae* homologue of *fem-2*:** *Cb-fem-2* was cloned using low stringency hybridization and the entire putative coding region, covering approximately 3.0 kbp, was sequenced. Northern blotting shows a single band at approximately 1.9 kb, suggesting that *Cb-fem-2* makes only one transcript, or more than one with very similar sizes (Figure 2). Comparison of the genomic sequence of *Cb-fem-2* to that obtained through 5′ RACE and RT-PCR reveals the presence of five introns (Figure 1), three of which are at the same location as those in *Ce-fem-2* (Figure 3A). The sequence of the 5′ RACE product contains a leader with the sequence 5′GGTTTAATTAC CCAAGTTGAG3′, confirming that the *Cb-fem-2* transcript is *trans*-spliced. The leader is similar to SL1 to which the *Ce-fem-2* transcript is spliced (Pilgrim *et al.* 1995).

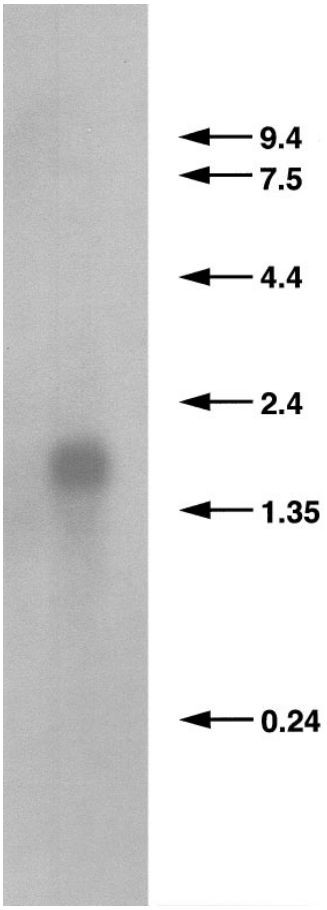


Figure 2.—Northern blot of *C. briggsae* mRNA. Ten micrograms of *C. briggsae* total RNA was hybridized with a ^{32}P-labeled probe generated from a *Ce-fem-2* RT-PCR product. The numbers represent relative location of RNA size standards in kb. Hybridization occurs only at a molecular weight of ~1.9 kb and suggests the presence of only one transcript or multiple transcripts of similar molecular weight. The blot was exposed at −80° for 8 days.

The predicted Cb-FEM-2 protein consists of 502 amino acids, 53 amino acids longer than Ce-FEM-2, due to an even longer domain amino terminal to the conserved phosphatase motifs (Figure 3A). Over 449 amino acids, the two proteins are 63% identical and

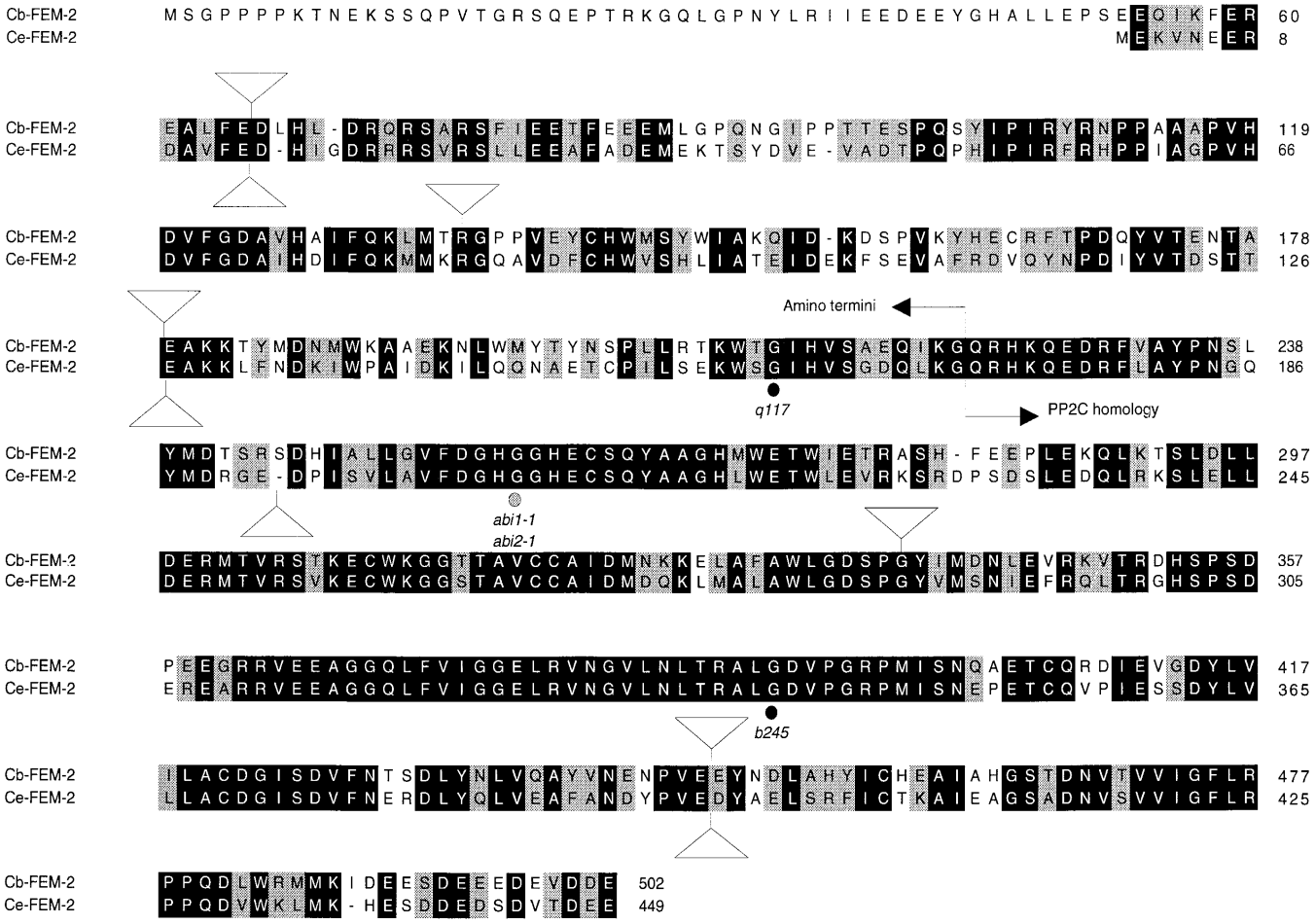


Figure 3.—Alignment of amino acid sequence of Cb-FEM-2 and Ce-FEM-2. (A) White letters on a black background identify identical amino acids while black letters on a gray background identify similar amino acids. Sequences were compared using the program ALIGN. Triangles show the locations of the predicted introns. Black circles show the locations of the lesions associated with the two temperature-sensitive alleles of *Ce-fem-2* (Pilgrim *et al.* 1995). The gray circle shows the location of the lesion associated with the Arabidopsis mutations *abi1-1* and *abi2-1* (Leung *et al.* 1994, 1997; Meyer *et al.* 1994). (B) The gray boxes represent the PP2C domains of the proteins. The striped boxes represent the portions of the amino-terminal domains that are common to each other. The exact location of the division between the gray and striped boxes is noted on part A.

85% similar. The degree of similarity is not consistent over the entire length of the protein (Figure 3). In the region common to PP2C enzymes (the carboxy 279 amino acids), the two proteins are 72% identical and 90% similar. The first 170 amino acids of Ce-FEM-2 and the equivalent region of Cb-FEM-2 are 49% identical and 80% similar (Figure 3B).

Comparison of ~400 bp of *Cb-fem-2* promoter sequence with the *Ce-fem-2* promoter revealed no significant similarity except for the 9-bp sequence 5′tctg catta3′. This sequence may be significant because it is at approximately the same location in both genes. This element starts 105 bp and 104 bp upstream of the *Ce-fem-2* and *Cb-fem-2* respective *trans*-splice sites. Some similarity is also seen in the 3′UTR of the two genes and will be discussed elsewhere.

Partial rescue of *Ce-fem-2(null)* with *Cb-fem-2*: While the identity of the phosphatase domains is high, the level of sequence divergence in the amino termini suggests that portions of the two proteins may be rapidly evolving (Figure 4). To determine if the two genes are functionally interchangeable, we introduced *Cb-fem-2* into *Ce-fem-2(null)* animals. Wild-type *XX* animals are normally self-fertile hermaphrodites, while wild-type *X*O

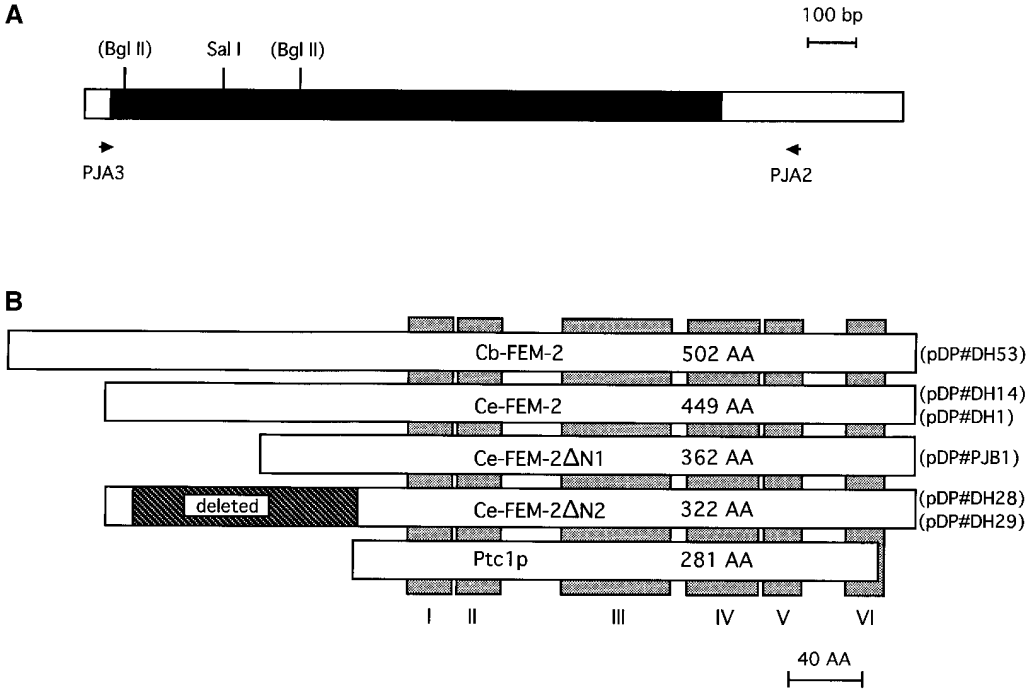


Figure 4.—The *Ce-fem-2* gene and construction of fusion proteins. (A) Restriction map of the transcribed region of *Ce-fem-2* (Pilgrim *et al.* 1995). The black box corresponds to the FEM-2 open reading frame, white boxes to the 5′ and 3′ untranslated regions. The *Bgl*II sites in brackets were introduced into the gene by *in vitro* mutagenesis as described in materials and methods, and were subsequently used to create an in-frame deletion that results in an amino-terminal deletion of 127 amino acids in Ce-FEM-2. Primers PJA2 and PJA3 used in the construction of pDP#DH14 and pDP#DH28 are indicated by arrows. The *Sal*I site shown was used in the construction of pDP#PJB1. (B) Alignment of the predicted Ce-FEM-2 and Ptc1p proteins, with the vertical shaded regions corresponding to the conserved sequence motifs, as described (Pilgrim *et al.* 1995). The hatched box in FEM-2ΔN2 shows the portion of FEM-2 missing because of the deletion using the *Bgl*II sites. The names of the plasmids encoding the proteins are in parentheses beside the respective protein. Ce-FEM-2 and Ptc1p share 30% identity and 50% similarity over the six motifs.

are male. *XX Ce-fem-2(null)* animals, which receive neither maternal nor zygotic *fem-2* product (m−z−), are self-sterile at both 21° and 25°, due to their inability to make sperm (Hodgkin 1986). *X*O m−z− *Ce-fem-2(null)* animals are feminized in both the germline and the soma. The degree of somatic feminization is temperature dependent, with the somatic tissue of *X*O m−z− animals being intersexual at 21° and female at 25° (Hodgkin 1986). Transgenic *Cb-fem-2* is unable to rescue the germline feminization defect of either *XX* or *X*O animals. *XX* m−z− animals carrying the *Cb-fem-2* array ($n > 60$) were unable to self-fertilize. After self-sterility was confirmed, two of these worms were mated to +/*fem-2* males and were able to produce progeny, confirming that oogenesis occurred normally in these worms. At 21°, *X*O m−z− *Ce-fem-2(null)* animals have an intersexual soma that includes a partially formed vulva, a hermaphroditic bilobed gonad and an incomplete male tail (Hodgkin 1986). *X*O m−z− *Ce-fem-2(null)* animals, either carrying the array or lacking it, were examined for somatic phenotype. It was found that all animals carrying the array lacked any obvious vulval tissue, had a single-lobed gonad and an incomplete male tail at both 21° ($n = 34$) and 25° ($n = 24$) (Figure 5). The germline was still feminized as evidenced by the presence of oocytes. Therefore, the conserved sequences in *Cb-fem-2* are sufficient to rescue, at least partially, the somatic feminization cause by *Ce-fem-2* mutations.

Lack of rescue using truncated *fem-2*: To determine if the amino-terminal domain is necessary for proper FEM-2 function in worms, a clone carrying a deletion of this region was injected into *Ce-fem-2(null)* worms. The portion of the protein deleted is identical to that deleted in FEM-2-ΔN2, which is able to rescue a PP2C-deficient yeast strain, suggesting that the phosphatase activity is still intact (discussed below). *X*O m−z− *Ce-fem-2(null)* animals ($n = 33$) carrying the array showed the same degree of somatic feminization as those not carrying the array. Germline rescue was not observed in m−z− *XX Ce-fem-2(null)* animals carrying the array ($n > 40$), as demonstrated by self-sterility. Western blotting was used to confirm that worms carrying the array were expressing the amino-truncated form of FEM-2 (Figure 8). Strain DP51 shows a band at the predicted size for FEM-2 (51 kD); however, strain DP151 (same as strain DP51 but carrying array with plasmid pDP#DH28) shows a band at both 51 kD and 36 kD (the expected size of Ce-FEM-2ΔN2). Yeast strain sDH5, which also expresses Ce-FEM-2ΔN2, shows a band of the same size (Figure 8). This suggests that the amino terminus is necessary for FEM-2 to function properly in worm sex determination, and that phosphatase activity alone is not sufficient.

Phosphatase activity of truncated FEM-2 protein: FEM-2 is predicted to have a longer amino terminus than either *S. cerevisiae* Ptc1p or its *Schizosaccharomyces pombe* homologue (Pilgrim *et al.* 1995), and it appears from the experiments described above that this extra

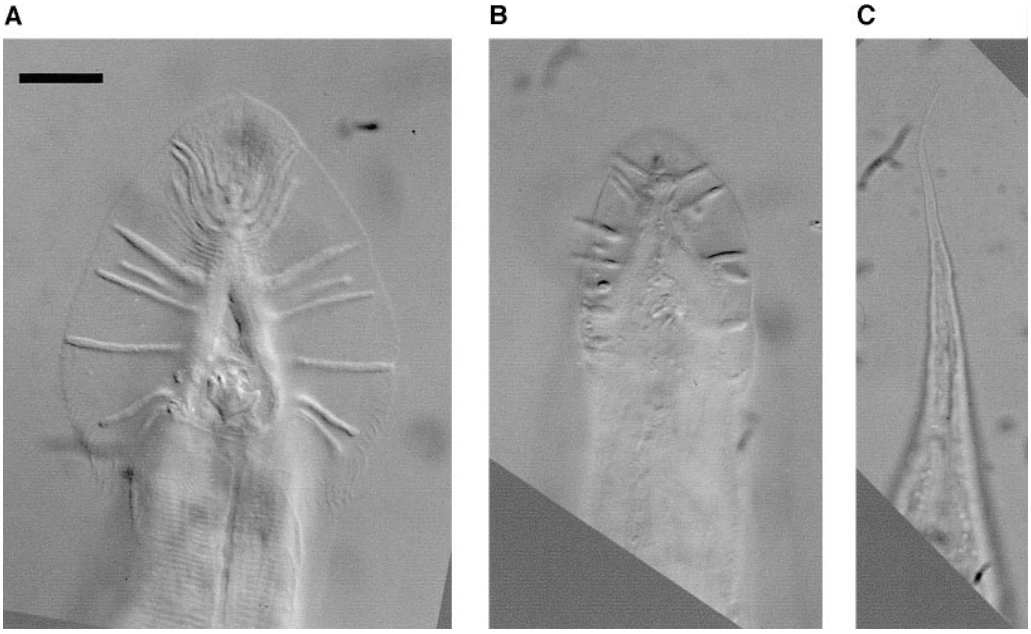


Figure 5.—Comparison of the male tail in *Ce-fem-2* and wild-type worms. (A) The tail of a wild-type male grown at 25°. (B) The tail of an *X*O m−z− *fem-2(null)* animal carrying *Cb-fem-2* in a transgenic array grown at 25°. (C) The tail of an m−z− *fem-2(null)* female grown at 25°. Both *XX* and *X*O m−z− *fem-2(null)* animals develop indistinguishably as females at 25° (Hodgkin 1986). Bar, 10 μm.

sequence encodes a domain necessary for sex-determining function in *C. elegans*. GST-FEM-2 exhibits phosphatase activity using ^{32}P-labeled casein as a substrate (Figure 6), as has been previously shown (Chin-Sang and Spence 1996). We tested the phosphatase activity of a truncated form of FEM-2 that lacks the first 87 amino acids (FEM-2NΔ1). The deleted protein retains the six conserved motifs found in all PP2C enzymes (Pilgrim *et al.* 1995). Bacterially expressed GST-fusion protein was purified as described, and tested for protein phosphatase activity in the same assay. As seen in Figure 6, FEM-2ΔN1 has indistinguishable phosphatase activity from intact FEM-2. Therefore, an intact amino terminus is not required for phosphatase activity *in vitro*.

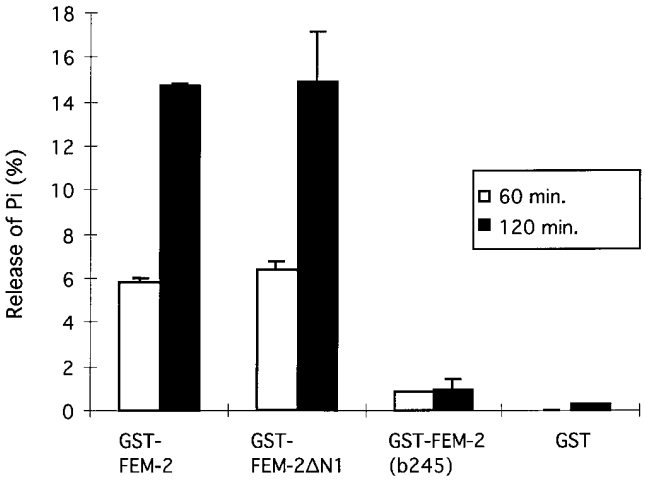
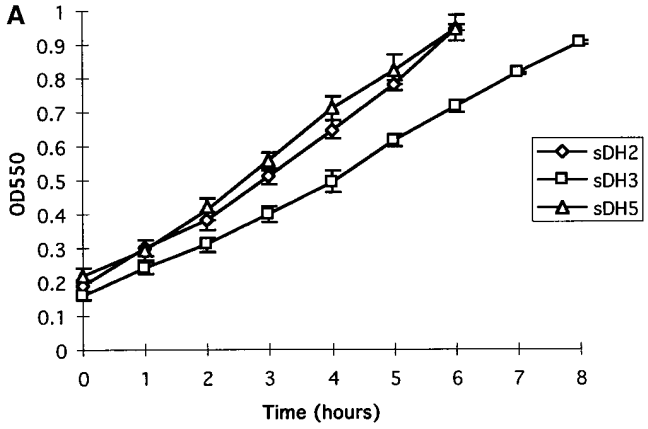


Figure 6.—*In vitro* phosphatase assays. GST-FEM-2 fusion proteins were purified from *E. coli* and assayed for casein phosphatase activity as described in materials and methods. All reactions were performed with 10 μg of test protein, except GST where 20 μg was used. Phosphatase activity was determined at 60 and 120 min for GST-FEM-2, GST-FEM-2ΔN2 and GST-FEM-2(*b245*). Bars represent the range of the results of the independent trials.

The *fem-2* gene was first discovered due to a temperature-sensitive mutation, *b245* (Kimble *et al.* 1984). This has subsequently been shown to be because of a missense mutation in conserved motif IV (Figure 3A; Pilgrim *et al.* 1995). This base pair change was introduced into the FEM-2 coding sequence on a plasmid containing GST-FEM-2, and this fusion protein (FEM-2-*b245*) was also expressed and purified. As predicted from its mutant phenotype, little activity above background could be detected (Figure 6). Since even null alleles of *fem-2* show temperature-dependent phenotypes (Pilgrim *et al.* 1995), the low level of phosphatase activity produced from FEM-2(*b245*) may be sufficient at lower temperatures but not at the higher restrictive temperature. Alternatively, GST-FEM-2(*b245*) may not have been folded properly in bacteria, but is in worms at permissive temperatures.

Rescue of yeast *ptc1* mutation: There may be some overlap in function between the various PP2C enzymes within a yeast cell (Maeda *et al.* 1994; Shiozaki and Russell 1995). Ce-fem-2 has 50% similarity with Ptc1p in the six conserved motifs of the phosphatase domain. Since a phenotype has been described for *S. cerevisiae ptc1* mutant cells (Maeda *et al.* 1993; Robinson *et al.* 1994; Huang and Symington 1995; Jiang *et al.* 1995), we tested whether the similarity between the two proteins is sufficient for Ce-FEM-2 to complement the yeast defects. ABI2 from Arabidopsis has recently been shown to be able to rescue *ptc1* cells (Leung *et al.* 1997). *ptc1* mutant cells show a temperature-dependent growth defect, where growth is significantly slowed at 37°, but not at 30°. We transformed *ptc1* yeast cells with a plasmid



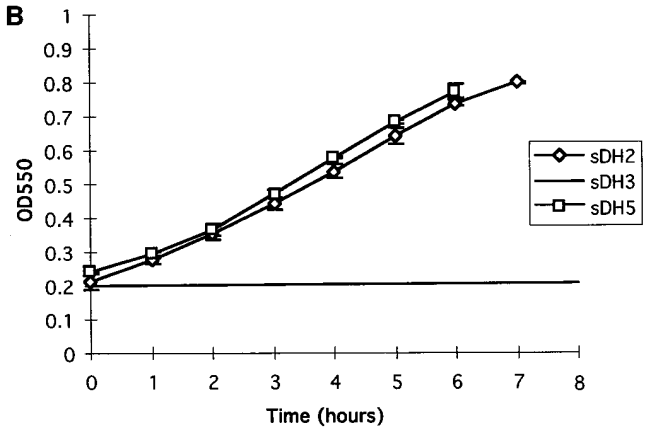


Figure 7.—Growth curves for yeast containing Ce-FEM-2. *S. cerevisiae ptc1* strains carrying an expression plasmid with Ce-FEM-2 (sDH2), Ce-FEM-2ΔN2 (sDH5) or vector alone (sDH3), were grown in selective liquid medium at (A) 30° or (B) 37° as described in materials and methods. Points represent the average of five independent experiments, and bars are ±1 SD. For sDH3 at 37°, no increase in OD_{550} was seen over 7 hr in any of the experiments, although enough growth was seen over a 5-day period to predict a growth rate of ~0.003 OD_{550}/hr. All OD_{550} readings were normalized to 0.2 at 0 hr.

containing the Ce-FEM-2 coding region under the control of the constitutive yeast ADH1 promoter. In transformants containing Ce-FEM-2, the growth arrest is not observed, but growth arrest is observed in transformants containing vector alone (Figure 7).

As an independent *in vivo* assay for the effect of the amino terminus on FEM-2 phosphatase activity, a second truncated form of Ce-FEM-2 was tested. FEM-2-ΔN2 contains an in-frame deletion of 127 amino acids, which includes the majority of the amino-terminal extension (Figure 4). When this truncated form of Ce-FEM-2 is expressed in yeast, it is also able to rescue the temperature-dependent growth defect (Figure 7), with no apparent difference from the rescue achieved by intact Ce-FEM-2. Strains sDH2 and sDH5 were confirmed to be expressing Ce-FEM-2 and Ce-FEM-2ΔN2, respectively, through Western analysis (Figure 8). Thus, the conserved motifs of FEM-2 are sufficient to rescue the growth defect caused by lack of Ptc1p PP2C activity in yeast.

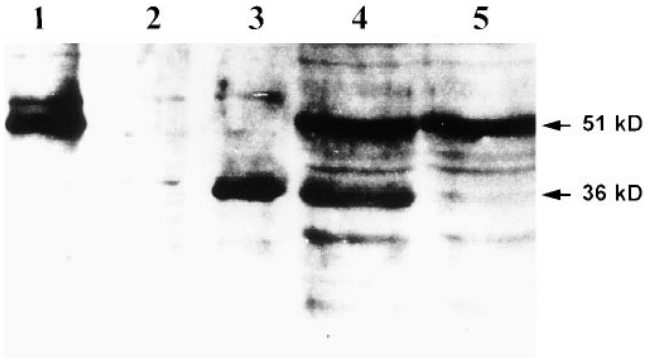


Figure 8.—Western blot of yeast and worm protein extracts. Lanes 1–3 are yeast protein extracts while lanes 4 and 5 are worm extracts. Proteins were detected using a polyclonal antibody raised against bacterially-expressed Ce-FEM-2. Lane 1, sDH2 that expresses full length Ce-FEM-2; lane 2, sDH3 that does not express FEM-2; lane 3, sDH5 that expresses Ce-FEM-2DN2; lane 4, DP151 that expresses wild-type Ce-FEM-2 and Ce-FEM-2ΔN2; lane 5, DP51 that only expresses Ce-FEM-2.

DISCUSSION

FEM-2 plays an important role in promoting male somatic cell fates and spermatogenesis. Its similarity to PP2C enzymes suggests that it performs this function, at least in part, through dephosphorylation, which has been supported with empirical evidence (Chin-Sang and Spence 1996). In order to gain more insight into the sequences necessary for *fem-2* to provide its masculinizing function, we cloned its *C. briggsae* homologue.

Very little is known about how the spatial or temporal expression of *fem-2* is regulated except that RNA transcription is detectable throughout development, being most abundant in adults (Pilgrim *et al.* 1995). Since *Cb-fem-2* can partially replace the function of *Ce-fem-2*, *Cb-fem-2* must contain elements that allow for its expression in *C. elegans* somatic tissue during development. The 9-bp sequence found at the same location in both upstream regions may be involved in this expression. Even though *Cb-fem-2* seems to function in the *C. elegans* soma, it is unable to promote spermatogenesis in the germline. This could be due to the inability of the array to be expressed in the germline; however, transgenic arrays carrying *Ce-fem-2* are able to rescue the germline defect and have never been shown to rescue only the somatic tissue (Pilgrim *et al.* 1995; D. Pilgrim, unpublished results). Genetic epistatic analysis predicts that the sex determination components downstream of *fem-2*

differ in the germline and soma. Perhaps there are two different targets of FEM-2, one in the germline and one in the soma. The germline target may have diverged more at the protein sequence level than the somatic target between the two species, and is unable to be dephosphorylated by Cb-FEM-2. Alternatively, the factors regulating the expression or activity of FEM-2 could be more diverged in the germline than the soma. Since hermaphrodites first produce sperm and then oocytes, the regulation of sex determination factors in the germline requires an additional level of temporal and spatial control that is not necessary in the soma. This additional level of control may complicate matters to the degree that Cb-FEM-2 is unable to functionally replace Ce-FEM-2 in the germline.

Ce-FEM-2 and Cb-FEM-2 are similar in amino acid sequence in both the amino-terminal and the phosphatase domains. Since Cb-FEM-2 contains a long amino extension that is similar in sequence to Ce-FEM-2's, this supports the idea that this domain is needed for FEM-2's role in sex determination. Indeed, *Ce-fem-2* transgenic constructs lacking a large portion of the coding region for the amino terminus are unable to rescue *Ce-fem-2* mutant animals. The long amino extensions of other Class A PP2C enzymes have been proposed to be involved in regulation of phosphatase activity (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Stone *et al.* 1994). This could also be the function of the FEM-2 amino terminus. However, it is unlikely to be directly involved in dephosphorylation because there is no difference in the ability of full-length and truncated forms of CeFEM-2 to rescue yeast cells deficient in Ptc1p. Also, another amino-truncated derivative of Ce-FEM-2 showed no difference in phosphatase activity *in vitro* as compared to full-length Ce-FEM-2. This also suggests that the amino terminus does not serve as a direct repressor of phosphatase activity, otherwise removal of this domain would have released it from repression and a higher level of activity would have been observed in its absence.

The amino terminus could bind to another protein(s), possibly to the protein to be dephosphorylated to provide target specificity. Alternatively, it could bind to a protein that sequesters FEM-2 to remove it from the dephosphorylation target. Indeed, a model has been proposed that involves the membrane-bound TRA-2A protein binding the FEM proteins in *XX* animals, preventing them from repressing the feminizing activity of TRA-1 (Kuwabara and Kimble 1992). A potential FEM-binding site has been predicted to exist in the carboxy terminus of TRA-2A (Kuwabara and Kimble 1995), but this region does not seem to be highly conserved in Cb-TRA-2 (Kuwabara 1996). Perhaps the regions of the sex determination proteins that interact with each other are evolving together. An amino acid change in one protein could be compensated for by a change in the other protein allowing the interaction to occur. Domains of proteins that are involved in binding other molecules, such as DNA (TRA-1) (De Bono and Hodgkin 1996), or a phosphate group (FEM-2), may be more constrained in their ability to evolve because the shapes of the targets are relatively fixed. This could explain why parts of FEM-2 seem to be evolving at different rates. The Cb-FEM-2 amino terminus is 32% longer than Ce-FEM-2's, and the region that is common between the two (170 aa) is only 49% identical. This degree of identity is similar to that seen with the *C. briggsae* and *C. elegans* homologues of TRA-1 and TRA-2, also involved in sex determination. However, the Ce-FEM-2 PP2C domain, which comprises ~60% of the protein, is 72% identical to the corresponding region of Cb-FEM-2. If the amino terminus is involved in binding to other sex determination proteins, a changed amino acid in one protein could put pressure on an interacting protein to also change to maintain the strength of the interaction. Thus, domains of protein-protein interaction, although functionally important, may not show a high degree of conservation between species. This does not explain why the degree of divergence in sex determination genes appears higher than in genes involved in other developmental processes. Sex determination factors in other systems (*SRY* in mammals and *transformer* in Drosophila) have also been shown to have unusually high divergence rates (O'Neil and Belote 1992; Tucker and Lundrigan 1993; Whitfield *et al.* 1993). For *SRY,* this has been suggested to be due to sequence-specific adaptive mutations as opposed to neutral evolution. This suggests that there is evolutionary pressure that causes sex determination proteins to evolve faster than proteins involved in other developmental decisions. Further analysis of the sequence and expression of sex-determining genes from a variety of species may help to determine why they are rapidly evolving.

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