Caenorhabditis elegans sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3

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Male sexual development in the nematode *Caenorhabditis elegans* requires the genes *fem-1*, *fem-2*, and *fem-3*. The current model of sex determination portrays the FEM proteins as components of a novel signal transduction pathway, but the mechanisms involved in signaling through the pathway are not understood. We report the isolation of *fem-2* cDNAs in a yeast two-hybrid screen for clones encoding proteins that interact with FEM-3. Association of FEM-3 and FEM-2 in two independent in vitro binding assays substantiates the interaction detected in the two-hybrid system. FEM-2 is related in sequence to protein serine/threonine phosphatases of Type 2C (PP2C). We demonstrate that FEM-2 exhibits magnesium-dependent casein phosphatase activity, typical of PP2C, in vitro. Point mutations that abolish the casein phosphatase activity of FEM-2 without affecting its FEM-3-binding activity reduce severely its ability to rescue male development in *fem-2* mutant nematodes. These results suggest that protein phosphorylation regulates sex determination in *C. elegans*.

[Key Words: Signal transduction; PP2C; development; protein interaction; phosphorylation; two-hybrid]

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The nematode *Caenorhabditis elegans* normally develops as either a self-fertile hermaphrodite or a male. Male development of both germ-line and somatic tissues requires three genes known as *fem-1*, *fem-2*, and *fem-3*. Mutants that lack the activity of any one of the *fem* genes develop as true females that differ from hermaphrodites only in that they do not produce any sperm (Doniach and Hodgkin 1984; Hodgkin 1986).

The primary sex-determining signal, which regulates the masculinizing activity of the *fem* genes, is the relative number of X chromosomes and sets of autosomes, known as the X/A ratio (Madl and Herman 1979). In diploid strains of *C. elegans*, males have one X chromosome (XO; X/A ratio of 0.5), whereas hermaphrodites have two (XX; X/A ratio of 1.0). The effect of the X/A ratio upon *fem* activity is indirect, involving a well-studied cascade of genetic interactions. A simplified form of that cascade is outlined in Figure 1A. The first portion, including the genes *xol-1* and *sdc-1*, *sdc-2*, and *sdc-3*, controls both sex determination and X chromosome dosage compensation. Control of these two processes diverges at the level of the *sdc* genes, and the remainder of the cascade shown in Figure 1A regulates sex determination exclusively (for review, see Hodgkin 1988; Villeneuve and Meyer 1990a).

Genetic mosaic analyses suggested that sex determination in *C. elegans* involves cell-cell communication (Villeneuve and Meyer 1990b; Hunter and Wood 1992). Molecular analysis supports a model of the sex-determining cascade as a cell-cell signaling pathway (Fig. 1B) in which the products of the *fem* genes act as signal-transducing molecules (for review, see Hodgkin 1992; Kuwabara and Kimble 1992). In XO animals, the low X/A ratio causes transcriptional derepression of *her-1* (Trent et al. 1991; Perry et al. 1993). The predicted product of *her-1* (HER-1) is a small cysteine-rich protein with a secretion signal sequence that is essential for its activity (Perry et al. 1993). HER-1 negatively regulates a predicted transmembrane protein encoded by *tra-2* (TRA-2A), perhaps by means of a direct ligand-receptor interaction (Kuwabara et al. 1992). TRA-2A is a negative regulator of at least one of the predicted intracellular proteins (FEM-1, FEM-2, and FEM-3) encoded by the *fem* genes (Spence et al. 1990; Ahringer et al. 1992; Kuwabara and Kimble 1995; Pilgrim et al. 1995). HER-1-mediated inactivation of TRA-2A relieves the negative regulation of the FEM proteins, and they in turn bring about male somatic development by negatively regulating the products of *tra-1*, one of which (TRA-1A) is a sequence-specific DNA-binding protein (Zarkower and Hodgkin 1992, 1993). The FEM proteins are essential for spermatogenesis even in the absence of *tra-1* activity, indicating that

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Figure 1. Model of somatic sex determination in *C. elegans*. (A) Genetic interactions between genes required for somatic sex determination. (Barred lines) Negative interactions; (arrows) positive interactions. The X/A ratio controls both X chromosome dosage compensation and sex determination via *xol-1* and the *sdc* genes (Villeneuve and Meyer 1990a). The regulatory pathway branches at the level of the *sdc* genes, and only the branch that controls somatic sex determination is shown. A low X/A ratio results in elevated activity of *her-1* and the *fem* genes, which bring about male development by regulating *tra-1* negatively. The *fem* genes have additional targets in the germ-line, because they are required for spermatogenesis irrespective of the state of *tra-1*. (B) Speculative molecular model of somatic sex determination. (Left) A low X/A ratio results in the synthesis of HER-1, a small, secreted protein that inactivates the membrane protein TRA-2A, thereby releasing the cytoplasmic FEM proteins from negative regulation. The FEM proteins then inhibit the activity of the sequence-specific DNA-binding protein, TRA-1A, by an unknown mechanism to cause male development. (Right) A high X/A ratio prevents HER-1 synthesis, allowing TRA-2A to inhibit the FEM proteins and TRA-1A to direct female somatic development.

they also have other, germ-line-specific targets (Doniach and Hodgkin 1984; Hodgkin 1986). Candidates for such targets include the products of the genes *fog-1* and *fog-3*, which are specifically required for spermatogenesis (Barton and Kimble 1990; Ellis and Kimble 1995).

Ignorance of the interactions and activities of the FEM proteins presently obscures the biochemical mechanism that transduces the sex-determining signal from HER-1 to TRA-1. Whether the FEM proteins act sequentially to elaborate a single regulatory activity, interact to form a regulatory complex, or employ independent, nonredundant regulatory mechanisms remains unknown. Their predicted sequences provide few clues as to their mechanisms of action. FEM-1 contains six copies of the cdc10-Swi6 (or ANK) motif, which in many other proteins mediates specific protein-protein interactions (Spence et al. 1990; Michaely and Bennett 1992). FEM-3

is a key target of regulation in the germ-line (Barton et al. 1987; Ahringer and Kimble 1991) and in somatic tissues (A. Mehra, L. Heck, P. Kuwabara, and A.M. Spence, in prep.), but its sequence is novel and provides no hint of its activity (Ahringer et al. 1992). With the view that the identification of FEM-3-interacting proteins would provide a starting point for investigating signal transduction in the sex determination pathway, we performed a two-hybrid screen (Fields and Song 1989; Durfee et al. 1993) for cDNAs that encode such proteins. In this paper, we report that FEM-3 interacts with FEM-2. The sequence of *fem-2*, reported recently by Pilgrim et al. (1995), predicts that FEM-2 is related in sequence to protein serine/threonine phosphatases of Type 2C. We show that FEM-2 is active as a protein phosphatase, and that its phosphatase activity is necessary for its role in promoting male development.

Results

FEM-3 and FEM-2 interact in the yeast two-hybrid system

We used the yeast two-hybrid system (Fields and Song 1989; Durfee et al. 1993) to identify genes encoding proteins that interact with FEM-3. A fusion protein consisting of FEM-3 and the DNA-binding domain of GAL4 (GAL4DB-FEM-3) served to screen a *C. elegans* cDNA library encoding fusion proteins containing the GAL4 activation domain (GAL4AD). Among 2.3 million yeast transformants bearing the plasmid encoding GAL4DB-FEM-3 and a library plasmid, we identified twenty that expressed *HIS3* and *lacZ* reporter genes under the control of the GAL4 binding site, UAS_G. Three transformants carried library plasmids that activated reporter gene expression only in the presence of GAL4DB-FEM-3.

DNA sequence analysis revealed that two of these three plasmids carry cDNAs from the sex-determining gene *fem-2*, the sequence of which was recently reported by Pilgrim et al. (1995). The third interacting clone will be described elsewhere. The *fem-2* cDNAs isolated in our screen differ only in the extent of the 3' untranslated sequence they include; both encode full-length FEM-2, consisting of 449 amino acid residues. Interaction between GAL4DB-FEM-3 and GAL4AD-FEM-2 in the yeast two-hybrid system stimulates the expression of a UAS_G-*lacZ* reporter gene 200-fold above background (Table 1). Neither fusion protein stimulates reporter gene expression when expressed alone or with a control fusion protein containing the complementary GAL4 domain.

Genetic studies have established that *fem-2* and *fem-3* function at the same level in the sex-determining pathway (Doniach and Hodgkin 1984; Kimble et al. 1984; Hodgkin 1986) (Fig. 1A), but previous work has not provided evidence of direct interactions between the products of the *fem* genes. To substantiate the interaction detected in the two-hybrid system, we tested for association between FEM-2 and FEM-3 in *in vitro* binding assays.

Table 1. Interaction of FEM-3 and FEM-2 in the yeast two-hybrid system

DNA-binding domain fusion	Activation domain fusion	Relative β -galactosidase activity ^a	
		mean	range
FEM-3	—	0.7	0.5–0.9
—	SNF4	0.7	0.5–0.9
—	FEM-2	0.7	0.5–1.0
FEM-3	SNF4	1.0	0.6–1.4
FEM-3	FEM-2	200	100–254

^a β -galactosidase activity was measured in liquid cultures of at least three independent transformants. Activity was normalized to the activity resulting from coexpression of a FEM-3 DNA-binding domain fusion and a SNF4 activation domain fusion.

Association of FEM-2 and FEM-3 in vitro

Two assays provided biochemical evidence for a protein–protein interaction between FEM-2 and FEM-3. In the first, we carried out coupled in vitro transcription and translation of *fem-2* cDNA, either alone or together with a clone directing the synthesis of a Myc epitope-tagged derivative of FEM-3 (Myc–FEM-3). We then tested the ability of the monoclonal antibody 9E10, which recognizes the Myc epitope (Evan et al. 1985), to immunoprecipitate the reaction products. The antibody exhibited no reactivity toward FEM-2 alone, but it coprecipitated FEM-2 and Myc–FEM-3 from reactions containing both proteins (Fig. 2).

In an independent test of the ability of FEM-2 to bind to FEM-3, we produced FEM-2 in *E. coli* as a soluble glutathione *S*-transferase fusion protein (GST–FEM-2). We bound GST–FEM-2 to glutathione–Sepharose and used it as a ligand for batch affinity chromatography of in vitro translation reactions containing ³⁵S–Myc–FEM-3. FEM-3 bound specifically to immobilized GST–FEM-2

and not to immobilized GST (Fig. 3). Analysis of bound and unbound fractions by SDS–PAGE and silver staining showed that unlabeled proteins in the reticulocyte lysate failed to bind to the GST–FEM-2 matrix, demonstrating its selectivity for FEM-3 (not shown).

FEM-2 exhibits protein phosphatase activity in vitro

FEM-2 is similar in sequence to protein serine/threonine phosphatases of Type 2C (PP2C), exhibiting 30–35% sequence identity with other members of the family over a region spanning ~260 amino acids (Pilgrim et al. 1995). FEM-2 differs from most PP2C in possessing an amino-terminal extension of 185 amino acids that is novel in sequence. Because the similarity between FEM-2 and the PP2C family suggests that protein phosphorylation might be involved in sex determination in *C. elegans*, we sought to determine whether FEM-2 possesses protein phosphatase activity.

The standard assay for PP2C activity uses ³²P-labeled, phosphorylated casein as a substrate (McGowan and Cohen 1988). In the presence of magnesium, GST–FEM-2, but not GST, readily dephosphorylated ³²P-casein (Fig. 4A). Magnesium dependence is one of the defining characteristics of Type 2C protein phosphatase activity (Cohen 1989). Omission of magnesium from the reaction abolished the casein phosphatase activity of GST–FEM-2 (Fig. 4B), supporting the conclusion that FEM-2 is a Type 2C protein phosphatase.

A typical PP2C requires magnesium for substrate binding (McGowan and Cohen 1988). GST–FEM-2 retained FEM-3-binding activity in the absence of magnesium (Fig. 3), indicating that the interaction between FEM-2 and FEM-3 differs from a canonical PP2C-substrate interaction. FEM-3 may not be a substrate for FEM-2, but we cannot rule out the possibility that it is (see Discussion).

Figure 2. Coimmunoprecipitation of FEM-2 with Myc–FEM-3. ³⁵S-labeled Myc–FEM-3 (lane 1), FEM-2 (lane 2), or both Myc–FEM-3 and FEM-2 (lane 3), synthesized by in vitro translation in reticulocyte lysates. Lanes 4–6 display proteins immunoprecipitated with anti-Myc monoclonal antibody 9E10 from the translation reactions shown in lanes 1–3, respectively. Proteins were analyzed by SDS–PAGE and fluorography.

Figure 3. Interaction of ³⁵S-labeled Myc–FEM-3 with GST–FEM-2-coated beads. Aliquots of an in vitro translation reaction containing ³⁵S–Myc–FEM-3 were incubated with beads coated either with GST (10 mg/ml) or with GST–FEM-2 (5 mg/ml). Binding reactions with GST–FEM-2 beads contained either 20 mM MgCl₂ (+ Mg²⁺, lanes 4,5) or 20 mM EDTA (– Mg²⁺, lanes 6,7). The beads were collected and the supernatant reserved as the unbound fraction. After repeated washing of the beads, they were boiled to release bound proteins. Equal proportions of load (L), unbound (U), and bound (B) fractions were analyzed by SDS–PAGE and fluorography.

Figure 4. Casein phosphatase activity of FEM-2. (A) Time course of phosphate release from ³²P-casein by GST (○), GST-FEM-2(+) (▲), GST-FEM-2(R336K) (■), and GST-FEM-2(R336A) (×). Assays were performed in triplicate with 2 μg of GST fusion protein. Radioactivity released from the substrate at each sampling time is plotted as a percentage of the input radioactivity. (B) Magnesium-dependence of the phosphatase activity of FEM-2. Assays were performed in triplicate with 1 μg of the indicated protein and either 20 mM MgCl₂ or 20 mM EDTA (–Mg²⁺), and reactions were stopped after 15 min. Soluble ³²P detected in the absence of added protein (3–5% of the total) was subtracted from the total to give the values shown.

Mutations affecting the casein phosphatase activity of FEM-2

To test whether male development in *C. elegans* requires the phosphatase activity of FEM-2, we produced mutant forms of the protein that lacked phosphatase activity but retained FEM-3-binding activity. Sequence conservation within the PP2C family provides the only available guide to the sequence requirements for PP2C activity, because the structure of the PP2C catalytic domain and the identities of its catalytically important amino acid residues are unknown. PP2Cs are unrelated in sequence to protein phosphatases of other families

(Shenolikar 1994). Comparison of 14 PP2C sequences from yeasts, protists, plants, and mammals reveals 22 invariant amino acid residues, eight of which lie within the 30-amino-acid region shown in Figure 5. The temperature-sensitive, partial loss-of-function *fem-2* allele, *b245*, carries a missense mutation that substitutes glutamate for the conserved glycine residue at position 339 (Pilgrim et al. 1995). The effects of the *b245* mutation on the phosphatase activity, folding, and stability of FEM-2 are not known, but because *fem-2(b245)* retains significant biological activity (Kimble et al. 1984), we chose to test the effects of mutations affecting a different conserved amino acid residue. We produced mutations causing the replacement of the invariant arginine residue at position 336 (R336) with either lysine (R336K) or alanine (R336A). These substitutions were intended to disrupt interactions involving the side chain of R336 but to have minimal steric or electrostatic effects on other interactions, so as to interfere minimally with the overall structure and stability of the protein (Cunningham and Wells 1989).

GST-FEM-2 fusion proteins carrying either the R336K or the R336A substitution were expressed as soluble proteins in *Escherichia coli*, suggesting that they were not severely misfolded. Both GST-FEM-2(R336K) and GST-FEM-2(R336A) exhibited FEM-3 binding activity similar to that of wild-type GST-FEM-2 in batch affinity chromatography experiments (Fig. 6), supporting the contention that neither mutation grossly perturbed the overall structure of FEM-2. Both mutations nevertheless abolished the casein phosphatase activity of FEM-2, as shown in Figure 4A.

Effects of phosphatase-inactivating mutations on the masculinizing activity of FEM-2

To assay the ability of wild-type and mutant *fem-2* cDNAs to promote male development, we placed them under the control of a *C. elegans* heat-shock promoter and introduced them into nematodes carrying a chromosomal null allele of *fem-2*, *e2105*. Animals homozygous

Rnpp2c α	174	RIQNAGGSV	..MIQ	RVNG	SLAVSRALGD	FDYK	203
Leishpp2c	157	RIENCAGRV	..ENN	RVDG	SLAVSRAFGD	REYK	186
Scptcl	178	RVEQAGGLI	..MKS	RVNG	MLAVTRSLGD	KFFD	207
Spptcl	219	RVTQLGGLM	..VQN	RING	VLAVTRALGD	TYLK	248
Abil	289	RIEAGGKVI	QWNGA	RVFG	VLAMSRSLGD	RYLK	321
FEM-2	311	RVEEAGGQLF	VIGGELRVNG	VNLNTRALGD	VPGR		340

Figure 5. Sequence alignment of FEM-2 with representative PP2C family members in the vicinity of Arginine 336 (indicated with *). The sequence of FEM-2 predicted from cDNA clones isolated in this study agrees with that reported by Pilgrim et al. (1995; GenBank accession no. U29515). Other sequences and their data-base accession numbers are: Rnpp2c, rat PP2Cα (J04503); Leishpp2c, *Leishmania chagasi* PP2C (L15559); Scptcl, *Saccharomyces cerevisiae* Ptc1 (L14593); Spptcl, *Schizosaccharomyces pombe* ptc1 (L26970); Abil, *Arabidopsis thaliana* Abil (X78886).

Figure 6. FEM-3-binding activity of FEM-2 variants lacking phosphatase activity. Beads coated with 3 mg/ml GST (lanes 2,3), 1.5 mg/ml GST-FEM-2(+) (lanes 4,5), GST-FEM-2(R336K) (lanes 6,7), or GST-FEM-2(R336A) (lanes 8,9) were tested for binding to 35 S-Myc-FEM-3 produced by in vitro translation. Equal proportions of the in vitro translation reaction (L, lane 1), the unbound (U) and bound (B) fractions were analyzed by SDS-PAGE and fluorography.

for *fem-2(e2105)*, if descended from a homozygous *fem-2* mother, develop as females at 25°C irrespective of their karyotype (Hodgkin 1986). We refer to these animals as *fem-2 m-z-* (following the convention of Hodgkin 1986) to indicate that they lack both maternal and zygotic *fem-2* activity. We produced *fem-2 m-z-* mutants carrying wild-type or mutant heat shock-*fem-2* (*hs-fem-2*) transgenes on extrachromosomal arrays. We then subjected them to heat shocks at intervals of 18 hr throughout development and determined the extent to which transgene expression rescued male development. To allow assessment of masculinizing activity in both XX and XO *fem-2 m-z-* animals, we used a strain homozygous for the *him-5(e1490)* mutation, which causes hermaphrodites to produce about 30% XO self-progeny (Hodgkin et al. 1979).

Heat shock-induced synthesis of amino-terminally Myc-tagged FEM-2 (HS-Myc-FEM-2) caused masculinization in 30% of transgenic *fem-2 m-z-*; *him-5* mu-

tant animals (Table 2). We observed extensive masculinization of the somatic gonad, ventral hypodermis, and the tail structures (Table 2; Fig. 7). Rescue of spermatogenesis was very infrequent, probably because the transgene was expressed poorly in the germ line. Several groups have reported previously that germ-line expression of transgenes controlled by the heat shock promoter is weak or undetectable (Stringham et al. 1992; Roehl and Kimble 1993; Kuwabara and Kimble 1995; Browning and Strome 1996). Because the fraction of transgenic animals exhibiting somatic masculinization corresponded closely to the fraction of XO animals produced by *him-5(e1490)* homozygotes, we conclude that transgene expression allowed at least partial rescue of male development in the majority of XO *fem-2 m-z-* individuals. The inference that all of the masculinized *fem-2*; *him-5* animals were of XO karyotype is supported by the observation that HS-Myc-FEM-2(+) did not cause inappropriate somatic masculinization of XX animals even when they were chromosomally *fem-2(+)* (Table 2).

Phosphatase-inactivating substitutions of arginine 336 compromised the rescuing activity of HS-Myc-FEM-2 severely. The alanine mutant, HS-Myc-FEM-2(R336A), was almost completely ineffectual at rescuing male development in *fem-2 m-z-*; *him-5* mutants. The only apparent sign of masculinization among 166 mutant animals expressing HS-Myc-FEM-2(R336A) was the presence, in the tails of two animals, of small refractile deposits that may have resulted from abortive spicule development (Table 2). Replacement of arginine 336 by lysine also impaired *fem-2* function, although somewhat less dramatically. HS-Myc-FEM-2(R336K) caused observable masculinization in only 7% of the transgenic *fem-2 m-z-*; *him-5* mutants examined after repeated heat shock, corresponding to about 1/4 of the expected number of XO animals in the sample. Among the few animals masculinized by HS-Myc-FEM-2(R336K), the

Table 2. Effect of phosphatase-inactivating mutations on the masculinizing activity of FEM-2

<i>fem-2</i> genotype	Karyotype ^a	HS-FEM-2 variant	Heat shock	% masculinized rollers ^b (n)	Tissues exhibiting masculinization (number with indicated masculinized feature/number of masculinized animals examined ^c)					
					vulva or absent or defective	somatic gonad	fan	tail rays	spicule material	sperm
+	XX	Myc-FEM-2(+)	+	0 (81)						
<i>e2105</i>	XX + XO	Myc-FEM-2(+)	+	30 (253)	39/47	31/47	46/47	38/47	47/47	1/47
			–	8 (381)	16/31	8/31	18/31	16/31	30/31	0/31
<i>e2105</i>	XX + XO	Myc-FEM-2(R336K)	+	7 (201)	5/15	5/15	8/15	3/15	10/15	0/15
			–	2 (327)	0/6	0/6	0/6	1/6	6/6	0/6
<i>e2105</i>	XX + XO	Myc-FEM-2(R336A)	+	1 (166)	0/2	0/2	0/2	0/2	2/2	0/2
			–	0 (337)						

^aTransgenic lines carrying *fem-2(e2105)* also carried *him-5(e1490)*, which caused the production of about 30% XO animals.

^bThe total number of masculinized animals was scored using a dissecting microscope (40× magnification). The total number of transgenic animals examined is given in parentheses.

^cAnimals exhibiting masculinization visible in the dissecting microscope were examined using Nomarski microscopy (400× magnification) to assess the masculinization of various tissues. All masculinized animals expressing mutant forms of Myc-FEM-2 and over half of the animals that were masculinized by expression of Myc-FEM-2(+) were examined in this way.

Figure 7. Rescue of male development by heat-shock expression of Myc-FEM-2. (A–C) Nomarski photomicrographs and tracings showing gonad morphology: (A) *fem-2* m–z– female, raised at 25°C (XX or XO), showing only the posterior gonad arm; (B) wild-type XO male; (C) *fem-2* m–z– XO intersex expressing HS-FEM-2(+), with rescued male gonad morphology. (dg) distal gonad arm (dorsal in female gonad in A, and ventral in male gonads in B and C); (v) vulva; (vd) vas deferens; (o) oocyte; (s) sperm. (D–H) Nomarski photomicrographs showing tail morphology. (D) *fem-2* m–z– female, raised at 25°C (XX or XO); (E) wild-type XO male; (F–H) *fem-2* m–z– XO animals expressing HS-FEM-2(+), showing variation in the extent of rescue of male tail development. Heat-shock expression of FEM-2(+) usually caused partial rescue of the tail fan, rays, and spicules, as in F and G. Expression of FEM-2(R336K) caused weaker and less frequent rescue of male tail development. Masculinized animals expressing FEM-2(R336K) generally exhibited masculinization similar to or weaker than the animal shown in H. Expression of FEM-2(R336A) rarely caused any detectable masculinization, as detailed in Table 2 and in the text.

average extent of male development was significantly lower than in mutants rescued by HS-Myc-FEM-2(+) (Table 2). HS-Myc-FEM-2(+) and both the R336K and R336A mutants accumulated to similar levels in nematodes following a single heat shock, arguing that the difference in their masculinizing activities did not result from differences in synthesis or stability (Fig. 8). Instead, we conclude that the protein phosphatase activity of FEM-2 is important for its normal role in promoting male development, and that the R336A and R336K substitutions impair the masculinizing activity of FEM-2 as a direct consequence of their effects on its phosphatase activity. Our results imply that signaling through the sex determination pathway in *C. elegans* involves the regulation of protein phosphorylation.

Discussion

The FEM proteins transduce an extracellular, masculin-

izing signal to bring about the negative regulation of TRA-1 in somatic tissues and the activation of other functions that are required for spermatogenesis. We have demonstrated a specific interaction between two of the FEM proteins, FEM-3 and FEM-2. We have shown that purified, bacterially expressed GST-FEM-2 exhibits magnesium-dependent casein phosphatase activity typical of PP2C, and we have provided evidence that the phosphatase activity of FEM-2 is required for male development.

Significance of the interaction between FEM-3 and FEM-2

Genetic analysis of *fem-2* and *fem-3* established that both are required for male development and that both act at the same level in the genetic cascade controlling sex determination (Hodgkin 1986). On the basis of their ability to associate in the yeast two-hybrid system and in

Figure 8. Expression of wild-type and mutant Myc-FEM-2 in transgenic nematodes. Lysates prepared from animals maintained continuously at 25°C (– heat shock; lanes 1,3,5,7), and from animals subjected to a 2-hr heat shock and a 2-hr recovery period at 25°C (+ heat shock; lanes 2,4,6,8), were analyzed by SDS-PAGE and immunoblotting with monoclonal antibody 9E10. Lysates were from transgenic nematodes carrying plasmids encoding Myc-FEM-2(+) (lanes 1,2), Myc-FEM-2(R336K) (lanes 3,4), or Myc-FEM-2(R336A) (lanes 5,6), or from nontransgenic *him-5* nematodes (lanes 7,8). Each Myc-FEM-2 variant (~55 kD Mr) accumulates to similar levels following heat shock.

vitro, we suggest that FEM-2 and FEM-3 interact directly in *C. elegans* to promote male development. Understanding the functional consequences of the interaction between FEM-2 and FEM-3 will require further analysis, but here we consider three models. In the first, FEM-3 regulates FEM-2 positively. FEM-3 binding is clearly not required in vitro for the phosphatase activity of FEM-2, but it may serve to target specific substrates for dephosphorylation in vivo. It could do so by localizing FEM-2 to a particular subcellular compartment or by increasing the affinity or specific activity of FEM-2 toward its substrates. In this case the role of FEM-3 would be analogous to the roles of the targeting subunits of Type 1 protein serine/threonine phosphatases (PP1) or the regulatory subunits of PP2A (Cohen 1989; Shenolikar 1994). Although canonical PP2C enzymes exist as monomers (Cohen 1989), FEM-2 is one of several members of the PP2C family that contain, in addition to the catalytic domain, an amino-terminal domain that might mediate associations with regulators (Leung et al. 1994; Meyer et al. 1994; Stone et al. 1994; Pilgrim et al. 1995).

A second possibility is that FEM-2 dephosphorylates FEM-3, activating it for binding to other target proteins. The interaction between FEM-2 and FEM-3 differs from other PP2C-substrate interactions (McGowan and Cohen 1988) in that it is magnesium independent. However, as FEM-2 has a specific developmental role, it might have a unique substrate recognition domain, perhaps involving its unique amino-terminal extension (Pilgrim et al. 1995). Because TRA-2A is the principal negative regulator of the FEM proteins (Kuwabara and Kimble 1995), FEM-3 phosphorylation, if it occurs, must be either TRA-2A-dependent or else insufficient to inhibit FEM activity in the absence of TRA-2A. TRA-2A does not resemble a kinase in sequence (Kuwabara et al.

1992), but it could recruit a kinase to FEM-3. A model in which FEM-2 activates FEM-3 by dephosphorylation, although subject to several constraints, cannot be excluded presently.

Finally, interaction between FEM-2 and FEM-3 need not alter the activity of either participant but may serve instead to facilitate their cooperation in regulating common targets. For example, dephosphorylation of a target protein by FEM-2 may allow FEM-3 to bind and regulate the activity of that target.

Rescue of the fem-2 mutant phenotype by a heat-shock promoter-driven cDNA

Extrachromosomal arrays expressing Myc-FEM-2(+) under the control of a *C. elegans* heat-shock promoter (Stringham et al. 1992; Mello and Fire 1995) allowed extensive rescue of XO male somatic development. However, we seldom observed complete somatic rescue. We obtained identical results with an untagged HS-FEM-2(+) transgene, establishing that the Myc epitope tag did not interfere with FEM-2 function (our study, unpubl.). Mitotic loss of the transgene, resulting in genetic mosaicism (Stinchcomb et al. 1985), may have limited the extent of rescue. In addition, pulsed expression of the transgene at regular intervals may not have provided adequate levels of FEM-2 at the correct times in all sexually dimorphic tissues.

Requirement for FEM-2 protein phosphatase activity in male development

Substitution of arginine 336 of FEM-2, a residue that is invariant within the PP2C family, with either lysine or alanine inactivates FEM-2 as a casein phosphatase and impairs dramatically its ability to rescue male development in *fem-2* m–z– XO mutants. Because the mutant proteins are expressed stably in transgenic nematodes, and because they retain FEM-3-binding activity similar to wild-type FEM-2 in vitro, we attribute the defect in their masculinizing activity primarily to their inactivity as protein phosphatases. FEM-2(R336K) and FEM-2(R336A) are not, however, equivalent with respect to their ability to promote male development. Although both mutant proteins lack detectable casein phosphatase activity, FEM-2(R336K) retains significantly more residual masculinizing activity than FEM-2(R336A), which is essentially inactive. It may be that FEM-2(R336K) retains significant phosphatase activity in vivo against its natural substrate, whereas FEM-2(R336A) does not. However, it is also possible that a phosphatase-independent mechanism can cause incomplete male development.

Genetic evidence suggests that limited male development can occur in the absence of *fem-2* function: The nonsense mutation *e2105*, which truncates the *fem-2* open reading frame near the beginning of the phosphatase domain (Pilgrim et al. 1995), causes only partial feminization of XO *fem-2* m–z– mutants at 15°C (Hodgkin 1986). The temperature sensitivity of *fem-*

2(e2105) is susceptible to two types of explanation: the first is that another protein phosphatase is able to substitute for FEM-2 at low temperature. This possibility should not be discounted, as partial or complete functional redundancy among protein phosphatases in signal transduction is not uncommon (Maeda et al. 1993; Hunter 1995; Shiozaki and Russell 1995). The second possibility is that FEM-1 and FEM-3 can bring about incomplete male development at 15°C by a phosphatase-independent mechanism. FEM-2(R336K), even if it lacks phosphatase activity utterly, might potentiate a phosphatase-independent regulatory activity of FEM-1 and FEM-3. For example, FEM-2(R336K) might mediate the formation of a complex involving FEM-3, FEM-1, and TRA-1, resulting in partial inhibition of TRA-1 even at 25°C, the incubation temperature in our experiments. This type of model would oblige us to suppose that FEM-2(R336A) is less effective than FEM-2(R336K) at stimulating the other FEM proteins because of a defect in an as-yet-unidentified interaction.

Candidate targets for the FEM-2 phosphatase

A requirement for the phosphatase activity of FEM-2 in sex determination, even if it is not an absolute requirement, implies that FEM-2 promotes male development by antagonizing the feminizing activity of a protein kinase. The identity of this kinase and the substrate that it shares with FEM-2 remain to be determined. Either of the other FEM proteins might be regulated negatively by phosphorylation. We have already discussed a model in which FEM-2 activates FEM-3 by dephosphorylation. An analogous model that invokes dephosphorylation as a mechanism for activating FEM-1 is subject to the same constraints.

TRA-1 proteins, as the principal targets of *fem* activity in somatic tissues, are candidate substrates for FEM-2-mediated dephosphorylation. Gain-of-function mutations in *tra-1* render its activity insensitive to regulation by the FEM proteins (Hodgkin 1987). Most are missense mutations that affect a small amino-terminal region common to TRA-1A and TRA-1B, referred to as the GF region (De Bono et al. 1995). Two *tra-1* splicing mutations that cause deletion of the GF region also have gain-of-function properties (De Bono et al. 1995). The GF region may be a target for a negative regulator, perhaps one of the FEM proteins. Models in which FEM-2 regulates TRA-1 by dephosphorylation in a manner sensitive to the GF region could take several forms. The GF region may constitute an intrinsically inhibitory domain of TRA-1, and phosphorylation of this domain might prevent it from exercising an inhibitory influence, perhaps by locking TRA-1 in an active conformation. In this case, dephosphorylation by FEM-2 would allow the GF region to reassert its inhibitory activity. Alternatively, phosphorylation at a site outside the GF region might be necessary for TRA-1 activation, but FEM-2-mediated dephosphorylation might require recognition of the GF region by FEM-2 itself or the other FEM proteins. In either of these models, mutations that alter or delete the GF

region could have the effect of preventing dephosphorylation and inactivation of TRA-1. A third possibility is that dephosphorylation of the GF region by FEM-2 may be a prerequisite for the binding of another negative regulator, perhaps another FEM protein, to that region.

The FEM proteins have targets other than TRA-1 in the germ-line (Doniach and Hodgkin 1984; Hodgkin 1986), any of which might also be a substrate for FEM-2. Candidates include the products of the genes *fog-1* and *fog-3* (Barton and Kimble 1990; Ellis and Kimble 1995), which have not been characterized molecularly.

Conclusion and future directions

Our results demonstrate that regulation of protein phosphorylation plays an important role in signal transduction through the sex-determining pathway in *C. elegans*. Enzymes of the PP2C family have been implicated previously in signal transduction pathways controlling osmoregulation in yeast (Maeda et al. 1994; Shiozaki and Russell 1995) and responses to abscisic acid in *Arabidopsis* (Leung et al. 1994; Meyer et al. 1994), but FEM-2 is the first example of a Type 2C protein serine/threonine phosphatase that is required specifically for the regulation of development in a metazoan. Progress in understanding the role of phosphorylation in sex determination will require identification of the endogenous substrates of FEM-2 and an analysis of the effects of FEM-3 binding on the subcellular distribution and specificity of FEM-2. At least one class of gene involved in sex determination likely awaits identification: at least one gene must encode a kinase that phosphorylates the substrate of FEM-2. Such a kinase is presumably necessary for the adoption of female cell fates, although its role need not be restricted to the regulation of sex determination. The sex-determining pathway in *C. elegans* may provide an example of a signal transduction pathway in which the regulation of protein phosphatase activity, rather than the activity of the opposing protein kinases, is paramount in determining the output of the pathway.

Materials and methods

C. elegans strains and culture

Methods for the culture and genetic manipulation of *C. elegans* were those described by Sulston and Hodgkin (1988), except that we used MYOB agar (Church et al. 1995) in place of NGM. We used the standard wild-type strain of *C. elegans* var. Bristol, N2, (Brenner 1974). Strain AS204, a derivative of N2, is of genotype *fem-2(e2105)/unc-45(r450) dpy-1(e1) III; him-5(e1490) V*. [*fem-2(e2105)*] (Hodgkin 1986) is a nonsense allele that is likely to be null (Pilgrim et al. 1995). *him-5(e1490)* (Hodgkin et al. 1979) causes homozygous hermaphrodites to produce about 30% XO self-progeny as a result of X chromosome nondisjunction. The *unc-45(r450)* and *dpy-1(e1)* marker mutations are described in Hodgkin (1988).]

Yeast two-hybrid screen

Yeast were grown on standard complete and selective media as appropriate. (Sherman 1991). Yeast transformation was per-

formed using the LiAc/single-stranded carrier DNA/PEG method (Schiestl and Gietz 1989).

We carried out a two-hybrid screen using the system described by Durfee et al. (1993). Dr. R. Barstead (Oklahoma Medical Research Foundation) generously provided a *C. elegans* cDNA library in λ ACT. The Cre-expressing *E. coli* strain RB4E (R. Barstead, pers. comm.), a derivative of BNN132 (Elledge et al. 1991), was used for conversion of λ ACT clones to plasmids. Yeast strain Y153 was transformed to tryptophan prototrophy with plasmid AS#1197, a pAS1 derivative that directs the expression of FEM-3 fused to the DNA-binding domain of GAL4 (GAL4DB-FEM-3). The resulting strain was transformed with library plasmid DNA, and Trp⁺, Leu⁺, His⁺, lacZ⁺ transformants were identified as described by Durfee et al. (1993). Library plasmids were recovered from these transformants, and the recovered plasmids were retested for interaction with GAL4DB-FEM-3 in Y153. Those that were positive upon retesting were introduced into Y153 alone or with pSE1112, which encodes a GAL4DB-SNF1 fusion (Durfee et al. 1993) that served as a negative control. Three plasmids that activated reporter gene expression only in the presence of GAL4DB-FEM-3 were retained for further analysis. β -galactosidase activity in liquid cultures was assayed as described (Ausubel et al. 1989). Three or more independent transformants of each type were assayed. Values were normalized to the level of activity resulting from coexpression of the FEM-3 DNA-binding domain fusion and a SNF4 activation domain fusion (Durfee et al. 1993).

DNA sequencing (Sanger et al. 1977) revealed that two of the three plasmids recovered in the two-hybrid screen carried *fem-2* cDNA inserts. Both *fem-2* cDNAs included the complete *fem-2* coding sequence and 5' untranslated region (which does not contain any in-frame stop codons), but they differed in the extent of the 3' untranslated sequence they contained. The *fem-2* cDNA carried by plasmid AS#1245 was used for further experiments.

Coimmunoprecipitations

The cDNA insert from plasmid AS#1245 was subcloned in plasmid T7 β Sal (Norman et al. 1988) to produce plasmid AS#1285. Transcription of AS#1285 with T7 RNA polymerase produces an mRNA encoding FEM-2 with an amino-terminal extension of 24 amino acid residues. Plasmid AS#1184 consists of a *fem-3* cDNA in a derivative of T7plink that carries the sequence encoding the 9E10 Myc epitope tag (Dalton and Treisman 1992; Bardwell and Treisman 1994). Transcription of AS#1184 with T7 RNA polymerase produces an mRNA that encodes aminoterminally Myc epitope-tagged FEM-3.

Plasmids AS#1184 and AS#1285 were transcribed and translated in the presence of [³²S]methionine using the T7 TNT system (Promega). A 5 μ l aliquot of a translation reaction containing either [³⁵S]-FEM-2, [³⁵S]-FEM-3, or both, was diluted to 100 μ l with Buffer A [10% glycerol, 20 mM HEPES (pH 7.9), 50 mM ammonium sulfate, 1 mM DTT, 1% Triton X-100, 0.1% SDS, 175 μ g/ml PMSF, and 10 μ g/ml each of aprotinin, leupeptin, and pepstatin; Kolodziej and Young 1991]. Subsequent steps were carried out at 4°C. The solution was mixed with 5 μ l of Protein A Sepharose CL-4B (Pharmacia) in Buffer A (100 mg/ml) for 30 min and centrifuged at 5000 rpm for 30 sec. A cell culture supernatant containing monoclonal antibody 9E10 (4 μ l) (Evan et al. 1985) was added to the supernatant and mixed for 1 hr. Protein A-Sepharose (25 μ l of a 100 mg/ml suspension) was added and mixed for 1 hr before being collected by centrifugation as above. The immunoprecipitates were washed twice in RIPA-500 buffer [50 mM Tris-HCl (pH 8), 500 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, and 1% Triton X-100], and once in

Buffer A. They were resuspended in SDS sample buffer, boiled, centrifuged briefly, and samples of the supernatants were analyzed on 10% SDS-polyacrylamide gels. Gels were fixed, treated with Amplify (Amersham) and exposed to X-ray film at -70°C.

Preparation of GST-FEM-2

The *fem-2* cDNA insert of plasmid AS#1245 was subcloned in pGEX-4T2 (Pharmacia) to produce AS#1252, which encodes GST-FEM-2. Synthesis of GST-FEM-2 was induced with 0.1 mM IPTG in *E. coli* DH5 cells carrying AS#1252. Cells were harvested by centrifugation, resuspended in PBST [150 mM NaCl, 3 mM KCl, 12 mM Na phosphate (pH 7.2), and 1% Triton X-100] and lysed by sonication. The lysate was centrifuged to remove debris, and the supernatant was incubated with glutathione-Sepharose beads (Pharmacia). The beads were collected by centrifugation and washed three times with PBS. GST-FEM-2 was eluted with 25 mM glutathione in 50 mM Tris-HCl, pH 8.0. After dialysis, the concentration of GST-FEM-2 was estimated by Bradford assay (Bio-Rad). Samples were subjected to SDS-PAGE and Coomassie staining to verify that full-length GST-FEM-2 was the predominant species.

Plasmids encoding GST-FEM-2(R336K or R336A) fusion proteins were produced in two steps: the pGEX-*fem-2* plasmid AS#1252 was digested with *Hind*III and recircularized to delete the 500 bp *Hind*III fragment of *fem-2* cDNA, and the corresponding *Hind*III fragment carrying the appropriate mutation was subcloned in the resulting plasmid.

Phosphatase assay

Casein was phosphorylated with [γ -³²P]ATP and protein kinase A, purified over Sephadex G-50, and used as a substrate to assay the phosphatase activity of GST-FEM-2, using the methods described by McGowan and Cohen (1988). Assays were performed in triplicate using 1–2 μ g of GST or GST-FEM-2 in a reaction volume of 50–200 μ l. Reactions contained 20 mM MgCl₂ or 20 mM EDTA (-Mg²⁺ in Figs. 3 and 4).

Batch affinity chromatography

Aliquots (25 μ l) of glutathione-Sepharose beads carrying either GST or GST-FEM-2 were suspended in 100 μ l of PBS containing 1% Triton X-100, 175 μ g/ml PMSF, and 10 μ g/ml each of pepstatin, leupeptin, and aprotinin. In initial experiments, GST-FEM-2 was bound to the beads at a concentration of 5 mg/ml, GST at 10 mg/ml. A 5- μ l aliquot of an in vitro translation reaction containing ³⁵S-labeled Myc-FEM-3 (load) was added to the suspension. After incubation at room temperature for 1.5 hr, the beads were collected by centrifugation, and the supernatant was saved as the unbound fraction. The beads were washed 4 times with RIPA-500 before being boiled in SDS sample buffer to release bound proteins. Equal proportions of load, unbound, and bound fractions were analyzed by SDS-PAGE and fluorography, as described above.

Tests of whether the R336K or R336A mutations affected FEM-3 binding to GST-FEM-2 were performed with all GST-FEM-2 proteins at a concentration of 1.5 mg/ml.

Mutagenesis of codon 336 of *fem-2*

A 500-bp *Hind*III fragment of *fem-2* cDNA subcloned in pBlue-scriptII (Stratagene) was mutagenized with the degenerate oligonucleotide oAS150 [5'-CTAAATCTCACCA(A or G)(A or C)AGCACTCGGAG-3'] using the method of Kunkel et al. (1987).

Clones carrying mutations encoding R336K and R336A amino acid substitutions were identified by DNA sequencing. Mutant clones used for construction of expression plasmids were sequenced in their entirety.

Construction of plasmids for heat shock-induced expression of FEM-2 and Myc-FEM-2

The *fem-2* cDNA in plasmid AS#1245 was cloned in pPD49.83 (Mello and Fire 1995) to produce AS#1255, which directs the expression of wild-type FEM-2 under the control of the *hsp16-41* promoter.

A 640-bp *XhoI*–*Bam*HI fragment of AS#1245 was subcloned in pBluescriptII(KS)+. It was amplified by PCR using primers oAS157 (5'-GTGTGGTACCATGCGCGCCGCGAAAAAGTAAACGAGGAGC-3') and FP1 (5'-GTAAAACGACGGC-CAGT-3') to introduce an *NcoI* site at the start codon of *fem-2* and a *NotI* site immediately downstream. The amplification product was subcloned in pBluescriptII(SK)+ as a *KpnI*–*Bam*HI fragment. A cassette composed of annealed oligonucleotides oAS153 (5'-GGCCGAACAGAAGCTTATC-TCTGAGGAGATCTG-3') and oAS154 (5'-GGCCCAGATCTTCCTCAGAGATAAGCT-TCTGTTC-3'), encoding the 9E10 Myc epitope, was then inserted into the newly introduced *NotI* site in *fem-2*. [The *NotI* site in the pBSII(SK)+ polylinker was first eliminated.] The entire insert was sequenced. A three-way ligation involving the 300-bp *NcoI*–*Sall* fragment of this plasmid, a 1.4-kb *Sall*–*SmaI* fragment of AS#1255, carrying the remainder of *fem-2* cDNA, and the 3.8-kb *NcoI*–*EcoRV* fragment of pPD49.83 (Mello and Fire 1995) produced plasmid AS#1315. The *hsp16-41* promoter in AS#1315 directs the expression of a mRNA encoding full-length FEM-2 bearing an amino-terminal Myc tag. Homologous plasmids directing the synthesis of FEM-2(R336K) and FEM-2(R336A) were constructed in the same way, except that the 1.4-kb *Sall*–*SmaI* fragment in each case was from a *fem-2* plasmid carrying the relevant mutation.

Heat shock rescue experiments

Nematode transformation was carried out using the methods described by Mello and Fire (1995). Hermaphrodites of either strain N2 or AS204 were injected with a DNA mixture containing 50 µg/ml of plasmid pRF4, which carries the transformation marker *rol-6(su1006dm)* (Mello et al. 1991), 20 µg/ml of the heat shock-*fem-2* plasmid being tested, and 30 µg/ml of pBluescript to bring the total DNA concentration to 100 µg/ml. Transgenic lines carrying the injected DNA on extrachromosomal arrays were established from F2 Rol progeny of injected animals.

Lines derived from AS204 were used to test the ability of each heat shock-*fem-2* construct to rescue *fem-2(e2105)*. Each plasmid was tested in at least three independent transgenic lines. Single Rol, non-Dpy, non-Unc XX animals were picked as L4 larvae to separate plates at 25°C. As *fem-2* exhibits maternal rescue (Hodgkin 1986), those animals that matured into self-fertile hermaphrodites were of two classes: one-third were *fem-2(e2105)* homozygotes, whereas the remaining two-thirds were heterozygous. Each Roller hermaphrodite was transferred to a new plate every 12–24 hr. Embryos deposited on one plate were allowed to develop at 25°C to determine the genotype of the mother. The progeny of *fem-1(e2105)* homozygotes develop as females at 25°C (Hodgkin 1986). Embryos deposited on a second plate were subjected to a 1-hr heat shock at 33°C, followed by 20 min at 20°C, followed by 1 hr at 33°C. The same heat treatment was repeated at intervals of 18 hr. Animals were maintained at 25°C at other times. Heat-shocked transgenic animals de-

scended from *fem-2* homozygotes were scored for heat shock-induced masculinization upon reaching maturity. The total numbers of masculinized animals observed with a dissecting microscope (40× magnification) are listed in column 5 of Table 2. Samples of masculinized animals were examined by Nomarski microscopy (400× magnification) to determine the extent of male development in various tissues (Table 2, columns 6–11). The following features were taken as indicative of masculinization: an asymmetric, unlobed somatic gonad; failure of vulval development, resulting in a pseudovulva or the complete absence of a vulva; presence of a tail fan, rays, or spicules; presence of sperm.

Western blotting

We assayed expression of HS-Myc-FEM-2 and its mutant derivatives by immunoblotting with monoclonal antibody 9E10. Twenty Rol, non-Dpy, non-Unc hermaphrodites carrying a given HS-Myc-FEM-2 transgene in the genetic background of strain AS204 were picked to each of two plates and allowed to lay eggs for 3–4 days. The progeny on one plate were then subjected to a 2-hr heat shock at 33°C and allowed to recover for 2 hr at 25°C. The other plate was incubated at 25°C continuously. Nematodes were harvested in M9 buffer, rinsed in M9 and again in water, and lysed by boiling in SDS sample buffer. Half of the resulting lysate was electrophoresed on an SDS polyacrylamide gel and transferred to nitrocellulose. Blotto [5% skim milk powder in TBST: 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween 20] was used for blocking blots and diluting antibodies, and washes were carried out with TBST (Harlow and Lane 1988). Monoclonal antibody 9E10 was used as a 1:50 dilution of cell culture supernatant. Peroxidase-conjugated donkey anti-mouse secondary antibody (Jackson Laboratories) was used at a 1:30,000 dilution, and it was detected using the ECL system (Amersham).

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