Molecular cloning and transcript analysis of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*

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The *fem-3* gene is required for specification of the male fate in the nematode *Caenorhabditis elegans*: XO males need *fem-3* for male differentiation in both soma and germ line; XX hermaphrodites need it for the production of sperm. We have cloned *fem-3* by transposon tagging. Among eight spontaneous *fem-3* mutations generated in a strain in which the transposable element Tc1 is mobile, six contain Tc1 insertions in a 2-kb region of the genome. From this region, we have identified three *fem-3* transcripts. Two, 1.7 kb and 1.62 kb, are presented in embryos, and two, 1.62 kb and 1.55 kb, are present in L4s and adults. The *fem-3* transcripts are not XO specific; however, in XX adult hermaphrodites, they appear to be limited to the germ line—a tissue involved in male development (both for spermatogenesis and for the maternal contribution of *fem-3* to the embryo). The amount of *fem-3* RNA in XO embryos is approximately sixfold greater than in XX embryos. The significance of this difference in specifying male development in XO but not in XX embryos is discussed.

[Key Words: Sex determination; *C. elegans*; *fem-3*; transposon tagging; maternal effect gene]

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During the development of multicellular organisms, cells are regulated to differentiate as one sex or another. Genes that control the sexual fate have been identified in the nematode *Caenorhabditis elegans* (e.g., Hodgkin 1987a), in *Drosophila* (e.g., Baker and Belote 1983), and in several mammals including man (e.g., Goodfellow and Darling 1988). By what molecular mechanisms do these regulatory sex-determining genes act? In *Drosophila*, molecular analyses of several sex-determining genes suggest that they are controlled at the level of RNA processing (Baker et al. 1987; Boggs et al. 1987; T. Cline and P. Schedl, pers. comm.). In man, the nucleotide sequence of the putative testis-determining factor (TDF) gene is likely to encode a protein with multiple Zn²⁺ finger domains and, therefore, may act by binding nucleic acid (Page et al. 1987). In *C. elegans*, though the sex-determining genes have been genetically characterized in detail, the molecular mechanism(s) by which they function are not known.

The initial signal for sex determination in *C. elegans* is the ratio of X chromosomes to sets of autosomes (Madl and Herman 1979). The self-fertilizing hermaphrodite has two X chromosomes (XX); the male has only one (XO). The X:A ratio is assessed by genes that regulate both the sexual phenotype and dosage compensation (Villeneuve and Meyer 1987; B. Meyer, pers. comm.). Then, seven autosomal genes (Klass et al. 1976; Hodgkin and Brenner 1977; Nelson et al. 1978; Hodgkin 1980, 1986, 1987b; Doniach and Hodgkin 1984; Kimble et al. 1984; Barton et al. 1987) regulate sexual differen-

tiation per se. These genes were initially identified by loss-of-function (*lf*) mutations that lead to sexual transformation of the animal. Four of them are further represented by gain-of-function (*gf*) mutations that lead to a sexual transformation opposite that of their *lf* counterparts; *her-1* (Trent et al. 1983), *tra-1* (Hodgkin 1983), *tra-2* (Doniach 1986), and *fem-3* (Barton et al. 1987). Genes with *lf* and *gf* alleles that result in opposite transformation phenotypes are likely to be central 'switch genes' in the pathway of sex determination.

Among the sex-determining genes of *C. elegans*, three *fem* genes, *fem-1*, *fem-2*, and *fem-3*, specify the male pathway of development: Mutations that eliminate any of the *fem* genes cause both XX and XO animals to develop as spermless hermaphrodites (females). Each of these *fem* genes has a maternal effect. Therefore, in hermaphrodites, the *fem* genes function to specify male development in the germ line for production of sperm and, by a maternal contribution, to specify male development in their progeny. In males, the *fem* genes are required to specify the male fate in all tissues. The wild-type function of the *fem* genes appears to vary, depending on the tissue. In the soma, they regulate the activity of *tra-1* negatively, whereas in the germ line, they regulate spermatogenesis positively (Doniach and Hodgkin 1984; Hodgkin 1986).

We have begun a molecular analysis of sex determination in *C. elegans* by cloning the *fem-3* gene. Two features of *fem-3* are of particular interest. First, the maternal effects of *fem-3* (Hodgkin 1986; Barton et al. 1987) are complex. Not only can maternal wild-type *fem-3*

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partially rescue *fem-3/fem-3 XO* progeny, but also this maternal contribution is necessary for the proper sexual development of *fem-3/+ XO* progeny. Second, the *fem-3* gene (or its product) appears to be controlled in the hermaphrodite germ line to permit the switch from spermatogenesis to oogenesis typical of that sex (Barton et al. 1987). Rare *gf* mutations in *fem-3*, which appear to alter its regulation, result in XX animals with a typical hermaphrodite soma but with a masculinized germ line that produces only sperm (Barton et al. 1987).

In this paper, we report the cloning of *fem-3* by transposon tagging. Transposon tagging is a powerful method for cloning genes in *C. elegans* (Greenwald 1985; Moerman et al. 1986). In addition, we describe three *fem-3* RNAs, their developmental profile, and their tissue and sex specificity. This work provides a necessary first step toward a biochemical understanding of the regulation of *fem-3* and the regulation of sexual differentiation by *fem-3* during development.

Results

Identification and cloning of *fem-3* DNA

To clone *fem-3*, we first isolated spontaneous *fem-3* mutations that carry insertions of the transposable element Tc1 (Emmons et al. 1983; Rosenzweig et al. 1983; Emmons and Yesner 1984; Ruan and Emmons 1984; Eide and Anderson 1985a) in the *fem-3* gene. *C. elegans* strains differ in their rate of germ-line transposition of Tc1 and in the number of copies of Tc1 per haploid genome. In the standard laboratory strain, Bristol, which has about 30 copies of Tc1, germ-line transposition has not been detected ($\leq 5 \times 10^{-9}$ for Tc1 insertions into *unc-54*) (Eide and Anderson 1985b). However, in TR403, an isolate from the wild that carries several hundred copies of Tc1, germ-line transposition has been observed at low frequency [2×10^{-6} for *unc-22* (J. Collins and P. Anderson, pers. comm.); 10^{-6} for *fem-3*, this paper]. In TR679 [*mut-2(r459)*], a strain derived after ethylmethane sulfonate (EMS) mutagenesis, germ-line transposition occurs at a much higher frequency (2×10^{-3} for *unc-54*) (Collins et al. 1987).

We have isolated spontaneous *lf* alleles of *fem-3* in TR403 or TR679 using the genetic selection diagrammed in Figure 1. Briefly, hermaphrodites homozygous for a temperature-sensitive *gf fem-3* mutation, *fem-3(q20)*, are self-fertile at permissive temperature (15°C), but are sterile, producing only sperm, at restrictive temperature (25°C) (Barton et al. 1987). However, a *fem-3(q20)/fem-3(lf)* heterozygote is self-fertile at 25°C. Therefore, *fem-3(lf)* mutations can be generated as intragenic suppressors of the sterile phenotype of *fem-3(q20)* homozygotes.

Strains homozygous for *fem-3(q20)* were constructed in which most of the genome was TR403 or TR679; only the region carrying *fem-3* and closely linked genes on chromosome IV remained Bristol (see Materials and methods). These *fem-3(q20)* homozygotes were raised at permissive temperature and then shifted to restrictive temperature as adults. Their F1 progeny were sterile un-

Figure 1. Selection scheme used to isolate spontaneous *fem(lf)* mutants (for details, see text).

less a suppressor mutation had been induced. Such a suppressor might either be a *fem-3(lf)* mutation induced in *cis* to *fem-3(q20)* or a dominant extragenic suppressor of *fem-3(q20)*. In this way, five spontaneous *fem-3(lf)* alleles, *q100*, *q102*, *q104*, *q137*, and *q138*, were isolated in TR403, and three, *q107*, *q135*, and *q136*, were isolated in TR679. In addition, two *tra-2(gf)* alleles, *q101* and *q103*, were isolated in TR403 as extragenic suppressors of *fem-3(q20)*.

If a *fem-3(lf)* mutation results from Tc1 insertion into *fem-3*, DNA from the suppressed strain would be expected to contain a Tc1 element at a novel site. In principle, this new Tc1 should be detectable on Southern blots with a Tc1 probe, but the presence of many copies of Tc1 in TR403 (or TR679) makes detection impractical in the original strains. Therefore, we reduced the background of Tc1 elements by crossing each of three *fem-3(lf)* mutations, *q100*, *q102*, and *q104*, into a Bristol genetic background. We then compared the Tc1-bearing restriction fragments in the *fem-3(lf)* mutant DNAs to those in Bristol DNA. Our results with *fem-3(q20q102)* are shown in Figure 2. In this Southern blot, several Tc1-bearing fragments not found in wild-type Bristol DNA (lane 1) are observed in two independently backcrossed lines of *fem-3(q20q102)* (lanes 2 and 3). To eliminate TR403-derived Tc1 elements closely linked to, but not in, *fem-3*, we picked recombinants between *fem-3* and a Bristol chromosome bearing a morphological marker less than 1 map unit on either side of *fem-3*. Comparison of the Tc1-bearing fragments in DNA from the strain carrying the recombined chromosome to those of Bristol DNA reveals only one novel Tc1 (Fig. 2, lane 4, large arrow). This Tc1 resides in a 3.1-kb *EcoRI* fragment. An *EcoRI* fragment of similar size was also observed as a novel Tc1 in *fem-3(q20q104)* (data not shown). Because Tc1 is 1.6 kb and because *EcoRI* does not cut within most Tc1 elements, this 3.1-kb fragment must contain 1.5 kb of wild-type *C. elegans* DNA in addition to Tc1.

We cloned the novel 3.1-kb *EcoRI* band from *fem-3(q20q102)* DNA. Unlike most *fem-3(lf)* alleles, *fem-3(q20q102)* is leaky and can be grown as a homo-

Figure 2. Identification of a TcI element associated with *fem-3(q20q102)*. DNAs were digested with *Eco*RI, electrophoresed in a 0.5% agarose gel, and blotted to nitrocellulose. The filter was hybridized to a 32 P-labeled TcI probe. (Lane 1) Bristol [*nuc-1(e1392)*] DNA; (lanes 2–4) DNAs from *fem-3(q20q102)* lines that had been independently crossed from the TR403 to the Bristol backgrounds. The strain represented in lane 4 was constructed by recombination on either side of *fem-3* to remove closely linked TcIs (see Materials and methods). The large arrow indicates the position of a novel 3.1-kb TcI-containing fragment that is not present in wild type but is present in all three independently backcrossed *fem-3* strains. Small arrows indicate positions of TR403-derived TcI elements not present in all backcrossed strains.

zygous stock. Therefore, in this strain, the novel TcI element is equimolar with other TcI elements in the genome. The novel fragment was first cloned into λ vector NM641 (Murray et al. 1977) and then subcloned

into plasmid vector pBI20, adjacent to a T7 promoter. This recombinant plasmid is designated pJK11.

If the TcI in pJK11 were an insertion into *fem-3*, as expected, the unique DNA adjacent to it should be *fem-3* DNA. Probes produced from this flanking DNA should therefore detect TcI insertions or DNA alterations in other *fem-3* alleles. We therefore synthesized a 32 P-labeled RNA probe from DNA flanking the novel TcI found in pJK11. (This probe was later shown to correspond to the *Eco*RI/*Hind*III fragment designated probe F in Fig. 4.) We hybridized this probe to Southern blots of DNAs from strains containing other spontaneous mutations of *fem-3* and, as controls, to DNAs from Bristol, TR403, and TR679 (Fig. 3, lanes 1–3). Insertions of 1.6 kb, the size of TcI, were detected in *fem-3(q20q104)* and *fem-3(q20q107)* (Fig. 3, lanes 4 and 5). These data strongly suggest that the TcIs associated with *q102*, *q104*, and *q107* are physically near to each other and that all three are in *fem-3*. To isolate the wild-type *fem-3* gene, the same probe was used to isolate phage from a genomic library of wild-type *C. elegans*, var. Bristol DNA. Three phage, λ JK2, λ JK3, and λ JK4, were isolated that contain overlapping inserts covering a continuous stretch of 26 kb of genomic DNA (Fig. 4C).

To localize the *fem-3* gene in *C. elegans* genomic DNA, additional *fem-3(lf)* mutants were analyzed by Southern blotting, using λ JK2, λ JK3, or plasmids derived from them as hybridization probes. We found insertions of 1.6 kb associated with the spontaneous mutations *fem-3(q20q135)*, *fem-3(q20q137)*, and *fem-3(q20q138)*. Further restriction digests showed that these insertions are TcI and located their approximate position in the genomic DNA (Fig. 4A). Thus, in all, we found that six out of eight spontaneous *fem-3* mutations were associated with TcI insertions within a 2-kb region (Fig. 4A). In addition, we discovered DNA rearrangements associated with one other spontaneous mutation, *fem-3(q20q136)*, an EMS-induced allele, *fem-3(e2040)*, and an X-ray-induced allele, *fem-3(e2062)* (Fig. 4A). The precise structure of these rearrangements is not known. For all nine *fem-3* mutations that possess a change detectable on a Southern blot, that change falls within a 4.5-kb region defined by two adjacent *Hind*III fragments. We conclude that the *fem-3* gene resides within this segment of cloned DNA.

Identification of two *fem-3* transcripts in adult hermaphrodites

We next identified transcripts from the *fem-3* region. 32 P-Labeled RNA probes were prepared from each of four continuous *Hind*III fragments (labeled A–D, Fig. 4B). Together, these encompass 9 kb of DNA and include all of the putative *fem-3* gene plus flanking sequences. The probes were single-stranded and, therefore, complementary to only one strand or the other of the region (arbitrarily designated plus (+) or minus (–) in Fig. 5).

Each probe was hybridized to a Northern blot of poly(A)⁺ RNA isolated from adult hermaphrodites. We expected the *fem-3* transcript to be present in adult her-

maphrodites because of the maternal effects of *fem-3* (Hodgkin 1986; Barton et al. 1987). Probes from the + strand of fragments B and C, which contain all of the *fem-3* insertion mutants, detected a transcript of about 1.6 kb (Fig. 5A). This band could be resolved into two transcripts of ~1620 and 1550 nucleotides on a higher percentage gel (Fig. 5B). In addition, two transcripts were detected from the region to the right of the TcI insertions [with probes C(-) and D(-)], and one was detected from the region to the left of the TcI insertions [with probe A(+)] (Fig. 5A). The two transcripts detected by probe C(-) were not observed with a - probe from fragment E (Fig. 4B; data not shown). Therefore, the two transcripts of 1.62 and 1.55 kb are the only transcripts detected using probes specific for the *fem-3* region, as defined by TcI insertions. These two RNAs are likely to be *fem-3* transcripts.

To confirm the identity of the two putative *fem-3* transcripts, we examined RNAs from mutants with a DNA rearrangement in *fem-3*. Using a *fem-3* cDNA probe, pJK50 (see Materials and methods), the expected 1.62- and 1.55-kb RNAs were observed in wild-type RNA (Fig. 6A, B, lanes 1), but in heterozygotes for either of two *fem-3* mutants with DNA rearrangements (*e2040/+* and *e2062/+*), novel-sized RNAs were observed in addition to the wild-type RNAs (Fig. 6A, lanes 2 and 3). In RNA from *fem-3(e2040)* homozygotes, both wild-type RNAs are replaced by the two larger novel RNAs (Fig. 6B, lane 2). A similar result has been observed for *fem-3(e2062)* homozygotes (data not shown). Furthermore, the sizes of the poly(A)⁺ RNAs produced from regions flanking *fem-3* are not altered in either *e2040* or *e2062* (data not shown). These results confirm that both the 1.62- and 1.55-kb transcripts seen in wild-type RNA are indeed produced from the region identified as the *fem-3* gene.

Developmental profile of *fem-3* transcripts in XX and XO animals

To learn what *fem-3* RNAs are present during development, we probed Northern blots containing RNAs from developmentally staged worms. We first examined tran-

scripts from the XX self-progeny of hermaphrodites (Fig. 7). *fem-3* RNA is easily detectable in embryo, L4, and adult hermaphrodites but is also present at a low level in L1, L2, and L3 animals (Fig. 7A). The 1.62- and 1.55-kb *fem-3* transcripts are found in both L4 and adult hermaphrodites, though the 1.62-kb transcript is increased relative to the 1.55-kb transcript in adults. In embryos, the 1.55 kb *fem-3* transcript is missing, and a larger (1.7-kb) *fem-3* transcript is present (Fig. 7A). Like the 1.62- and 1.55-kb transcripts, the 1.7-kb embryonic transcript is altered in *e2040/+* and *e2062/+* (the *fem-3* mutants

Figure 3. The mutations *fem-3(q20q104)* and *fem-3(q20q107)* both contain insertions of 1.6 kb that are closely linked to the insertion found in *fem-3(q20q102)*. DNAs were digested with *Hind*III, electrophoresed in a 0.7% agarose gel, and transferred to nitrocellulose. The filter was probed with ³²P-labeled RNA transcribed by T7 RNA polymerase from unique sequences flanking the TcI element associated with *fem-3(q20q102)* (probe F, Fig. 4B). The first three lanes contain DNAs from parental strains. (Lane 1) Bristol [*nuc-1(e1392)*]; (lane 2) TR403; (lane 3) *fem-3(q20)* crossed into a TR679 background; (lane 4) *fem-3(q20q104)/fem-3(q20)*; (lane 5) *fem-3(q20q107)/fem-3(q20)*. The small arrow indicates the wild-type band found in Bristol DNA in all parental strains and in heterozygous *fem-3(lf)/fem-3(q20)* strains. The large arrow indicates the bands in *fem-3(q20q104)* and *fem-3(q20q107)* which are 1.6 kb larger than the wild-type band.

Figure 4. (A) Restriction map of the *fem-3* genomic region. The positions of 1.6-kb insertions associated with six spontaneous *fem-3* alleles are indicated above the line (▽). Each insertion was shown to be a Tc1 element by its size of 1.6 kb and its near precise removal by *EcoRV*, which cuts 17 bp from each end of Tc1 (Rosenzweig et al. 1983). The insertion point was also mapped by *EcoRV* digestion. Below the line are regions of genomic DNA that are affected by rearrangements in three *fem-3* alleles (for details, see text). (H) *HindIII*; (RV) *EcoRV*; (R) *EcoRI*. (B) (Probes A–D) Four contiguous *HindIII* fragments; (Probe E) an *EcoRI* fragment; (Probe F) a *HindIII*–*EcoRI* fragment. Another probe used was derived from a *fem-3* cDNA clone, pJK50 (see Materials and methods), and is not shown here; pJK50 includes coding sequences contained in probes B and E (T. Rosenquist, unpubl.). (C) Phage λJK2, λJK3, and λJK4 contain overlapping inserts of *fem-3* genomic DNA.

with novel *fem-3* RNAs), indicating that it is a bona fide transcript of *fem-3* (data not shown). Rehybridization of this blot with an *act-1* probe indicates that the difference in levels of *fem-3* RNA during development is not a loading artifact (Fig. 7B). Production of *fem-3* RNA in L4 and adult hermaphrodites corresponds well to the results of temperature-shift experiments and maternal effect tests (Hodgkin 1986; Barton et al. 1987). Shift experiments indicate that *fem-3* acts in late larval stages to direct spermatogenesis in hermaphrodites, and maternal effect tests indicate that maternal *fem-3* activity is contributed to the embryo.

To examine *fem-3* transcripts in XO males of different stages, RNAs were extracted from synchronized progeny of a wild-type mating population. These progeny included both XX and XO animals. (It is not technically feasible to obtain pure XO progeny at early developmental stages in sufficient numbers for Northern analysis.) When hybridized with a *fem-3* probe, we found the same developmental profile of *fem-3* transcripts as observed with XX animals (data not shown). *fem-3* RNAs were present at relatively high levels in embryos, L4s, and adults, barely detectable in L1, L2, and L3 animals, and transcripts were of the same size as in XX animals alone. Consistent with this finding of no XO-specific *fem-3* transcript, we have compared *fem-3* transcripts from XX embryos to those from XX plus XO embryos in numerous experiments (see below) and found no XO specific transcript. Figure 8, which is discussed in more detail below, shows a typical result from one of these experiments. In addition, we have compared RNAs from

adult hermaphrodites to those of pure adult males (RNA was prepared from wild-type adult XO males hand-picked to eliminate hermaphrodites from the population) (data not shown). Again, no XO specific transcript was observed.

Thus, in numerous experiments, examining RNAs of various developmental stages, no XO-specific *fem-3* transcript has been detected. The function of *fem-3* is to direct male development, and superficially it might seem surprising that no XO-specific transcript was found. However, limited male development (spermatogenesis) occurs in hermaphrodites, and hermaphrodites provide a maternal contribution of *fem-3* to direct male development in the embryo. Therefore, our inability to detect an XO-specific *fem-3* transcript is not unexpected.

A quantitative comparison of fem-3 transcripts in hermaphrodite and male embryos

We next compared the quantity of *fem-3* transcripts found in embryos of each sex (Fig. 8). Embryos were selected for this comparison because sex-specific differences are already established in embryos and newly hatched L1 worms (Sulston and Horvitz 1977; Sulston et al. 1983). Furthermore, because no sex-specific difference is known in the number of cells produced during embryogenesis or in embryonic muscle differentiation, an *act-1* probe could be used to standardize the amounts of RNAs isolated from each population of embryos.

The amount of *fem-3* RNA produced in XX embryos

Figure 5. Transcription from the *fem-3* region. (A) Poly(A)⁺ RNA from adult hermaphrodites was electrophoresed in a 1.5% denaturing agarose gel for 17 hr at .75 V/cm and transferred to nitrocellulose. Strand-specific ³²P-labeled RNA probes were made by T7 or SP6 RNA polymerase from each *Hind*III fragment (A–D). (+) Probes synthesized from left to right on the map; (–) probes synthesized from right to left. (∇) The positions of TcI insertions in *fem-3*. Transcripts are indicated by wavy lines above the *Hind*III fragment(s) to which they hybridize. The direction of transcription is indicated by arrowheads. (B) Poly(A)⁺ RNA from adult hermaphrodites was electrophoresed in a 2.0% denaturing gel for 17 hr at 1.5 V/cm. The RNA was transferred to nitrocellulose and hybridized to a strand-specific probe, (C+). Two RNAs are resolved from the single band observed in panel A.

cannot be compared directly to that produced in XO embryos, because it is not technically feasible to obtain a pure population of XO embryos. However, a pure population of XX embryos can be derived from self-fertilizing hermaphrodites. Therefore, we could compare the amount of *fem-3* RNA in XX embryos to that of XO embryos by subtracting the *fem-3* contributed by XX embryos in a given mixed population (for further explanation, see the footnote to Table 1). One of the Northern blots used to obtain densitometry tracings for this comparison is shown in Figure 8. In each experiment, the fractions of XX and XO embryos for a given RNA prep were known directly by permitting some embryos to grow up. The results of multiple experiments are presented in Table 1. We have consistently found that XO embryos possess about sixfold more *fem-3* RNA than XX embryos.

Tissue-specificity of fem-3 transcripts in hermaphrodites

The maternal effects of *fem-3* (Hodgkin 1986; Barton et

al. 1987) imply that a *fem-3* gene product is present in oocytes. To determine whether *fem-3* is transcribed specifically in the germ-line tissue of adult hermaphrodites, we compared RNAs from mutant worms, that contain very few germ cells, to RNAs from wild-type worms, that contain about 2500 germ cells (Fig. 9). Two temperature-sensitive mutants, which can be grown at permissive temperature as homozygotes, drastically reduce the number of germ-line cells when grown at restrictive temperature. In one of the mutants, SS104(*bn2*), germ-line proliferation is blocked prior to entry into meiosis; *bn2* homozygotes raised at restrictive temperature contain only 10–20 premeiotic germ cells per animal (S. Strome, pers. comm.). In the other mutant, *glp-1(q224)*, germ line proliferation is blocked by premature entry of mitotic cells into meiosis; homozygotes raised at restrictive temperature contain only 16–32 sperm per animal (Austin and Kimble 1987). In both mutants, XO male development is unimpaired, and in *glp-1* mutants, a few sperm are made prematurely. Therefore, the expression of *fem-3* per se appears to be unaffected in both mutants. RNA isolated from adult hermaphrodites ho-

Figure 6. Novel RNAs associated with *fem-3* mutations. Hermaphrodite RNAs were separated by electrophoresis in a 2.0% denaturing agarose gel, transferred to nitrocellulose, and hybridized to 32 P-labeled RNA transcribed from *fem-3* cDNA clone, pJK50. (A) (lane 1) Wild-type poly(A)⁺ RNA; (lane 2) *fem-3*(*e2040*)/+ poly(A)⁺ RNA; (lane 3) *fem-3*(*e2062*)/+ poly(A)⁺ RNA. (B) (Lane 1) Wild-type poly(A)⁺ RNA; (lane 2) total RNA from 1000 adult *fem-3*(*e2040*) homozygotes. (A and B) Arrows indicate wild-type *fem-3* RNAs; small arrowheads, *e2040* *fem-3* RNAs; large arrowhead, *e2062* *fem-3* RNA. The *fem-3*(*e2040*) RNAs are about 600 bases, and the *fem-3*(*e2062*) RNAs are about 150 bases larger than wild-type *fem-3* RNAs.

mozygous for either *bn2* or *glp-1* contained no detectable *fem-3* transcript, even though an excess of RNA from these strains was present (Fig. 9A). The excess of RNA on the filter was confirmed by rehybridization of the same blot to an *act-1* probe (Fig. 9B). The most straightforward explanation of the lack of *fem-3* transcripts in these mutants that lack a germ line is that *fem-3* is normally transcribed in the adult hermaphrodite germ line. Another possible explanation is that *fem-3* RNA is synthesized in a somatic tissue, but its synthesis (or stability) depends on the presence of a normal germ line.

Discussion

In this paper, we describe the molecular cloning of the *fem-3* gene and an analysis of its transcripts during development. We report five main conclusions. (1) At least three poly(A)⁺ RNAs are synthesized from *fem-3*; (2) two *fem-3* RNAs are stage specific—the 1.7-kb RNA is found only in embryos, and the 1.55-kb RNA is found only in L4 larvae and adults; (3) the production of *fem-3* RNA in adult hermaphrodites depends on the presence

Figure 7. Developmental profile of *fem-3* transcripts. Poly(A)⁺ RNAs were isolated from developmentally staged hermaphrodites and were separated by electrophoresis in a 2.0% denaturing agarose gel and transferred to nitrocellulose. The filter was hybridized to a *fem-3* probe (A) (cDNA clone pJK50, see Materials and Methods) and then rehybridized to an *act-1* probe (B). The amount of *act-1* RNA in total RNA appears to be constant throughout postembryonic development (Krause and Hirsh 1984); therefore, the *act-1* signal serves as an internal control for the amount of RNA loaded. Arrows indicate *fem-3* transcripts. (A and B) (Lane E) Embryonic RNA; (lanes L1–L4) RNAs from first through fourth larval stages, respectively; (lane A) Adult RNA.

Figure 8. XO embryos possess about sixfold more *fem-3* RNA than XX embryos. RNAs were electrophoresed in a 2.0% denaturing agarose gel and transferred to nitrocellulose. The filter was first hybridized to a *fem-3* probe (cDNA pJK50) (A) and then rehybridized to an *act-1* probe (B). Arrows indicate *fem-3* transcripts. (A and B) (Left lane) Poly(A)⁺ RNA from hermaphrodite (XX) embryos, self-progeny of hermaphrodites; (right lane) poly(A)⁺ RNA from embryos, self-hermaphrodite (XX) and male (XO) plus cross-progeny of wild-type hermaphrodites and males. This figure shows data for experiment III, Table 1, where the XX + XO lane contains 29% XO embryos.

Table 1. Relative amounts of *fem-3* RNA in XO and XX embryos

Experiment	RNA source ^a	Percent males ^b	<i>fem-3</i> signal ^c	<i>act-1</i> signal ^d	Relative amount ^e
I	wild-type XX	0	32.5	131	1
	wild-type XX + XO	30	198	302	6.5
II	wild-type XX	0	62.5	32	1
	<i>him-5</i> XX + XO	33	305	70	4.7
III	wild-type XX	0	33.2	221	1
	wild-type XX + XO	29	81.5	204	6.7
	<i>him-8</i> XX + XO	39	42.5	95	6.1

^a Wild-type XX, hermaphrodites; wild-type XX + XO, cross-progeny of mating wild-type hermaphrodites and males; *him-5* or *him-8* XX + XO, self-progeny of *him* mutants.

^b For each RNA prep, some embryos were allowed to develop to adulthood to score the percentage of males by visual inspection.

^c Mean value in arbitrary units determined by scanning laser densitometry of autoradiograms of Northern blots probed with a *fem-3*-specific probe, as described in Materials and methods. These values cannot be compared between experiments.

^d Mean value in arbitrary units determined by scanning laser densitometry of autoradiograms of Northern blots reprobed with an *act-1*-specific probe. Under the conditions used, the preexisting *fem-3* signal was insignificant compared to the *act-1* signal. This *act-1* value is used as an internal control to correct for the varying amount of RNA used within each experiment.

^e The value in the first row of each experiment is the level of *fem-3* RNA in XX embryos and is arbitrarily set to 1. The value in the second (and third) row in each experiment is the level of *fem-3* RNA in XO embryos relative to that in XX embryos of the same experiment. The level of *fem-3* RNA in XO embryos was calculated from the equation $(XX + XO) = f_{xx}(XX) + f_{xo}(XO)$, where $XX + XO$ is the *fem-3* signal from XX + XO embryos normalized to the *act-1* signal [i.e., *fem-3* (XX + XO) observed multiplied by XX *act-1*/(XX + XO) *act-1*]; f_{xx} and f_{xo} are fractions of XX and XO embryos, respectively; XX is the observed *fem-3* signal of XX embryos, and XO is the calculated *fem-3* signal of XO embryos.

Figure 9. The presence of *fem-3* RNAs in adult hermaphrodites is dependent on the presence of a germ line. RNAs were isolated from temperature-sensitive mutants, SS104(*bn2*) or *glp-1*(*q224*), and grown at restrictive temperature to eliminate most germ cells. Poly(A)⁺ RNAs were separated by electrophoresis in a 2.0% denaturing agarose gel and transferred to nitrocellulose. The filter was first hybridized to a *fem-3* probe (A) (cDNA pJK50, see Materials and methods), and then rehybridized to an *act-1* probe (B). (A and B) (Lane 1) Wild-type (N2) RNA; (lane 2) SS104(*bn2*) RNA; (lane 3) *glp-1*(*q224*) RNA. The mutants lack *fem-3* RNA but possess actin RNA, showing that RNA was present in each mutant lane; wild-type animals possess *fem-3* but appear to lack actin at this exposure due to the difference in the amount of RNA loaded in each lane.

of the germ line, suggesting that it is made only in that tissue; (4) more *fem-3* RNA is present in XO embryos than in XX embryos; and (5) no XO-specific *fem-3* transcript is detected at any time during development.

In *C. elegans*, XX animals are hermaphrodite (females that produce a limited amount of sperm), and XO animals are male. From the central role played by *fem-3* in sex regulation, one might have expected that *fem-3* would produce a sex-specific transcript. However, XX hermaphrodites make sperm and are therefore partially male. Because *fem-3* promotes masculine development in both hermaphrodites and males, an XO-specific transcript is not expected. We have compared RNAs from XX and XO animals throughout development and, indeed, find no XO-specific transcript. Nevertheless, it remains possible that XO-specific RNAs exist if their sizes are not sufficiently different from those of other *fem-3* RNAs for detection by Northern analysis.

Although both hermaphrodites and males seem to produce the same *fem-3* RNAs, the amount of these RNAs differs in embryos of the two sexes: XO male embryos have approximately sixfold more *fem-3* RNA than XX hermaphrodite embryos do. The presence of more *fem-3* RNA in male than in hermaphrodite embryos is consistent with the role of *fem-3* to direct male development. This quantitative difference might be due to differential *fem-3* transcription or RNA stability in XX and XO embryos. The greater amount of *fem-3* transcript in XO embryos may be critical to regulation of the sexual phenotype. Genetic evidence suggests that the dose of *fem-3* affects its ability to direct male development (Hodgkin 1986; Barton et al. 1987). Therefore, a sixfold difference in *fem-3* transcript could provide the critical difference in the amount of *fem-3* necessary to specify

male development in *XO*, but not in *XX*, embryos. Furthermore, because the sixfold difference is based on RNAs extracted from embryos of all ages, this difference may reflect an average of early embryos with, e.g., nearly equal amounts of *fem-3* RNA, and later embryos with vastly different amounts of *fem-3* RNA. Similarly, because the sixfold difference is seen with RNAs from all embryonic tissues, this difference may reflect a tissue-specific difference. For example, male development in *XX* hermaphrodites is limited to the germ line, and *fem-3* RNA in *XX* embryos may be similarly limited to the germ-line precursor cells. On the other hand, the sixfold difference in *fem-3* RNA may not be the key to *fem-3* regulation of male development. It remains possible that other mechanisms (e.g., posttranscriptional regulation) control the activity of *fem-3* during embryogenesis. Clearly, more experiments are required to assess the significance of the sixfold difference reported here.

The maternal effects of *fem-3* (Hodgkin 1986; Barton et al. 1987) suggest that *fem-3* is present in the adult hermaphrodite germ line. We find that *fem-3* transcripts are present in wild-type adult hermaphrodites but are missing in mutant adult hermaphrodites that lack a germ line. These molecular data suggest that the presence of *fem-3* transcripts in hermaphrodites is due to accumulation of *fem-3* RNA in the germ line. Alternatively, this accumulation may represent expression of *fem-3* in somatic tissues that depends on the presence of a germ line. However, given the maternal effects, the simplest explanation is that only the germ line produces *fem-3* RNA. Such tissue-specific control of *fem-3* expression may explain the germ-line-specific masculinization by *fem-3(gf)* mutations (Barton et al. 1987). If *fem-3* activity is limited to the germ line in adult hermaphrodites, a *fem-3(gf)* mutation may only be able to masculinize that tissue.

The relative abundance of *fem-3* transcripts in embryos, L4s, and adults, and the existence of stage-specific *fem-3* RNAs suggest that *fem-3* expression is controlled temporally. However, an alternative explanation of this developmental profile is that *fem-3* is constitutively expressed by germ-line cells. Because only two germ cells are present at hatching, and growth of the germ line is exponential, the production of *fem-3* may be barely detectable during L1, L2, and L3 due to paucity of germ cells but may become easily detectable in L4 and adult stages when many more germ cells are present. By this model, embryonic *fem-3* RNAs would be maternal RNAs produced by oocytes, and the larger embryo-specific transcript would be a modified form (e.g., polyadenylated or *trans*-spliced) of a preexisting RNA.

In hermaphrodites, only the germ line requires *fem-3* activity. Sperm are male germ cells, and their production depends on the function of *fem-3*. Furthermore, although oocytes are female germ cells, they are the most likely source of a maternal contribution of *fem-3* to the embryo. Based on the lack of *fem-3* RNA in hermaphrodites with no germ line, we have suggested that *fem-3* RNA is only made by the germ line in hermaphrodites. Therefore, although *fem-3* transcripts are not limited to

XO individuals, they appear to be limited in *XX* hermaphrodites to tissues that direct masculine development.

In conclusion, we have cloned the *fem-3* gene and identified its transcripts during development. This provides a necessary first step in the analysis of the molecular mechanism of sex determination in *C. elegans*. In addition, we have analyzed the presence of the *fem-3* transcripts during development and have begun to investigate their tissue and sex-specificity. The *fem-3* transcripts, though not *XO* specific, are likely to be specific to tissues involved in male development. In *XX* hermaphrodites, *fem-3* RNAs appear to be limited to the germ line—a tissue that makes some sperm and then contributes male-directing activity to embryos. Now that *fem-3* is cloned, several problems of developmental regulation become accessible. How does *fem-3* specify male development? How does *fem-3* function differ in somatic and germ-line tissues? How is *fem-3* regulated to achieve the switch from spermatogenesis to oogenesis in the hermaphrodite germ line? And, finally, how does maternal *fem-3* direct male development only in the germ line of *XX* embryos but in all tissues of *XO* embryos?

Materials and methods

Strains

C. elegans, strain Bristol [N2], was obtained from S. Brenner. TR403 and TR679 were obtained from P. Anderson. Strains *unc-24(e138)* and *dpy-20(e1282)*, *him-8(e1489)*, *him-5(e1490)*, and *nuc-1(e1392)* are described in Swanson et al. (1984). *glp-1(q224)* (Austin and Kimble 1987) and *fem-3(q20)* (Barton et al. 1987) are also described. SS104 (*bn2*) was provided by S. Strome. *fem-3(lf)* alleles, *e2040* and *e2062*, were obtained from J. Hodgkin and are described (Hodgkin 1986).

Worm culture

Worms were grown routinely on agar-filled petri dishes seeded with *Escherichia coli* (Brenner 1974). Large-scale cultures were grown in liquid (Sulston and Brenner 1974). Animals were synchronized by isolating eggs after hypochlorite digestion of gravid hermaphrodites (Emmons et al. 1979). Embryos of both sexes (*XX* and *XO*) were obtained as cross-progeny of wild-type (N2) mating adults or as self-progeny of *him-8* or *him-5* hermaphrodites. *him* hermaphrodites produce a high incidence of males among their self progeny due to a defect in disjunction of the *X* chromosome (Hodgkin et al. 1979).

Isolation of spontaneous *fem(lf)* mutations

The strain JK925 was constructed to select for *fem-3(lf)* mutations in TR403. *fem-3(q20) dpy-20*, originally in a Bristol genetic background, was crossed to TR403 males, and *dpy-20* homozygotes were reisolated from the F2. This cross and reisolation was repeated six times. The resultant hybrid strain contains Bristol DNA from the *fem-3* region of chromosome IV, but the rest of the genome is mostly TR403. The presence of *fem-3(q20)* was confirmed by its temperature-sensitive sterile phenotype. Another strain, JK708, was similarly constructed by crossing *fem-3(q20) dpy-20* into the TR679 background.

Cultures (500-ml) were seeded with about 10³ adult her-

maphrodites of strain JK925 or JK708 and were shaken at 25°C for about a week. Worms were harvested, and any eggs—the result of suppression of the sterile *fem-3(q20)* phenotype—were isolated by hypochlorite treatment. The suppressor was identified by a combination of mapping and complementation tests. The *fem-3(q20) dpy-20 sup* hermaphrodites were crossed with wild-type (N2) males, and F2 Dpy animals were examined. The presence of Dpy females suggested that the suppressor was *fem-3(lf)*. To confirm the identity of a new *fem-3(lf)* allele, a complementation test was performed. F2 females were crossed with *fem-3(e1996) dpy-20/+ +* males, and the cross-progeny examined for Dpy females. *e1996* is a putative null allele of *fem-3* (Hodgkin 1986).

Two unlinked suppressors of *fem-3(q20)*, *q101* and *q103*, were isolated from JK925. These yielded non-Dpy F2 females after crossing to N2. After a second cross with N2 males, both *q101* and *q103* produced F1 females. The dominant feminizing activity of both suppressors was mapped to chromosome II near the *tra-2* gene. The feminizing phenotype of these suppressors resembles that described for *tra-2 gf* mutants (Doniach 1986). *q101* was confirmed to be a *gf* allele of *tra-2* by isolating an EMS-induced *tra-2(lf)* allele (*q105*), tightly linked to *q101*. *q105* eliminates the feminizing activity of *q101*. Both *q101* and *q103* have been shown to contain TcI insertions in the *tra-2* gene (P. Okkema and J. Kimble, pers. comm.).

For three of the spontaneous *fem-3(lf)* alleles isolated, several (two to four) independent lines were crossed into a Bristol background. After the sixth cross to N2, the chromosome bearing *fem-3(lf)* was recombined on each side of *fem-3* to replace closely linked TR403 DNA with Bristol DNA. From the progeny of *+fem-3(lf) dpy-20/unc-24 + +* worms, UncFem Dpy animals were picked; and from *unc-24 fem-3(lf) dpy-20/+ +* worms, Unc Fem non-Dpy animals were picked. The *fem-3(lf)* mutations were maintained as *fem-3(lf)/fem-3(q20)*.

General procedures for manipulation of nucleic acids

Preparation of nematode DNA was as described (Emmons and Yesner 1984). General methods for manipulating DNA and RNA relied on procedures described by Maniatis et al. (1982). ³²P-Labeled RNA probes were synthesized with SP6, T7, or T3 RNA polymerase by methods described for SP6 RNA polymerase (Melton et al. 1984). The plasmid pJK9 contains a complete copy of TcI and 0.9 kb of the *unc-54* gene inserted in the *Bam*HI site of pBR322. TcI probes were produced by nick translation of pJK9. Plasmid vectors pIBI20 and pIBI76, which contain T7 and SP6 RNA polymerase promoters for the transcription of insert DNA, were purchased from International Biotechnologies, Inc. The *act-1* plasmid pT7/T3 18-103 was the gift of M. Krause; this *act-1* probe is specific for *act-1*, one of the muscle actin genes of *C. elegans* (Files et al. 1983). NM641 was obtained from W. Dove. A genomic library of *C. elegans* DNA in the λ vector EMBL3 was the gift of C. Cummins and P. Anderson.

For preparation of RNA, worms were collected by centrifugation, washed several times in M9 buffer to remove contaminating *E. coli*, and resuspended in 4 M guanidinium isothiocyanate, 100 mM Tris (pH 7.5), 10 mM Sarkosyl, and 10 mM β -mercaptoethanol. Some preparations include 10 μ g/ml aurintricarboxylic acid. An equal volume of siliconized sand was added, and worms were lysed by vortexing for 5 min at room temperature. The lysate was extracted three times with phenol:chloroform and once with chloroform. RNA was precipitated with 0.025 volumes of 1 M acetic acid and 0.75 volumes of ethanol and stored at -20°C . Some RNA preps were further purified by proteinase K and DNase I treatment.

Poly(A)⁺ RNA was selected by a single round of selection on an oligo(dT) cellulose column. Northern blots were performed by electrophoresing RNA in agarose-formaldehyde gels, followed by transfer of the RNA to nitrocellulose. Blots were probed as described in the figure legends and were washed in $0.2\times$ SSC, 0.1% SDS at 65°C.

Cloning

DNA isolated from *fem-3(q20q102)* homozygotes was digested with *Eco*RI and electrophoresed in a 0.8% low-melting-temperature agarose gel. DNA from 2–4 kb was purified from the gel and ligated into *Eco*RI-digested NM641 (Murray et al. 1977). Recombinant phage were screened by hybridization to a TcI probe (nick-translated pJK9). The 3.1-kb *Eco*RI insert in these phage was subcloned into pIBI20 to generate the plasmid pJK11. To synthesize a probe to unique *C. elegans* DNA that flanked the TcI within this 3.1-kb fragment, the plasmid pJK11 was digested with *Hind*III and a ³²P-labeled RNA probe was generated by transcription with T7 RNA polymerase. This probe was used to obtain genomic clones by screening a *C. elegans* library in EMBL3. The clones, λ JK2, λ JK3, and λ JK4, were isolated by hybridization to this probe. *Hind*III and *Eco*RI fragments were subcloned from λ JK2 and λ JK3 into pIBI76. A cDNA subclone, pJK50, was isolated from a mixed-stage cDNA library in λ gt10 (J. Ahringer and D. Lawson, pers. comm.) by hybridization to probe E (Fig. 4). Antisense ³²P-labeled RNA was synthesized from pJK50 with T7 RNA polymerase. Southern blots were probed as described in the figure legends and washed at high stringency: $0.2\times$ SSC, 0.1% SDS at 65°C. RNA sizes were estimated relative to *C. elegans* ribosomal and actin RNAs.

Quantitation of Northern blots

Quantitation of transcript bands on autoradiographs was performed using a Zeineh model SL-504-XL scanning laser microdensitometer. Each transcript band was scanned in three locations, and a mean peak area was calculated to use in the comparison between different RNA preparations. The areas reported in Table 1 are given in the arbitrary units provided by the integrator. When possible, two exposures of each autoradiograph were traced to eliminate any complication because of potential nonlinearity of the film.

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