Post-transcriptional Regulation of Sex Determination in *Caenorhabditis elegans*: Widespread Expression of the Sex-determining Gene *fem-1* in Both Sexes

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Submitted March 20, 1996; Accepted May 3, 1996
Monitoring Editor: Judith Kimble

The *fem-1* gene of *C. elegans* is one of three genes required for all aspects of male development in the nematode. Current models of sex determination propose that the products of the *fem* genes act in a novel signal-transduction pathway and that their activity is regulated primarily at the post-translational level in somatic tissues. We analyzed the expression of *fem-1* to determine whether it revealed any additional levels of regulation. Both XX hermaphrodites and XO males express *fem-1* at approximately constant levels throughout development. Somatic tissues in hermaphrodites adopt female fates, but they nonetheless express *fem-1* mRNA and FEM-1 protein, suggesting that the regulation of *fem-1* activity is post-transcriptional and probably post-translational. A compact promoter directs functional expression of *fem-1* transgenes, as assayed by their masculinizing activity in *fem-1* mutants. Activity also requires any two or more introns, suggesting that splicing may enhance *fem-1* expression. The minimal noncoding sequences required for activity of *fem-1* transgenes suffice to direct expression of a *fem-1::lacZ* reporter gene in all somatic tissues in both sexes. Many *fem-1* transgenes, including those that rescue male somatic development in *fem-1* mutants, paradoxically feminize the germline. We suggest that they do so by interfering with the germline expression of the endogenous *fem-1* gene.

INTRODUCTION

In the nematode *Caenorhabditis elegans*, the primary sex-determining signal is the ratio of the number of X chromosomes to the number of sets of autosomes (the X/A ratio) (Madl and Herman, 1979). Diploid animals with only one X chromosome (XO) develop as males, whereas those with two X chromosomes (XX) develop as self-fertile hermaphrodites. The hermaphrodite is somatically female, but it briefly produces sperm before making oocytes.

Mutations that cause sexual transformation define a number of genes that control the choice between male and female cell fates in response to the X/A ratio. Epistatic interactions among these genes suggest that in somatic tissues they act in the regulatory cascade illustrated in Figure 1A (reviewed by Hodgkin, 1988;

Villeneuve and Meyer, 1990). In this model, genes at each level in the cascade inhibit the activity of the genes at the next level downstream.

In the context of the regulatory relationships outlined in Figure 1A, the sequences of the sex-determining genes suggest that these genes encode the components of a cell-cell signaling pathway (reviewed by Hodgkin, 1992b; Kuwabara and Kimble, 1992). Figure 1B summarizes a current model of the pathway. In XX animals the cytoplasmic domain of the transmembrane protein TRA-2A, encoded by *tra-2*, interacts with and inhibits the activity (Kuwabara *et al.*, 1992; Kuwabara and Kimble, 1995) of at least one of the three intracellular proteins, FEM-1, FEM-2, and FEM-3, encoded by the *fem* genes (Spence *et al.*, 1990; Ahringer *et al.*, 1992; Pilgrim *et al.*, 1995). When FEM activity is inhibited, a sequence-specific DNA-binding protein encoded by *tra-1*, TRA-1A, (Zarkower and Hodgkin, 1992, 1993) directs female development,

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Figure 1. Model of somatic sex determination in *C. elegans*. (A) Formal model of the genetic interactions between the sex-determining genes, after Hodgkin (1992a). Bold type indicates high gene activity, normal type indicates low activity, barred lines denote negative interactions, arrows indicate positive ones. Several genes that act upstream of *her-1* are not shown. They also regulate X-chromosome dosage compensation via a second pathway (Villeneuve and Meyer, 1990). Sex determination in the germline involves additional germline-specific regulators that are not shown here (Clifford *et al.*, 1994). (B) Molecular model of somatic sex determination, after Kuwabara and Kimble (1992). This speculative model is based on the predicted sequences of the sex-determining proteins, the order of their genetic interactions (outlined in A), and the noncell-autonomous behavior of *her-1* in genetic mosaics. In XX animals the integral membrane protein TRA-2A inhibits the FEM proteins, perhaps by sequestering one or more of them (Kuwabara *et al.*, 1992; Kuwabara and Kimble, 1995). The sequence-specific DNA-binding protein TRA-1A (Zarkower and Hodgkin, 1992, 1993) brings about female development by regulating the transcription of target genes. In XO animals the secreted protein HER-1 (Hunter and Wood, 1992; Perry *et al.*, 1993) inactivates TRA-2A and relieves the inhibition of the FEM proteins. The FEM proteins then inhibit TRA-1A by an unknown mechanism, preventing it from regulating the transcription of downstream genes. Inhibition of TRA-1A results in male development.

probably by regulating the transcription of other genes. In XO animals, expression of the *her-1* gene produces a secreted protein HER-1 (Perry *et al.*, 1993) that inactivates TRA-2A, perhaps by means of a direct interaction. The noncell-autonomous behavior of *her-1* in genetic mosaics (Hunter and Wood, 1992) is consistent with the proposal that its product acts as a secreted masculinizing signal. Inactivation of TRA-2A

freed the FEM proteins to inhibit TRA-1A by an unknown mechanism, allowing male development to proceed in all somatic tissues.

In the germline, the regulatory cascade involves additional, germline-specific factors. The *fem* genes are essential for spermatogenesis in both males and hermaphrodites (Doniach and Hodgkin, 1984; Hodgkin, 1986). In contrast to their function in somatic tissues, the role of the *fem* genes in the germline is not only to inhibit *tra-1*, because mutants that lack *tra-1* activity still require *fem* activity for spermatogenesis (Doniach and Hodgkin, 1984; Hodgkin, 1986). The genes *fog-1* and *fog-3* are required specifically for spermatogenesis and may be targets for activation by the FEM proteins (Barton and Kimble, 1990; Ellis and Kimble, 1995). Other germline-specific sex-determining genes, including *fog-2* and the *mog* genes, regulate *fem* activity in the hermaphrodite germline to allow a brief period of spermatogenesis followed by a switch to oogenesis (Schedl and Kimble, 1988; Graham and Kimble, 1993; Graham *et al.*, 1993; for a review of germline sex determination, see Clifford *et al.*, 1994).

The model of somatic sex determination diagrammed in Figure 1B portrays the FEM proteins as signal-transducing molecules that act cell autonomously to bring about the adoption of male cell fates, and it suggests that TRA-2A regulates their activity at a post-translational level. A cell-autonomous role for the *fem* genes implies that, at a minimum, they must be expressed in every lineage that produces cells with male-specific fates. Because ~40% of the somatic cells in the male, including representatives of every tissue, are sexually specialized, the model leads us to expect that the *fem* genes should be widely expressed (Hodgkin, 1988).

If control over *fem* activity were exclusively post-translational, the expression patterns of the *fem* genes should be identical in both sexes. Both hermaphrodites and males express *fem-2* and *fem-3* transcripts (Rosenquist and Kimble, 1988; Pilgrim *et al.*, 1995), and Northern blots do not reveal sex-specific transcripts of either gene. The levels of *fem-2* and *fem-3* mRNAs increase during postembryonic development in hermaphrodites (Rosenquist and Kimble, 1988; Pilgrim *et al.*, 1995). The increase in *fem-3* mRNA levels is likely to result from transcription in the germline, because adults of either sex in which germline proliferation is blocked contain very low levels of *fem-3* mRNA (Rosenquist and Kimble, 1988; Rosenquist, 1989). Genetic analysis suggests that all three *fem* genes are expressed in the adult hermaphrodite germline, because all exhibit maternal rescue: Male development in homozygous *fem* mutants descended from heterozygous mothers can be rescued to varying degrees, depending on the gene in question, by wild-type product contributed to the embryo via the oocyte (Doniach and Hodgkin, 1984; Hodgkin, 1986).

Maternal expression of *fem-3* is necessary but not sufficient for normal male development (Hodgkin, 1986), and that of *fem-1* and *fem-2* is neither necessary nor sufficient (Doniach and Hodgkin, 1984; Hodgkin, 1986). Zygotic expression of *fem-1* and *fem-2* must precede the time at which decisions concerning sexual fate are made. Sexual dimorphism first becomes apparent in late embryogenesis when certain neurons undergo sex-specific-programmed cell death, but most sexually dimorphic structures develop after hatching (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Temperature-shift experiments with conditional alleles of *fem-1* and *fem-2* suggest that their expression or activity is required during the first two larval stages for the development of the male somatic gonad and tail and for hermaphrodite spermatogenesis (Nelson *et al.*, 1978; Kimble *et al.*, 1984). Males require the activities of *fem-1* and *fem-2* continuously for sustained spermatogenesis (Kimble *et al.*, 1984). Repression of yolk protein synthesis in the male gut requires *her-1* activity during adulthood (Schedin *et al.*, 1994), suggesting that the activities of the *fem* genes through which *her-1* acts probably are also required in the adult male gut. Expression of the *fem* genes should therefore commence during embryogenesis, and *fem* products should persist until adulthood, at least in the germline and the gut.

We have examined the spatial and temporal expression pattern of *fem-1* to determine whether it meets the expectations arising from our current model of sex determination or whether it reveals additional levels of regulation that are not accounted for in the model. We used *fem-1::lacZ* reporter-gene constructs in transgenic animals as indicators of the spatial expression pattern of the endogenous *fem-1* gene. To assess their validity, we determined the sequence requirements for generation of the observed pattern of reporter-gene expression and compared them with the requirements for rescue of the *fem-1* mutant phenotype by *fem-1* transgenes.

MATERIALS AND METHODS

Caenorhabditis elegans Strains and Culture Methods

All of the strains used in this study were derived from the standard *C. elegans* wild-type strain N2 (Brenner, 1974). Genetic nomenclature follows Horvitz *et al.* (1979), with the following additional conventions. Gain-of-function mutations are distinguished with the suffix *gf* (Barton *et al.*, 1987). Because *fem-1* has a maternal effect (Doniach and Hodgkin, 1984), homozygous *fem-1* mutants descended from homozygous mothers are denoted *fem-1(m-z-)* (Hodgkin, 1986) to distinguish them from the homozygous mutant progeny of heterozygous mothers [*fem-1(m+z-)*]. Extrachromosomal arrays are designated *idEx*[], with the constituents of the array listed between brackets. Integrated arrays are designated *ids*, followed by a number. The mutant alleles used in this study are listed below. Those not explicitly referenced can be found in Brenner (1974) or Hodgkin *et al.* (1988).

LGI, *dpy-5(e61)*; *smg-1(e1228)* (Hodgkin *et al.*, 1989); *glp-4(bn2ts)* (Beanan and Strome, 1992)

LGII, *bli-2(e768)*; *tra-2(b202ts)* (Klass *et al.*, 1976)

LGIII, *unc-32(e189)*; *fem-2(b245ts)* (Kimble *et al.*, 1984); *glp-1(e2144ts)* (Priess *et al.*, 1987)

LGIV, *unc-5(e53)*; *fem-1(e1991)* (Doniach and Hodgkin, 1984); *fem-1(e2044)* (Hodgkin, 1986); *fem-1(e2382)* (Spence *et al.*, 1990); *unc-24(e138)*; *mor-2(e1125)*; *fem-3(e1996)* (Hodgkin, 1986); *fem-3(q20 gf,ts)* (Barton *et al.*, 1987); *dpy-20(e1282ts)*

LGv, *dpy-11(e224)*; *him-5(e1490)* (Hodgkin *et al.*, 1979)

LGX, *lon-2(e678)*

Standard methods of culture and genetic crossing of *C. elegans* were used (Sulston and Hodgkin, 1988), except that MYOB agar (Church *et al.*, 1995) was used in place of NG agar in some experiments.

Large-scale cultures for RNA extraction were grown in liquid S medium. Embryos were collected by hypochlorite treatment of populations containing many gravid adults (Sulston and Hodgkin, 1988). They were either frozen in liquid nitrogen for the later extraction of RNA or allowed to hatch in sterile M9 buffer for the preparation of synchronous populations of L1 larvae. Synchronous samples of later stages were obtained by putting these L1 larvae into liquid culture until they had reached the desired stage.

Strains carrying the temperature-sensitive mutations listed above were maintained at 15°C. Cultures from which RNA or protein was to be extracted were grown at 25°C.

Homozygotes for *fem-1* deletion alleles *e2044* and *e2382* were obtained in quantities sufficient for Western blot analysis from heterozygotes of genotype + *fem-1(e2044* or *e2382)* *mor-2(e1125)* *unc-24(e138)* *fem-3(q20 gf)* +/*unc-5(e53)* + + + + *dpy-20(e1282)*. The heterozygotes were allowed to self-fertilize, and their Unc-24, Mor F1 progeny were picked to new plates in groups of 10. Most of these animals were homozygous for *fem-1* and *fem-3(gf)*, and they were self-fertile, because *fem-3(gf)* enhances maternal rescue of *fem-1* (Barton *et al.*, 1987). They produced F2 broods consisting entirely of females, which were harvested and analyzed as described below (Western analysis). Recombinants that were heterozygous for *fem-1* were recognized easily, because they segregated the *unc-5* marker, which is very tightly linked to *fem-1* (Doniach and Hodgkin, 1984), and because they produced an F3 generation. Plates that carried any such recombinants were discarded.

General Molecular Biological Methods

Procedures for the handling and analysis of nucleic acids, when not described below or attributed to other sources, were those described by Sambrook *et al.* (1989).

Preparation and Analysis of RNA

Nematode RNA was prepared by using the procedure described by Hope (1994) with minor modifications. Total RNA (20 µg) was electrophoresed on 1% agarose gels containing 0.5 M formaldehyde, transferred to Hybond N (Amersham, Arlington Heights, IL), and hybridized to randomly primed ³²P-DNA probes. Probes were made from the *fem-1* cDNA clone AS#1000 or the *act-1*-specific clone pT3/T7-18-103, provided by M. Krause (National Institutes of Health, Bethesda, MD). The intensity of the hybridization signals was measured by densitometry of autoradiographic images (Molecular Dynamics, Sunnyvale, CA). The *fem-1* signal in each sample was normalized to the *act-1* signal.

Primer extension mapping of the 5' end of the *fem-1* transcript was performed with oligonucleotide oAS101 (5'-CTCTGCAAATT-TCCAACC-3'). Each primer extension reaction used 20–40 µg of total RNA.

fem-1 Plasmids

Throughout this paper, positions in the genomic sequence of *fem-1* are numbered in relation to the A of the *fem-1* initiation codon, which is designated position +1.

Construction of the *fem-1::lacZ* Fusion Plasmid AS#1209

Our approach in constructing the *fem-1::lacZ* translational fusion plasmid AS#1209 was similar to that described by Wilkinson *et al.* (1994). A *Bam*HI site was introduced at position +3 of *fem-1* by mutagenesis with oligonucleotide oAS140 (5'-ATGTCCATTTG-GATCCATACCGCCA-3') (Kunkel, 1985). Sequencing of the mutagenized fragment by the method of Sanger *et al.* (1977) confirmed that no extraneous mutation had been introduced. A *Bgl*II-*Bam*HI fragment extending 1.9 kb upstream from this *Bam*HI site was inserted into the *Bam*HI site of the *lacZ* fusion vector pPD16.43 (Fire *et al.*, 1990), placing the *fem-1* ATG codon in frame with the *lacZ* coding sequence. A 5.9-kb *Eco*RV-*Spe*I fragment extending from position +202 in exon 2 of the *fem-1* gene to position +6095, 1.4 kb 3' to the site of polyadenylation of *fem-1* mRNA, was then ligated between the *Stu*I and *Spe*I sites downstream of the *lacZ* stop codon. AS#1209 contains all sequences present in a genomic DNA fragment capable of rescuing *fem-1* mutants except for a 200-bp region extending from within exon 1 to exon 2, that region having been replaced by the *lacZ* coding sequence. The plasmid encodes a modified β -galactosidase containing a nuclear localization signal to facilitate the identification of cells expressing it (Fire *et al.*, 1990).

Fusion of *fem-1* Regulatory Sequences to Intron-containing *lacZ* Genes

AS#1269 and AS#1263 are *fem-1::lacZ* translational fusions at position +3 in *fem-1*, like AS#1209. AS#1269 carries 1.9 kb of *fem-1* sequence upstream of the initiation codon, whereas AS#1263 has only 335 bp of *fem-1* sequence. Both differ from AS#1209 in several respects. First, they lack all *fem-1* sequence downstream of position +3; second, they carry a 3' fragment of the *unc-54* gene, including a polyadenylation signal, downstream of the *lacZ* stop codon; third, the *lacZ* gene in each of these plasmids is interrupted by 12 synthetic introns. Both plasmids were constructed by inserting the appropriate fragment of *fem-1* 5' flanking DNA into plasmid pPD95.03, which carries the intron-containing *lacZ* gene and *unc-54* 3' end region. pPD95.03 was generously provided by Dr. A. Fire (Carnegie Institution of Washington, Baltimore, MD) (A. Fire, S. Xu, J. Ahnn, and G. Seydoux, personal communication).

Construction of *fem-1* Minigenes

AS#1000 is a pBluescript (Stratagene, La Jolla, CA) clone of the full-length *fem-1* cDNA insert from lambda clone CB#1185 (Spence *et al.*, 1990). Appropriate restriction fragments of AS#1000 and the rescuing genomic clone CB#1095 (Spence *et al.*, 1990) were combined to produce a series of *fem-1* minigenes containing different subsets of *fem-1* introns. Table 2 indicates the intron content of each construct. All of the minigenes carry 486 bp of *fem-1* sequence 5' to the start codon and contain the complete 3' untranslated region of *fem-1*.

Transformation of *C. elegans*

Nematode transformation was performed by germline microinjection with the method described by Mello *et al.* (1991). DNA for injections was prepared as described previously (Spence *et al.*, 1990). Transgenic lines were established from the F2 descendants of animals injected with a mixture containing the plasmid of interest at a concentration of 2, 10, or 50 μ g/ml and the plasmid pRF4 at 50 μ g/ml. The total DNA concentration in the mixture was adjusted to 100 μ g/ml with pBluescript. pRF4 carries the dominant *rol-6* allele *rol-6(su1006)* that serves as a transformation marker (Mello *et al.*, 1991). In experiments in which only the F1 progeny of injected animals were analyzed, the injected DNA mixture contained no marker plasmid.

Analysis of *fem-1::lacZ* Expression

Analysis of the *fem-1::lacZ* expression pattern was performed either by staining the F1 brood of injected animals or by staining animals from established transgenic lines. Animals to be stained were raised at 25° (Way and Chalfie, 1989; Hamelin *et al.*, 1992).

For F1 *lacZ* assays, 15–20 adult hermaphrodites were injected on each of three or four consecutive days. The injected animals and their F1 broods, containing animals of all developmental stages, were then collected and stained for β -galactosidase activity (Fire, 1992; Xie *et al.*, 1995). Animals stained for β -galactosidase activity were contained with 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) to allow visualization of cell nuclei.

The predicted transcript of the *fem-1::lacZ* fusion in AS#1209 has a stop codon at the end of the *lacZ* open reading frame followed by a very long 3' untranslated region. Because such transcripts are often unstable (Wilkinson *et al.*, 1994), we assayed *lacZ* expression from AS#1209 both in a wild-type genetic background and in a *smg-1(e1228); him-5(e1490)* background. The *smg-1* mutation stabilizes aberrant transcripts (Pulak and Anderson, 1993). The *him-5* mutation increases the frequency of XO males among the self-progeny of hermaphrodites to ~30% from the wild-type level of 0.2% (Hodgkin *et al.*, 1979) and facilitates analysis of expression in both sexes.

Chromosomal Integration of *fem-1::lacZ* Transgenes

We used gamma irradiation to integrate two different AS#1209 extrachromosomal arrays into chromosomal sites (Way *et al.*, 1991; Mello and Fire, 1995). We started with strains of genotype *unc-24(e138) fem-3(q20 gf); idEx[rol-6(su1006dm) fem-1(AS#1209)]*, because *fem-3(q20 gf)* suppressed the germline-feminizing effects of the extrachromosomal arrays and simplified strain propagation. After integration of each array, the *unc-24 fem-3(gf)* chromosome was eliminated by outcrossing for several generations, first to N2 and then to *smg-1; him-5*. We demonstrated linkage of integrated array *idls6* to chromosome II by showing that heterozygotes of genotype *idls6 +/+ bli-2 II* rarely segregated wild-type animals. Integrated array *idls5* showed linkage to chromosome V: Heterozygotes of genotype *idls5 +/+ dpy-11 V* failed to segregate wild-type animals. Neither array showed linkage to any other chromosome.

Transformation Rescue of *fem-1* Mutants

Extrachromosomal arrays carrying *fem-1* promoter-deletion constructs or minigene derivatives were tested for their ability to rescue the phenotype of animals carrying the *fem-1* amber allele *fem-1(e1991)*. At least two extrachromosomal arrays were tested for each construct and for each concentration at which that construct was injected. The *unc-24(e138)* mutation, which maps ~1.5 cM from *fem-1* (Hodgkin *et al.*, 1988), served as a marker allowing identification of *fem-1* homozygotes independently of their sexual phenotype.

Males of genotype + *fem-1 unc-24* were crossed to transgenic Rol XX animals [genotype; +; *idEx(rol-6(dm) fem-1(AS#nnnn))*]. Because extrachromosomal arrays carrying *fem-1* constructs often caused strong feminization of the germline (see RESULTS), the F1 Rol progeny of this cross were often female. F1 Rol females were backcrossed to + *fem-1 unc-24* males, and Unc, Rol animals [*fem-1 unc-24; idEx(rol-6(dm) fem-1(AS#nnnn))*] were picked from among the F2. When the array being tested did not feminize the germline, Unc, Rol F2 were picked from among the self-progeny of F1 Rol hermaphrodites. The Unc, Rol F2 were almost invariably female, because germline rescue was infrequent (see RESULTS). They were individually back-crossed to several + *fem-1 unc-24* males. Broods were scored under a dissecting microscope for the Unc and Rol phenotypes and for sexual phenotype. Unc Rol animals were then examined by using Nomarski optics ($\times 400$ magnification) to determine the extent of masculinization. Animals were scored for gonad morphology, presence of a vulva, production of sperm, and tail anatomy (including the presence of rays, fan, hook, and spicules and the absence of a tail spike). Animals with complete masculin-

ization of all examined somatic structures were scored as males. Animals showing incomplete masculinization were scored as intersexes. Broods in which >5% of non-Rol Uncs were male (+ *unc-24/fem-1 unc-24*) were assumed to result from crosses involving one or more +/+ *unc-24* recombinant male parents and were not included in the data set.

FEM-1 Antibodies

Plasmid AS#1007 was constructed by cloning a 2.1-kb *EcoRV*–*Bam*HI fragment of the *fem-1* cDNA from AS#1000 into the *Bam*HI site of the T7 expression vector pET3b (Studier *et al.*, 1990). AS#1007 encodes a fusion protein (T7FEM-1) including all but the first 49 amino acids of FEM-1. Synthesis of T7FEM-1 was induced in *Escherichia coli* strain BL21(DE3)pLysS with 0.4 mM IPTG. Inclusion bodies containing the fusion protein were purified and used to immunize rabbits. Primary immunization and boosts were performed by subcutaneous injection of 0.2 mg of antigen. Boosts were administered at monthly intervals, and blood was collected 2 wk after each boost. Each serum sample was first tested for reactivity against T7FEM-1 fusion protein on Western blots. Antisera were affinity purified against T7FEM-1 bound to nitrocellulose strips (Smith and Fisher, 1984). Bound anti-T7FEM-1 antibodies were eluted by rinsing the strips in three 100- μ l aliquots of 100 mM glycine-HCl, pH 2.3. Each fraction was neutralized immediately by adding Na_2HPO_4 to a final concentration of 100 mM. Eluates were pooled and stored in 0.1% bovine serum albumin and 0.02% sodium azide. In some experiments, antisera were preadsorbed against an *E. coli* lysate before affinity purification.

Western Analysis

Nematode lysates were prepared by harvesting worms from plates in M9 buffer, rinsing three times in M9 to remove bacteria, rinsing once in water, and boiling in SDS sample buffer. Samples containing ~150 μ g of protein were electrophoresed through 10% SDS-polyacrylamide gels and electroblotted to nitrocellulose (0.22 μ m). Blots were blocked in Blotto (5% skim milk powder in Tris-buffered saline with Tween [TBST]: 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) for 1 h before being incubated overnight at room temperature with affinity-purified antisera diluted 1:200 in Blotto. After 3–4 washes in TBST, the blots were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (GIBCO BRL, Grand Island, NY), diluted 1:3000 in Blotto. Blots were washed four times in TBST and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase buffer, as described by Harlow and Lane (1988).

RESULTS

fem-1 Is Expressed throughout Development in Both Sexes

Previous work showed that a *fem-1* probe recognizes a single transcript of ~2.4 kb on a Northern blot of total RNA from a mixed-stage hermaphrodite population (Spence *et al.*, 1990). Analysis of RNA from staged, synchronous populations reveals that the 2.4-kb mRNA is present throughout hermaphrodite development (Figure 2). The steady-state level of *fem-1* mRNA in relation to *act-1* mRNA varies by no more than twofold, except in L4 animals, when *fem-1* mRNA is fivefold more abundant than at other stages in relation to *act-1* mRNA (average of 2 experiments). Adult males contain a *fem-1* transcript of the same size and

Figure 2. Expression of *fem-1* mRNA during development. The top shows a Northern blot of total RNA from staged populations of embryos (E), larvae of stages L1–L4, adult hermaphrodites (A), and adult males (A) hybridized with a *fem-1* cDNA probe. The bottom illustrates the same blot after stripping and rehybridizing with a probe from the *act-1* gene. The sizes, in kb, of single-stranded DNA markers are listed on the right.

relative abundance as that detected in adult hermaphrodites (Figure 2). Primer extension mapping located the 5' end of *fem-1* mRNA at position –94 in both sexes (Figure 3). Many *C. elegans* mRNAs are trans-spliced at their 5' ends to a 22-nucleotide-untranslated leader sequence known as SL1 (Zorio *et al.*, 1994), but the *fem-1* transcript apparently is not. The closest candidate splice-acceptor sequence upstream of *fem-1* is at position –120, 30 nucleotides upstream of the 5' end of the transcript as determined by primer extension. Reverse transcription of *C. elegans* RNA followed by PCR amplification with a *fem-1* antisense primer and an SL1 primer consistently failed to yield a *fem-1* product (our unpublished observations), supporting the conclusion that *fem-1* transcripts do not undergo trans-splicing.

Antibodies against a bacterial FEM-1 fusion protein detect a protein of 74 kDa, the predicted size of FEM-1,

Figure 3. Primer extension mapping of the 5' end of *fem-1* mRNA. Lanes 1–4 display the DNA sequence flanking the 5' end of *fem-1* as read from a primer complementary to the sense strand of the first exon. Lanes 5 and 6 show the product of extension of the same primer on RNA from mixed-stage hermaphrodites (lane 5) or adult males (lane 6). Primer extension yields a single product that maps to position –94 with respect to the *fem-1* start codon.

on immunoblots of lysates of wild-type nematodes. The 74-kDa protein is absent from *fem-1(m-z-)* mutants carrying *fem-1* deficiency alleles (Figure 4). It is detectable at all stages of hermaphrodite development and does not vary appreciably in abundance. Immunoblot analysis provides no indication of stage- or sex-specific isoforms of FEM-1: Lysates prepared from staged hermaphrodite populations or from adult males contain a single FEM-1 band of the same molecular weight and abundance.

Expression of *fem-1* in hermaphrodites is not limited to the germline. Both *fem-1* mRNA and FEM-1 are readily detectable in *glp-1* or *glp-4* hermaphrodites in which the germline fails to proliferate, indicating that *fem-1* is expressed in female somatic tissues (Figure 5, A and B).

According to the model of sex determination shown in Figure 1, male and female cell fates result from reciprocal activity states of the genes in the sex-determining cascade. It follows that if FEM-1 protein accumulates at the same steady-state levels in both male and female tissues, its accumulation is not regulated by the other members of the cascade. This inference is supported by the observation that mutations that reduce the activity of *tra-2*, *fem-2*, or *fem-3* have no effect on FEM-1 as assayed by Western blotting (Figure 5C). Gain-of-function mutations in *fem-3*, which increase the activity of *fem-3* in the hermaphrodite germline,

Figure 4. Expression of FEM-1 during development. The top panel is an immunoblot of lysates from wild-type nematodes (lanes 1 and 2) or females homozygous for the indicated *fem-1* deficiencies (lanes 3 and 4) probed with anti-FEM-1 antiserum. The bottom panel is a similarly treated immunoblot of lysates from staged populations of wild-type hermaphrodite embryos (E), larvae of stages L1–L4, adult hermaphrodites (A), adult males (A♂), and *fem-1(e2382)* homozygotes. Each lane contains ~150 μ g of total protein.

Figure 5. *fem-1* expression in mutants. (A) Northern blot of total RNA (20 μ g per lane) probed with *fem-1* cDNA. RNA was from mixed-stage, wild-type hermaphrodites (lane 1), sterile *glp-1(e2144ts)* adults (lane 2), sterile *glp-4(bn2ts)* adults (lane 3), or adult wild-type hermaphrodites (lane 4). RNA in lanes 2–4 was isolated from animals raised at 25°C. Sizes, in kb, of single-stranded DNA markers are indicated on the right. (B) Anti-FEM-1 immunoblot of lysates from wild-type mixed-sex population (lane 1), *glp-4(bn2ts)* sterile adults (lane 2), *glp-1(e2144ts)* sterile adults (lane 3), *fem-1(e2044)* homozygotes (lane 4), and adult wild-type hermaphrodites (lane 5). Each lane contains ~150 μ g of total protein. Lysates in lanes 2 and 3 were prepared from animals raised at 25°C. Marker sizes in kDa are indicated on the right. (C) Anti-FEM-1 immunoblot of lysates from adults homozygous for mutations in the indicated sex-determining genes: Lane 1, *tra-2(b202ts)*; lane 2, *fem-3(q20 gf,ts)*; lane 3, *fem-3(e1996)*; lane 4, *fem-2(b245ts)*; lane 5, *fem-1(e2044)*; lane 6, wild-type strain N2. Each lane contains ~150 μ g of total protein. Lysates in lanes 1, 2, and 4 were prepared from animals raised at 25°C. Marker sizes in kDa are indicated on the right.

are similarly without effect on the size or abundance of FEM-1 (Figure 5C).

fem-1::lacZ Expression

To examine the spatial pattern of *fem-1* expression throughout development, we used a *fem-1::lacZ* fusion, AS#1209, as a reporter gene in transgenic nematodes. The structure of the reporter gene is diagrammed in Figure 6A. It includes all portions of a genomic DNA fragment capable of rescuing *fem-1* mutants, except for a fragment extending from position +4 in exon 1 to position +201 in exon 2. That fragment was replaced by the coding sequence of a modified *lacZ* gene. The first intron of *fem-1*, missing from AS#1209, is not required for *fem-1* activity (see below).

AS#1209 exhibited the same overall pattern of *lacZ* expression in both F1 “transient transformants” and in established transgenic lines. Individual animals in any given line or F1 brood seemed to be mosaics, because they expressed the transgene in different subsets of cells. Integration of the reporter transgene into either

Figure 6. Structure and activity of *fem-1::lacZ* reporter constructs. (A) The prototypical construct AS#1209 is described in detail in the text. Other constructs were derived from it by deletion of various restriction fragments. Filled boxes represent *fem-1* exons, shaded boxes represent the *lacZ* coding sequence, and open boxes represent *fem-1* introns and flanking sequences. The extent of *fem-1* sequence included in each construct is indicated with respect to the start codon, the A of which is position +1. Staining was assessed in populations of stained F1 transgenic animals. +++ indicates extensive staining of somatic tissues; - indicates rare staining of a few cells, usually in the pharynx. (B) Structure and activity of *fem-1::lacZ* reporter constructs based on intron-containing *lacZ* vector provided by Dr. A. Fire. The shaded box represents the *lacZ* coding sequence, and the white bars that interrupt it represent synthetic introns. The hatched box indicates a fragment from the 3' end of the *unc-54* locus that includes a polyadenylation signal (Fire *et al.*, 1990). Activity was evaluated as in A.

of two chromosomal sites did not alleviate the problem, suggesting that mosaic expression did not result entirely, or even primarily, from mitotic loss of the transgene. (See MATERIALS AND METHODS for information on transgene integration.) Others have reported similar observations of mosaic expression of reporter transgenes (Krause *et al.*, 1994). Because mosaicism of expression of *fem-1::lacZ* was more severe in transgenic lines than in F1 transformants, we relied for the most part on the latter.

Despite the unusual structure of the predicted *fem-1::lacZ* transcript of AS#1209 and the fact that transcripts with long 3' untranslated regions are often unstable in *C. elegans* (Pulak and Anderson, 1993; Wilkinson *et al.*, 1994), expression of AS#1209 was detectable in a wild-type genetic background. We have not determined whether the actual structure of the transcripts of AS#1209 conforms to our prediction. Strains carrying a *smg-1* mutation, which stabilizes mRNAs of aberrant structure (Pulak and Anderson, 1993), exhibited somewhat higher levels of *fem-1::lacZ* expression: Histochemical staining required an incubation of 3–4 h in *smg-1* animals, compared with 6–10 h in *smg-1(+)* animals. On average, individual *smg-1* animals exhibited more extensive staining than *smg-1(+)* animals.

Embryos carrying AS#1209 exhibited β -galactosidase activity in many and perhaps all cells, beginning at approximately the 100-cell stage. Extensive, diffuse X-Gal staining persisted through the remainder of embryogenesis and the first larval stage (L1) (Figure 7). The diffuse quality of the staining prevented us from excluding the possibility that some cells might have failed to stain in embryos and L1 larvae. We do not

know why the nuclear localization signal contained in the β -galactosidase encoded by AS#1209 was ineffectual in embryos and L1 larvae. At later stages of development, we observed nuclear staining of all somatic tissues in samples of both males and hermaphrodites. Nuclei of hypodermal cells, muscles, and neurons most frequently exhibited staining, whereas staining of the gut and somatic gonad was less common (Figure 7). We never observed β -galactosidase expression in the germline. Inactivity in the germline seems to be a general property of *lacZ* reporter fusions in *C. elegans* (Mello and Fire, 1995).

Regulatory Sequence Requirements for *fem-1::lacZ* Expression

Implicit in our use of a *fem-1::lacZ* fusion as a reporter of the expression pattern of *fem-1* was the assumption that its expression depended on the same regulatory sequences as the expression of *fem-1* itself. To test this assumption, we determined which sequences were required for widespread expression of the reporter gene and compared them with the regulatory sequences required for rescue of the *fem-1* mutant phenotype by a *fem-1* transgene.

To delimit the promoter sequence required to drive expression of *fem-1::lacZ*, we made deletions of the *fem-1* 5' flanking sequence in AS#1209. Plasmids containing 332 bp (AS#1238) or 170 bp (AS#1239) of *fem-1* sequence 5' to the initiation codon directed *lacZ* expression in the same pattern as AS#1209 (Figures 6A and 7). However, an analogous plasmid that retained *fem-1* sequence extending upstream only as far as po-

Figure 7. Expression pattern of *fem-1::lacZ* reporter constructs. (A–F) Expression of AS#1209 in (A) embryo of ~100 cells, (B) L1 larva, (C) L3 hermaphrodite, (D and E) L4 hermaphrodites exhibiting different mosaic patterns of expression, and (F) L4 male. bm, Body wall muscle; g, gut; h, hypodermis; nc, ventral nerve cord; ph, pharynx; sh, gonadal sheath cell; v, vulval cells. (G) Expression of AS#1239 in L4 hermaphrodite. (H) Expression of AS#1238 in L4 hermaphrodite. Scale bar, 50 μ m. Animals are oriented with anterior to the left and dorsal up. (Those in C and G were inverted during photographic processing.)

sition –112 (AS#1243) was inactive except for occasional expression in the pharynx.

We also tested whether *fem-1* sequence located 3' to *lacZ* in AS#1209 was important for the observed pattern of *lacZ* expression. Deletion of a fragment extending from exon 2 of *fem-1* to exon 7 (AS#1213) or an overlapping fragment extending from exon 7 to exon 10 (AS#1214) had no discernible effect on the expression of the reporter. However, a construct lacking both regions (AS#1215) was very poorly and infrequently expressed (Figure 6A). Animals carrying AS#1215 showed occasional pharyngeal staining and, rarely, staining of one or two hypodermal or body-wall muscle cells. The inactivity of AS#1215 suggests that widespread expression of the *fem-1::lacZ* fusion carried by AS#1209 requires elements downstream of the *fem-1* start codon, but because both AS#1213 and AS#1214 direct β -galactosidase expression in the same pattern as AS#1209, either of two regions of *fem-1* apparently can fulfill this requirement.

Okkema *et al.* (1993) demonstrated that introns stimulate expression of the *unc-54* myosin gene of *C. elegans* in a sequence-independent manner. The same laboratory has produced plasmids in which the *lacZ* gene is interrupted by up to 12 synthetic introns. The multiply interrupted *lacZ* gene serves as a sensitive reporter for assaying the promoter activity of upstream DNA sequences (Fire, Xu, Ahnn, and Seydoux, personal communication). We fused sequences from

the 5' end of the *fem-1* gene to a *lacZ* gene containing 12 introns, kindly provided by Dr. A. Fire. Plasmids containing *fem-1* 5' flanking sequence extending to position –1866 (AS#1269, Figure 6B) or position –332 (AS#1263, Figure 6B) directed β -galactosidase expression in the same pattern as that of AS#1209. Animals carrying either of these constructs stained more intensely than animals carrying AS#1209, and individual animals tended to exhibit more extensive staining. We interpret these results to mean that a fragment of *fem-1* DNA extending upstream from position +3 to position –332 contains all of the *fem-1* sequences specifically required for the widespread somatic expression of a *lacZ* reporter gene. Because the requirement for sequences downstream of the *fem-1* start codon suggested by the inactivity of AS#1215 can be fulfilled or bypassed by the provision of synthetic intron sequences in the reporter gene, it probably reflects a nonspecific stimulatory effect of splicing on gene expression similar to that observed by Okkema *et al.* (1993).

Regulatory Sequence Requirements for *fem-1* Rescue

Plasmid CB#1095 carries the intact *fem-1* gene with 486 bp upstream of the *fem-1* start codon, and it can rescue all aspects of the *fem-1* mutant phenotype (Spence *et al.*, 1990). Plasmids derived from CB#1095 by the deletion of varying amounts of *fem-1* 5' flanking DNA

allowed us to determine whether the minimum promoter sequence required for rescue of *fem-1* mutants corresponded to the minimum required for *fem-1::lacZ* expression. A plasmid carrying *fem-1* flanking sequences extending to position -332 (AS#1236) rescued the somatic phenotype of *fem-1* mutants as effectively as CB#1095 (Table 1). Plasmid AS#1237, with 5' flanking sequence extending to position -170, was considerably less active, and a plasmid that retained only 112 bp upstream of the *fem-1* start codon (AS#1303) was almost inactive, resulting in only very weak masculinization of approximately one-third of the *fem-1* XO animals examined.

To test the requirement for internal noncoding sequences in rescuing *fem-1* mutants, we constructed *fem-1* minigenes in which portions of the *fem-1* gene were replaced by the corresponding portions of a *fem-1* cDNA to eliminate subsets of *fem-1* introns. To aid in comparing the efficiency with which different plasmids rescued *fem-1* mutants, we injected each at three different concentrations while keeping the total concentration of injected DNA constant. Because extrachromosomal arrays assemble by homologous recombination among molecules in the injected DNA mixture (Mello *et al.*, 1991), the copy number of a particular plasmid in an array is related to the relative concentration of that plasmid in the mixture (Mello and Fire, 1995).

Three minigenes that retained only a single *fem-1* intron (AS#1006, AS#1233, and AS#1234) were relatively ineffective at rescuing *fem-1* mutants (Table 2). AS#1006, for example, completely rescued the somatic

tissues of only 19% of transgenic XO animals when injected at the highest concentration tested. In contrast, minigenes that retained two or more introns, including combinations of introns that were individually inactive, rescued the XO soma as effectively as the genomic clone CB#1095 at all concentrations tested (Table 2). (Effects of *fem-1* transgenes on the germline are described below.) These results suggest that expression of a *fem-1* transgene at levels adequate to rescue mutants requires more than one intron but that no specific intron sequence is required.

Effects of *fem-1* Transgenes on the Germline

In an earlier study, injection of *fem-1* genomic clones into *fem-1* mutant females, followed by selection for fertility, yielded extrachromosomal arrays that rescued all aspects of the *fem-1* phenotype (Spence *et al.*, 1990). Our approach differed in that we first established transgenic lines in a *fem-1*(+) genetic background on the basis of the phenotype conferred by a cotransformation marker. We then tested the extrachromosomal arrays carried in these lines for their ability to rescue *fem-1* mutants. Most arrays that rescued the somatic tissues of *fem-1* XO animals failed to rescue the germline of either sex. This finding is consistent with the earlier observation that, among the F1 of *fem-1* females injected with *fem-1*(+) genomic clones, somatically rescued XO males occurred more frequently than either fertile males or self-fertile hermaphrodites (Spence *et al.*, 1990). We were surprised to find, however, that most extrachromosomal arrays carrying *fem-1* plasmids had the paradoxical effect of eliminating spermatogenesis in wild-type XX and XO animals, causing a Fog (feminization of the germline) phenotype.

We made mixed extrachromosomal arrays carrying various concentrations of the *fem-1* genomic clone CB#1095 with either of two transformation markers, and we tested the ability of these arrays to rescue the germline of *fem-1* mutants and to cause feminization of the wild-type germline. Neither of the transformation markers *rol-6*(*su1006*) and *dpy-20*(+) affected the sex of the germline when injected alone. Some arrays, formed from the injection of CB#1095 at intermediate concentrations (5–10 μ g/ml) with either marker, rescued spermatogenesis in *fem-1* mutants. Higher concentrations of CB#1095, although more effective at rescuing XO somatic tissues (see Table 2), invariably caused germline feminization in a *fem-1*(+) genetic background and very rarely rescued spermatogenesis in *fem-1* mutants. Lower concentrations of CB#1095 feminized the wild-type germline less severely but were also less effective at rescuing the *fem-1* phenotype in either soma or germline.

Most of the plasmids that we tested caused feminization of the germline, and we have not quantified the

Table 1. Deletion mapping of the 5' boundary of the *fem-1* promoter

Plasmid ^a	Extent of <i>fem-1</i> sequence 5' to ATG	Sexual phenotype of transgenic <i>fem-1</i> mutants ^b		
		Female	Masculinized	Percent male ^c
		n	n	
CB#1095	-486	51	45	49
AS#1236	-332	153	144	47
AS#1237	-170	119	129	11
AS#1303	-112	273	60 ^d	0

^a The test plasmids were injected at 50 μ g/ml to establish the transgenic lines used to generate the data in this table.

^b *unc-24*(*e138*) served as a marker for *fem-1*(*e1991*), and *rol-6*(*su1006dm*) marked the extrachromosomal array carrying the *fem-1* transgene. See MATERIALS AND METHODS for a complete description of the cross.

^c "Percent male" indicates the percentage of masculinized animals that exhibited normal male somatic development in all tissues examined.

^d The ratio of masculinized Unc Rol animals to Unc Rol females was significantly <1.0 in this cross (ratio = 0.22; $p < 0.005$), indicating that the transgene failed to masculinize many of the XO animals.

Table 2. Rescue of the *fem-1* somatic phenotype by *fem-1* minigenes

Plasmid	Introns present	Sexual phenotype of transgenic <i>fem-1</i> mutants ^a								
		Plasmid @ 50 µg/ml			Plasmid @ 10 µg/ml			Plasmid @ 2 µg/ml		
		Female	Masculinized	Percent male ^b	Female	Masculinized	Percent male ^b	Female	Masculinized	Percent male ^b
		n	n		n	n		n	n	
CB#1095	1–10 (all)	51	45	49	71	61	31	105	124	3
AS#1212	1, 7–9	72	76	50	117	115	43	119	120	17
AS#1235	2–6	52	58	47	53	64	30	35	44	7
AS#1223	1, 6	57	64	52	55	67	27	55	46	17
AS#1224	1, 7	38	39	67	39	44	11	56	51	10
AS#1225	1, 10	50	46	50	66	64	45	62	68	16
AS#1006	1	54	67	19	61	71	0	56	37 ^c	0
AS#1233	6	61	70	24	41	53	4	48	48	0
AS#1234	7	42	54	17	57	60	3	39	28	4

^a *unc-24(e138)* served as a marker for *fem-1(e1991)*, and *rol-6(su1006dm)* marked the extrachromosomal array carrying the *fem-1* minigene. See MATERIALS AND METHODS for a complete description of the cross.

^b "Percent male" indicates the percentage of masculinized animals that exhibited normal male somatic development in all tissues examined.

^c The ratio of masculinized Unc Rol animals to Unc Rol females was significantly <1.0 in this cross (ratio = 0.66; *p* < 0.05), indicating failure of the transgene to cause detectable masculinization in some XO animals.

severity of their effects. Certain plasmids, however, were noteworthy because they did not affect the germline. Their features provide some indications, discussed below, as to the basis of the germline-feminizing effects of the remaining plasmids. Of the *fem-1* plasmids listed in Tables I and II, only one, AS#1303, did not feminize the wild-type germline when injected at high concentration (50 µg/ml). AS#1303 was also the least active of the plasmids tested at rescuing the *fem-1* somatic phenotype, because it lacks a functional *fem-1* promoter.

Arrays formed from the *fem-1::lacZ* fusion plasmid AS#1209 (injected at 50 µg/ml) invariably feminized the germline. In contrast, *fem-1::lacZ* fusion plasmids AS#1269 and AS#1263, which carry the *fem-1* promoter and 5' untranslated region but lack *fem-1* sequences downstream of position +3, did not cause germline feminization.

DISCUSSION

fem-1 Expression

Our results suggest that, despite differences in the requirements for *fem-1* in males and hermaphrodites, the two sexes are indistinguishable with respect to the steady-state levels of *fem-1* products. Analysis of endogenous *fem-1* expression by Northern and Western blotting suggests that *fem-1* mRNA and protein are present throughout hermaphrodite development at approximately the same level as that in adult males. Neither *fem-1* mRNA nor FEM-1 is limited to the

germline in adult hermaphrodites, suggesting that fully differentiated female somatic tissues express *fem-1*, apparently superfluously.

A *fem-1::lacZ* reporter gene behaves in a manner consistent with these observations, exhibiting widespread expression in both males and hermaphrodites from approximately the 100-cell stage of embryogenesis to adulthood. Embryos and L1 larvae stain diffusely, making it impossible to identify any nonstaining cells. At later stages, all tissues except the germline are capable of expressing *fem-1::lacZ* fusions, although in any given animal only a subset of somatic tissues exhibit staining.

Mosaic expression of *lacZ* fusion genes previously has been reported in *C. elegans* (Way and Chalfie, 1989; Aamodt *et al.*, 1991; Okkema *et al.*, 1993; Krause *et al.*, 1994), and others have observed, as we have, that chromosomal integration of the transgenes does not necessarily relieve the problem (Okkema *et al.*, 1993; Krause *et al.*, 1994). Extrachromosomal arrays and chromosomally integrated transgenes derived from such arrays have highly repetitive structures (Mello *et al.*, 1991). In *Drosophila*, repeated arrays can induce heterochromatin formation and silencing of the genes within the arrays, leading to phenotypic mosaicism (Dorer and Henikoff, 1994). Mosaicism of transgene expression in *C. elegans* may be the consequence of a similar process of repeat-induced silencing. Variability in mosaic expression patterns among individuals could result from stochastic heterochromatinization of arrays during development. Maintenance of the het-

erochromatic state in the descendants of the cells in which it was established would cause mosaic expression patterns to follow lineal boundaries, as we and others (Krause *et al.*, 1994) have observed.

The uniform failure of *fem-1::lacZ* transgenes to be expressed in the germline stands in contrast to the variability of somatic mosaicism of expression. The *C. elegans* germline similarly fails to express other *lacZ* fusion genes (Mello and Fire, 1995). We cannot firmly exclude the possibility that our *fem-1::lacZ* fusion genes lack some element important for germline expression. However, the genomic DNA fragment from which the *fem-1::lacZ* fusions were derived can rescue the germline of *fem-1* mutants when it is injected at intermediate concentrations, suggesting that it contains all elements essential for expression in the germline. A more likely explanation is that the *fem-1::lacZ* transgene undergoes silencing in the germline by mechanisms distinct from those affecting its behavior in somatic tissues. We return to this point below.

The observation that both sexes accumulate similar levels of *fem-1* mRNA and FEM-1 has two important implications for the control of sex determination. First, the primary sex-determining signal does not regulate the steady-state levels of *fem-1* products, either directly or indirectly. It is therefore not surprising that mutations in the other *fem* genes or in the gene encoding the principal regulator of *fem* activity, *tra-2*, have no effect on FEM-1 levels. Second, synthesis of FEM-1, although essential for the adoption of male cell fates, is not sufficient to activate male development. Negative regulation of *fem* activity is likely to be post-translational with respect to *fem-1*, although FEM-1 protein itself need not be a direct target for any negative regulator. Negative regulation of *fem-2*, *fem-3*, or their products in XX animals would constitute indirect negative regulation of FEM-1, because all three FEM proteins are essential for male development (Doniach and Hodgkin, 1984; Hodgkin, 1986).

Genetic evidence suggests that regulation of *fem-3* restricts male development in XX animals to the immature germline. Gain-of-function mutations in *fem-3* masculinize the XX germline, causing constitutive spermatogenesis (Barton *et al.*, 1987). These mutations alter the sequence of the 3' untranslated region of *fem-3* and result in increased polyadenylation of its transcript (Ahringer and Kimble, 1991). They suggest the existence of a negative regulator that interacts with the 3' untranslated region of *fem-3* mRNA to limit its polyadenylation and probably its translation, thereby allowing the normal switch from spermatogenesis to oogenesis.

In XX somatic tissues, another mechanism must prevent male development, because *fem-3(gf)* mutations do not masculinize the XX soma. The predicted transmembrane protein TRA-2A is sufficient to regulate *fem* activity in somatic tissues and might do so by seques-

tering one or more of the FEM proteins at the cell membrane (Kuwabara *et al.*, 1992; Kuwabara and Kimble, 1995). This model for the action of TRA-2A predicts that at least one of the FEM proteins should be membrane associated in XX animals. The association should be *tra-2* dependent and regulated by the X/A ratio. We have attempted without success to analyze the subcellular localization of FEM-1 by immunofluorescence with the FEM-1 antibody described in this paper. Efforts to determine the localization of an active, epitope-tagged derivative of FEM-1 with the use of a monoclonal antibody against the epitope tag have, unfortunately, also gone unrewarded. Which of the FEM proteins is the direct target of TRA-2A remains an open question, but the data presented in this paper suggest that *fem-1* activity is limited in XX animals, whether directly or indirectly, by a post-translational mechanism.

Sequences Required for *fem-1* Expression: the *fem-1* Promoter

We determined the noncoding sequence requirements for *fem-1* activity by assaying the ability of various *fem-1* plasmids to rescue male somatic development in *fem-1* mutants. Our experiments identified a short promoter region upstream of the coding region and a sequence-nonspecific requirement for introns. Analysis of the sequences required for widespread somatic expression of *fem-1::lacZ* reporter genes gave nearly concordant results.

Sequences necessary for *fem-1* activity extend no more than 332 bp upstream of the initiation codon. Detailed analyses of the regulatory regions of several other *C. elegans* genes have revealed that these regions also lie close to or within the transcribed regions (Aamodt *et al.*, 1991; Way *et al.*, 1991; MacMorris *et al.*, 1992; Stringham *et al.*, 1992a; Okkema *et al.*, 1993; Jones *et al.*, 1995). Relatively compact regulatory regions may be typical of *C. elegans* genes, because the average gene density in the nematode genome is high (Wilson *et al.*, 1994). The coding region of the upstream neighbor of *fem-1* begins only 634 bp upstream of the *fem-1* initiation codon on the opposite DNA strand (Washington University Genome Sequencing Centre, personal communication).

Deletion of sequence upstream of position -170 from a *fem-1* genomic clone significantly reduces its activity in rescuing the *fem-1* mutant phenotype, suggesting that some element of the promoter lies between positions -170 and -332. The region includes one copy of a WGATAR sequence (Figure 8), a potential target of the *elt-1* and *elt-2* transcription factors (Spieth *et al.*, 1991; Hawkins and McGhee, 1995), but we have not determined whether this sequence plays any role in augmenting *fem-1* activity. Expression of a *fem-1::lacZ* reporter gene seems insensitive to deletion

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-333  ccatgggagt tcctatacaa ttttgcatac ttctgcgtag tttgtttcaa acaatgatac
-273  aaacagtaac gatctttgag atttttacgg aaaaatcgat aaaattgagg caaaagttga
-213  caaaaccgac gggaaaatct ctgatatccg atatttccgc cgcggcgctg aactcaaaat
-153  ttcgagcact ccgcataaat cctcacgtca gattgcagac cgtgcaccct atccgacga
-93   aaatcacgag gagtattttc ttttcttttt cttctcgaat ttctgcaatt tctattaaa
-33   ttcgccagc ttttttttgg aaaaattggc ggtATGACAC CAAATGGACA TCATTTTCGA
+28   ACAGTAATTT ATAATGCTGC GGCGGTTGGA AATTGTCAGA GAATTAAAg aagcaaaaag
      <-----oAS101----->

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of sequence upstream of position -170 . The discrepancy may be an artifact of threshold effects or differences in sensitivity between the two assays, neither of which is strictly quantitative. Alternatively, the GC-rich sequence that spans position -170 might be important for *fem-1* expression, and differences in vector sequence adjacent to the -170 deletion breakpoints in the plasmids used in the two assays (AS#1237, Table 1; AS#1239, Figure 6A) may have caused a difference in activity.

Constructs lacking *fem-1* sequence upstream of position -112 are nearly inactive in both assays, suggesting the existence of essential promoter elements between positions -112 and -170 . One candidate is an AT-rich sequence AATAAT at position -139 , which may act as a TATA element (Figure 8). Primer extension experiments suggest that transcription of *fem-1* initiates 40 nucleotides downstream at position -94 . The untranslated leader region includes a 16-nucleotide inverted repeat that could potentially form a stem-loop structure. Such a structure could affect the stability or translation of *fem-1* mRNA, but any such effect is unlikely to be modulated, in view of the constancy of FEM-1 levels during development and in both sexes. An unrelated inverted repeat of similar size occurs in the 5' untranslated region of *fem-3* mRNA and has been proposed to regulate its translation (Ahringer *et al.*, 1992).

The levels of *fem-1* mRNA during development and the pattern of expression of *fem-1::lacZ* reporter genes provide little indication that the *fem-1* promoter responds to lineage-, cell-type-, stage-, or sex-specific regulators. Deletion analysis did not reveal any tissue-specific regulatory elements associated with the gene. Its expression probably depends on ubiquitous factors, and its promoter might be expected to resemble those of constitutive "housekeeping" genes. Few such genes have been subjected to analysis of their regulatory sequences in *C. elegans*, and their regulatory regions do not seem to be related to that of *fem-1* (Stringham *et al.*, 1992b; Jones *et al.*, 1995). The *ubq-2* gene, for

example, exhibits constitutive expression in all somatic tissues throughout development, but its expression results from the combined effects of regulatory sequences upstream and downstream of the transcribed region, including at least one tissue-specific element (Jones *et al.*, 1995).

Intron Dependence of *fem-1* Expression

Optimal activity of either a *fem-1* minigene or a *fem-1::lacZ* reporter requires two or more introns, but we found no evidence of a requirement for specific intron sequences. Even synthetic introns unrelated in sequence to any *fem-1* intron permitted constitutive expression of *lacZ* under the direction of the *fem-1* promoter. A single synthetic intron stimulates the expression of *lacZ* from the promoters of the *C. elegans* myosin genes *unc-54* and *myo-2* (Okkema *et al.*, 1993), and several examples exist of genes from other organisms that require at least one intron for their efficient expression (Nesic *et al.*, 1993 and references cited therein). Multiple introns can increase the efficiency of splicing of a particular intron and the efficiency of transcript 3' end generation in mammalian cells (Neel *et al.*, 1993; Nesic and Maquat, 1994). The importance of splicing for gene expression varies with different promoters (Neuberger and Williams, 1988; Okkema *et al.*, 1993), and the need for multiple introns to assure expression of *fem-1* may arise from some limitation imposed by the *fem-1* promoter or leader sequence.

Germline Feminization by Extrachromosomal Arrays Carrying *fem-1*

Extrachromosomal arrays carrying *fem-1* sequences, including even intact copies of *fem-1*, often abolish spermatogenesis in otherwise wild-type transgenic animals, suggesting that they interfere with an endogenous masculinizing activity in the germline. The germline-feminizing effect of these arrays seems to depend on the *fem-1* promoter, because arrays carrying a promoterless *fem-1* genomic clone (AS#1303, Ta-

Figure 8. Sequence of the minimal promoter region of *fem-1*. Upper case lettering indicates *fem-1* coding sequence. The oAS101 primer used for primer extension experiments was complementary to the sequence underlined with a half arrow. Filled arrows indicate an inverted repeat in the 5' untranslated region of *fem-1*. A shaded box indicates the transcriptional start site, and a candidate TATA sequence ~ 40 bp upstream is indicated in bold type and boxed. A dashed outline surrounds a consensus GATA sequence at positions -237 to -233 . The sequence shown here is available under GenBank Accession number J03172, nucleotides 154–573. (Numbering in the figure is with respect to the A of the *fem-1* start codon, which is $+1$.)

ble 1) do not feminize the germline. Germline feminization is not merely a consequence of the presence of multiple copies of the *fem-1* promoter or leader sequence, because clones in which the *fem-1* promoter and untranslated leader are fused to *lacZ* without other *fem-1* sequences (AS#1269, Figure 6B) also fail to affect germline sex. Instead, we suggest that those *fem-1* transgenes that cause germline feminization do so by directing the production of transcripts containing *fem-1* sequences but that are abnormal in structure or quantity. (We include antisense transcripts in the "structurally abnormal" group for the purposes of this discussion.) These transcripts might interfere with the germline function of the transgene and the endogenous *fem-1* gene, resulting in a Fog phenotype. Observations reported by Guo and Kemphues (1995) and Lin *et al.* (1995) provide evidence that exogenous RNA can interfere with gene function in the *C. elegans* germline. These authors found that the injection of either sense or antisense RNA transcripts of the maternal-effect genes *par-1* and *pop-1* into the *C. elegans* germline results in the production of progeny that precisely phenocopy the corresponding loss-of-function mutants.

The failure of any *fem-1::lacZ* fusion gene that we have tested to direct synthesis of β -galactosidase in the germline might reflect silencing of the transgene in the germline brought about by its own transcripts. We suggest that a fusion gene such as that carried by plasmid AS#1209, which causes germline feminization, interferes with both its own activity and that of the endogenous *fem-1* gene. A *fem-1::lacZ* fusion such as that carried by plasmid AS#1269, which does not feminize the germline, only silences itself, perhaps because its transcripts have less than some minimal region of homology with the endogenous gene.

Transgenes in plants often undergo silencing, and in some instances the endogenous homologues of these genes also suffer inactivation, a phenomenon referred to as cosuppression (reviewed by Flavell, 1994; Dougherty and Parks, 1995; Matzke and Matzke, 1995). In many cases silencing seems to be post-transcriptional. Models proposed to account for the phenomenon postulate destabilization of the transcripts of the transgene (and in some cases its endogenous homologues) by antisense RNA, by inefficient processing, or by titration of RNA-stabilizing factors. [See reviews cited above and Dehio and Schell (1994).] The germline-feminizing effects of *fem-1* transgenes and the ability of injected RNA to phenocopy maternal effect mutants suggest that similar silencing phenomena may occur in *C. elegans*. Genes that are active in the germline of *C. elegans* generally may be susceptible to this type of interference.

FEM-1 and Protein-Protein Interactions

Our analysis of *fem-1* expression supports and extends the results of earlier studies (Doniach and Hodgkin, 1984; Hodgkin, 1986) showing that *fem* activity is subject to post-transcriptional regulation. In addition, we argue that control of *fem* activity is at least partly post-translational with respect to FEM-1. An attractive possibility is that the masculinizing activity of FEM-1 depends on its physical association with other sex-determining proteins, because FEM-1 contains six copies of the cdc10/SWI6 (or ANK) motif (Spence *et al.*, 1990). In many other proteins ANK repeats mediates-specific protein-protein interactions (Michaely and Bennett, 1992). Analysis of interactions involving the ANK repeats of FEM-1 will be essential to a more complete understanding of the sex-determining signal transduction pathway in *C. elegans*.

ACKNOWLEDGMENTS

We are very grateful to Dr. A. Fire for providing us with his intron-containing *lacZ* vectors before publication. We also thank the Washington University Genome Sequencing Centre for the communication of sequence data before GenBank submission, I. Andrusis for the use of her densitometer, and M. Perry and J. Culotti for helpful comments on this manuscript. Some of the work described here was done at the Centre de Recherches de Biochimie Macromoléculaire du CNRS (CRBM) in Montpellier, France. A.M.S. thanks J. DeMaille of the CRBM for support, D. Casanova for assistance with the production of antisera, and especially Danielle Thierry-Mieg for generously sharing her laboratory. J.G. was supported by a National Sciences and Engineering Research Council (NSERC) postgraduate scholarship and an Ontario Graduate Scholarship. This research was supported by the Medical Research Council of Canada.

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