The EMBO Journal vol.11 no.6 pp.2303 - 2310, 1992

The *Caenorhabditis elegans* sex determining gene *fem-3* is regulated post-transcriptionally

Julie Ahringer[1], Thomas A.Rosenquist[2], David N.Lawson and Judith Kimble[3]

Department of Biochemistry, College of Agricultural and Life Sciences and Laboratory of Molecular Biology, Graduate School, University of Wisconsin-Madison, Madison, WI 53706, USA

[1]Present address: MRC-LMB, Hills Road, Cambridge CB2 2QH, UK
[2]Present address: Department of Anatomy, University of California-San Francisco, San Francisco, CA, USA
[3]Corresponding author

Communicated by J.Hodgkin

The *fem-3* gene of *Caenorhabditis elegans* is required for male development. Both maternal and zygotic *fem-3* activities are required for spermatogenesis in the XX hermaphrodite germline and for male development in somatic and germline tissues of XO (male) animals. Here we show that *fem-3* RNA is contributed to embryos as a maternal product and that this RNA is degraded early in embryonic development. The poly(A) tail of embryonic *fem-3* RNA is substantially longer than that of adult hermaphrodites which indicates that poly(A) tail lengthening probably occurs at or soon after fertilization. During subsequent development, *fem-3* poly(A) tails shorten. The amount of *fem-3* RNA in XX and XO embryos is equivalent, suggesting sex-specific regulation of maternal *fem-3* activity occurs post-transcriptionally. The sequence of *fem-3* predicts an open reading frame that could encode a soluble protein; putative *fem-3* null mutants truncate this open reading frame. We discuss the implications of these results for the regulation and function of *fem-3*.

Key words: C.elegans/fem-3/maternal RNA/polyadenylation/sex determination

Introduction

In the nematode *Caenorhabditis elegans*, the *fem-3* gene is required for specification of male development (Hodgkin, 1986; Barton *et al.*, 1987). Sex is initially determined by the ratio of X chromosomes to autosomes; animals with one X chromosome (XO) are males and those with two (XX) are hermaphrodites (Madl and Herman, 1979). In XO animals, *fem-3* directs male differentiation in all tissues, whereas in XX animals, *fem-3* specifies the first 80 or so germ cells to be sperm. Subsequently, the XX expression of *fem-3* is negatively regulated, which results in a switch from spermatogenesis to oogenesis (Barton *et al.*, 1987; Ahringer and Kimble, 1991). Hence XO animals are male, and XX animals become self-fertilizing hermaphrodites that produce sperm and then oocytes.

The *fem-3* gene acts within a regulatory cascade of sex-determining genes to influence the sexual phenotype (reviewed by Hodgkin, 1990). In somatic tissues, *fem-3*, together with *fem-1* and *fem-2*, negatively regulates *tra-1*, the control gene acting at the end of the cascade. Recent data suggest that *tra-1* may be a transcriptional regulator (D.Zarkower and J.Hodgkin, submitted). Because *tra-1* activity directs female somatic development (Hodgkin, 1980, 1987), *fem-3* is required to turn off *tra-1* in XO animals to permit male differentiation. Conversely, in the XX soma, *fem-3* itself must be turned off to allow *tra-1* to direct female development. In the germline, *tra-1* is not the terminal gene. Instead, *fem-3*, together with the other *fem* genes and *fog-1*, direct spermatogenesis as the final genes in the cascade (Doniach and Hodgkin, 1984; Kimble *et al.*, 1984; Hodgkin, 1986; Barton and Kimble, 1990; Barton *et al.*, 1987).

Wild-type *fem-3* activity must be contributed by both maternal and zygotic genomes for normal development. Lack of both maternal and zygotic *fem-3* activity transforms both XX and XO animals into females (spermless hermaphrodites) (Hodgkin, 1986). When maternal *fem-3* activity is absent, both XX and XO animals are partially feminized. XO animals require maternal *fem-3* activity for male differentiation of both somatic and germline tissues, whereas in XXs it is required only in the germline for spermatogenesis. The maternal requirement suggests that either *fem-3* RNA or *fem-3* protein (or both) is contributed to the embryo by the oocyte. Because this maternal *fem-3* product has a different role in XX and XO animals, it is likely to be controlled in a sex-specific manner, and this regulation probably occurs post-transcriptionally in the embryo.

The *fem-3* gene has been cloned and three *fem-3* RNAs detected (Rosenquist and Kimble, 1988). No sex-specific *fem-3* RNAs were observed, though a 6-fold difference in the level of *fem-3* RNA between XX and XO embryos was reported. In adult hermaphrodites, *fem-3* RNA was found primarily in the germline, consistent with its requirement for hermaphrodite spermatogenesis and *fem-3* maternal effects.

In this paper we extend our molecular characterization of *fem-3* RNA. We find that *fem-3* RNA in the early embryo is maternally derived and rapidly degraded, and that the levels of *fem-3* RNAs in XX and XO embryos, in contrast to our earlier report, are not significantly different. We analyse length differences of *fem-3* RNAs during development and show that these are due to changes in poly(A) tail lengths. In addition, we characterize the sequence and structure of the major *fem-3* transcript and present the molecular changes associated with some *fem-3* loss of function mutants. The implications of these results with respect to both the function and regulation of *fem-3* during development are discussed.

Results

Early embryonic fem-3 RNA is derived from the maternal genome

The activity of *fem-3* is required maternally (Hodgkin, 1986; Barton *et al.*, 1987) and *fem-3* RNA is found primarily in the germline of adult hermaphrodites and in embryos (Rosenquist and Kimble, 1988). Therefore, it seemed plausible that *fem-3* activity might be contributed to embryos as a maternal RNA. To test this possibility, we mated *fem-3(e2040)* mutant females, which make novel-sized *fem-3* RNA (Rosenquist and Kimble, 1988), with wild-type males to produce *fem-3(e2040)/+* embryos (Figure 1A). If an early embryo normally transcribes *fem-3* RNA from its zygotic genome, *fem-3(e2040)/+* embryos should contain both wild-type and mutant-sized *fem-3* RNAs. However, if *fem-3* RNA in early embryos is derived from the mother, these embryos should have only novel-sized *fem-3* RNA. In *fem-3(e2040)/+* embryos, we detected only the novel-sized *fem-3* RNA (Figure 1B, lane 4). The simplest interpretation of this result is that *fem-3* RNA is made in the hermaphrodite germline and packaged into oocytes for use in the early embryo. It is also possible that the maternal but not the paternal allele is transcriptionally active in early embryos; however, it has been argued that imprinting probably does not occur in *C.elegans* (Haack and Hodgkin, 1991).

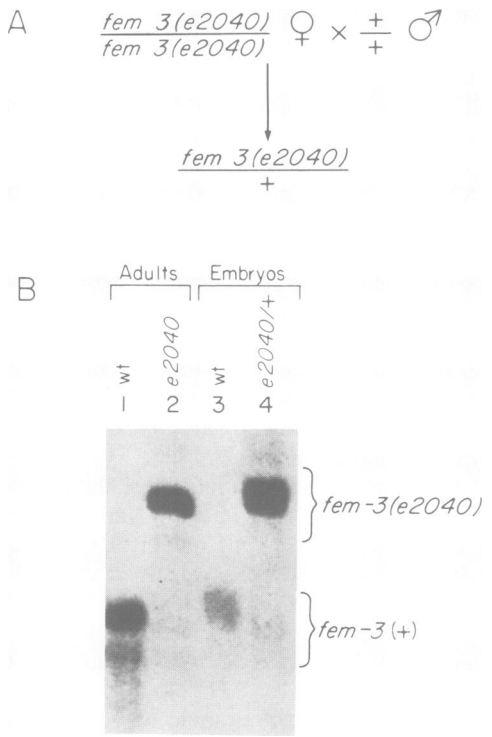

Fig. 1. Maternal *fem-3* RNA is contributed to embryos. **A**, scheme of the procedure used to obtain *fem-3(e2040)/+* embryos. *fem-3(e2040)* females were mated with wild-type males for 24 h before embryos were isolated. **B**, Northern blot of total RNA isolated from: lane 1, wild-type adult hermaphrodites; lane 2, *fem-3(e2040)* adult females; lane 3, wild-type embryos; lane 4, *fem-3(e2040)/+* embryos. Lane 4 has 3- to 5-fold more RNA than lane 3 (judged by reprobing the blot for *act-1* RNA; data not shown). Thus, the wild-type size *fem-3* RNA would have been readily detected in lane 4, if present. The small amount of signal at the wild-type position in lane 4 is probably due to trapping of probe in the ribosomal RNA which runs at this position since total, instead of poly(A)$^+$ RNA, was used to minimize losses. A similar result was obtained using *fem-3(e2062)* (not shown), which also has novel sized *fem-3* RNA (Rosenquist and Kimble, 1988).

Maternal fem-3 RNA is degraded early in embryogenesis

Embryos have substantially more *fem-3* RNA than L1 larvae (Rosenquist and Kimble, 1988). To determine when the steady-state level of *fem-3* RNA decreases during embryogenesis, we isolated staged embryos (see Materials and methods) and analysed their *fem-3* RNA by Northern blotting. The specific embryonic stages present in each preparation of embryos is shown in Table I. We found that the amount of *fem-3* RNA decreases dramatically from the 0 h to the 1 h time point and then remains at the same low level for at least 8 h (Table I, Figure 2). We estimate that 1- to 8-cell embryos have ~10-fold more *fem-3* RNA than >8-cell embryos (see legend to Table I for calculation). Because *fem-3* RNA is maternally derived, this decrease in abundance is likely to be due to degradation of maternal *fem-3* RNA during the first few cell divisions.

For comparison, the Northern blots were rehybridized with probes to *glp-1*, a message whose level is known to decrease during embryonic development (Austin and Kimble, 1989), and *act-1*, a *C.elegans* muscle actin gene (Files *et al.*, 1983). The *glp-1* signal decreases in parallel with that of *fem-3*, whereas the *act-1* signal is roughly constant for 4 h

Table I. Quantification of *fem-3*, *act-1* and *glp-1* RNA during embryonic development

Experiment	Time[a] (h)	Relative RNA levels[b]			Assessment of synchrony[c]		
		fem-3[d]	*act-1*	*glp-1*[d]	%1–8-cells	%9–28-cells	%>28-cells
A[e]	0	1.0	1.0	1.0	31	27	42
	1	0.24	0.71	0.30	0	34	66
	2	0.18	0.70	0.20	0	4	96
	3	0.19	0.78	0.11	nd	nd	nd
	4	0.16	0.83	0.04	nd	nd	nd
B	0	1.0	1.0	1.0	nd	nd	nd
	2	0.25	0.60	0.21	nd	nd	nd
	4.5	0.15	0.95	0.12	nd	nd	nd
	8	0.10	3.6	0.07	nd	nd	nd

[a]Number of hours of development after embryo isolation, 23°C.
[b]Band intensities on Northern blots were quantified using a Betagen beta-scanner. Both *fem-3* RNAs were included for *fem-3* quantification. Data are normalized so that 0 h is set at 1.0 for each RNA; signals from later time points are relative to the 0 h signal.
[c]Number of cells per embryo was determined by counting the number of nuclei/embryo in an aliquot of fixed embryos stained with DAPI (see Materials and methods).
[d]We calculate that 1- to 8-cell embryos have ~10 times more *fem-3* RNA than >8-cell embryos as follows. We assume that the >8-cell embryos in the 0 h time point have the same amount of *fem-3* RNA/embryo as the embryos in the 1 h time point. Calculation: 1 unit *fem-3* = (*X* units) (fraction 1- to 8-cell embryos) + (*Y* units) (fraction >8-cell embryos). *X* = *fem-3* RNA contributed by 1- to 8-cell embryos; Y = *fem-3* RNA contributed by >8-cell embryos = 0.24; fraction 1–8 cell embryos = 0.31; fraction >8-cell embryos = 0.27 + 0.42 = 0.69. So, 1 unit *fem-3* = (*X* units) (0.31) + (0.24) (0.69); *X* = 2.7; *X*:*Y* is 2.7:0.24 or 11:1, so 1- to 8-cell embryos have ~10 times more *fem-3* RNA than >8-cell embryos. This may be an underestimate for the decrease in abundance if *fem-3* RNA is degraded before the 8-cell stage. The analogous calculation for *glp-1* RNA is: 1 unit *glp-1* = (*X* units) (0.31) + (0.30) (0.69); *X* = 2.6, *Y* = 0.30; *X*:*Y* is 2.6:0.30 or 8.7:1.
[e]Experiment A corresponds to Figure 2.
nd, not determined.

and then increases several-fold by 8 h of development (Table I). Because *glp-1*, like *fem-3*, has maternal effects (Austin and Kimble, 1987; Priess *et al.*, 1987), embryonic *glp-1* RNA is likely to be maternally derived, and therefore the decrease in *glp-1* RNA abundance may also be due to degradation of maternal *glp-1* RNA. Early embryonic degradation may be a general control of maternal RNAs in *C.elegans*.

XX and XO embryos have the same amount of fem-3 RNA

Previously, we reported that XO (male) embryos contain six times more *fem-3* RNA than XX (hermaphrodite) embryos (Rosenquist and Kimble, 1988). Since the amount of *fem-3* RNA drops precipitously early in embryogenesis, this 6-fold difference might be explained by a small difference in the age distribution between the XX and XX + XO embryos in the original experiments. Therefore, we repeated these experiments with more carefully staged embryos. Embryos were obtained from wild-type hermaphrodites (wt XX), wild-type mating populations (wt XX + XO, which have <50% males) and two mutant male/female strains, *spe-8* (L'Hernault *et al.*, 1988) and *fog-2* (Schedl and Kimble, 1988), both of which have 50% XX and 50% XO progeny. To control for variability in the number of embryos in one preparation to the next, we measured the amount of *fem-3* RNA relative to *act-1* RNA. In contrast to previous results, we found no increase in the *fem-3/act-1* ratios in tightly synchronized preparations containing XO embryos (Figure 3 and Table II). Therefore, the previous results were probably due to a consistent difference in the age of embryos assayed rather than a difference in sex. We conclude that sex-specific regulation of maternal *fem-3* activity does not occur at the level of RNA accumulation, but rather, may occur translationally or post-translationally.

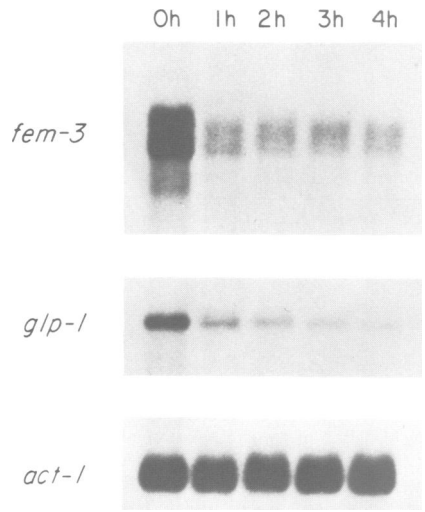

Fig. 2. The amount of *fem-3* RNA rapidly decreases during early embryonic development. Northern blot hybridized sequentially with probes to *fem-3*, *glp-1* and *act-1*. Early embryos were isolated from wild-type hermaphrodites and allowed to develop for the times shown. For each time point, one aliquot was frozen for RNA extraction and one aliquot was fixed for staging. In Table I, experiment A provides quantification of band intensities and stages present at each time point.

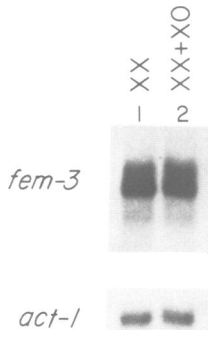

Fig. 3. XX and XO embryos have equivalent amounts of *fem-3* RNA. Northern blot of embryonic poly(A)$^+$ RNA isolated from: lane 1, wild-type XX embryos; lane 2, wild-type XX + XO embryos. In Table II, experiment B gives the ratio of *fem-3/act-1* band intensities for this blot.

Polyadenylation of fem-3 RNA is developmentally regulated

fem-3 RNAs of three sizes are detected during development (Rosenquist and Kimble, 1988). To find whether these RNAs differ primarily in polyadenylation state, we compared *fem-3* RNAs with and without a poly(A) tail by Northern blotting (Figure 4). To remove poly(A) tails, poly(A)$^+$ RNA was hybridized to oligo(dT) and then digested with RNase H. In contrast to the polyadenylated *fem-3* RNAs (Figure 4, lanes 1–5), only two *fem-3* RNAs were observed after poly(A) tail removal (Figure 4, lanes 6–10; data not shown). These two *fem-3* RNAs are 1.7 and 1.55 kb in length after poly(A) tail removal, and are present throughout development. The 1.7 kb RNA is consistently more abundant than the 1.55 kb RNA (Figures 1–4). Since this difference in abundance is similar before and after poly(A) tail removal, the larger *fem-3* RNA in the polyadenylated lanes (Figure 4, lanes 1–5) is unlikely to contain a hyperadenylated form of the 1.55 kb RNA. In this paper, we focus on the more abundant 1.7 kb *fem-3* RNA.

The poly(A) tail of the 1.7 kb *fem-3* RNA decreases in length during development (Figure 4, lanes 1–5). Poly(A) tails are longest on embryonic *fem-3* RNA (Figure 4, lane 1), intermediate in length during larval stages (Figure 4, lanes 2–4) and shortest in adults (Figure 4, lane 5). These results extend our earlier finding that *fem-3* poly(A) tails decrease in length from L4 to the adult stage (Ahringer and Kimble, 1991). We estimate the lengths of *fem-3* poly(A) tails to be 30–150 residues in embryos, 30–100 in larvae and 30–50 in adults (data not shown; Ahringer and Kimble, 1991). In adult hermaphrodites, most *fem-3* RNA is found in the germline (Rosenquist and Kimble, 1988), and is probably in oocytes as a maternal RNA (see above); this *fem-3* RNA has a short poly(A) tail. Because embryonic *fem-3* RNA has a long poly(A) tail, further adenylation of the short adult (presumably oocyte) poly(A) tail probably occurs at or after fertilization.

Structure and sequence of the 1.7 kb fem-3 RNA

The transcribed region of the *fem-3* gene is contained within two *Hin*dIII fragments (Rosenquist and Kimble, 1988; Figure 5A). The DNA sequence of this 4.8 kb genomic region is available through EMBL. We have determined the structure of the 1.7 kb *fem-3* RNA (Figure 5B) by a comparison of genomic and cDNA sequences plus S1 and primer extension analyses. The structure of the 1.55 kb

Table II. Relative amount of *fem-3* RNA in XX and XO embryos

Experiment	Strain[a]	%XO[b]	*fem-3/act-1*[c]	Time[d] (h)	%1–8-cells[e]	%9–28-cells[e]	%>28-cells[e]
A	wt XX	0.2	1.0	0	62	33	5
	wt XX+XO	26	1.0	0	56	39	5
	spe-8	50	1.0	0	45	34	21
	fog-2	50	0.82	0	54	30	16
B[f]	wt XX	0.2	1.0	0	59	31	10
	wt XX+XO	27	0.85	0	61	25	14
C	wt XX	0.2	1.0	0	43	30	27
	wt XX+XO	19	0.98	0	38	31	31
	wt XX	0.2	0.23	2	0	0	100
	wt XX+XO	19	0.24	2	0	0	100

[a]Embryos were isolated from: wild-type hermaphrodites (wt XX), wild-type mating populations of hermaphrodites and males (wt XX+XO), *spe-8(hc53)* and *fog-2(q71)*.
[b]Preparations with only XO embryos cannot be isolated. %XO in wt XX+XO was determined by counting the number of males and hermaphrodites in an aliquot of the preparation grown to adulthood. Wild-type hermaphrodites produce 0.2% males due to non-disjunction of the X chromosome (Brenner, 1974). *spe-8(hc53)* (L'Hernault *et al.*, 1988) and *fog-2(q71)* (Schedl and Kimble, 1988) are obligate male/female strains which produce 50% XX and 50% XO progeny. Both *spe-8* and *fog-2* XX animals are cross-fertile females; *spe-8* females produce defective sperm whereas *fog-2* produces no sperm. Both *spe-8* and *fog-2* males are fertile.
[c]*fem-3/act-1* is the ratio of *fem-3* signal to *act-1* signal obtained by quantifying signals on Northern blots using a Betagen beta-scanner. For each experiment, the wt XX *fem-3* and *act-1* signals were set to 1.0; signals from the other lanes were normalized to the wt XX signal. Both *fem-3* RNAs were included for *fem-3* quantification.
[d]See Table I, footnote a.
[e]See Table I, footnote c.
[f]Experiment B corresponds to Figure 3.

fem-3 RNA has not yet been fully determined (see legend to Figure 5).

The DNA sequence corresponding to the 1.7 kb *fem-3* RNA is shown in Figure 6. Of several *fem-3* cDNAs isolated, we sequenced the two largest ones. These cDNAs probably correspond to the 1.7 kb *fem-3* RNA based on S1 analysis (data not shown). The longest cDNA contains a putative cleavage and polyadenylation signal, AATAAA, located 16 bp upstream of 16 A residues. We propose that this is the 3′ end of the 1.7 kb *fem-3* RNA (Figure 6).

Comparison of cDNA and genomic sequences identified six exons and five introns. The presence and size of exons 2–6 was confirmed by S1 analysis (data not shown; Rosenquist, 1989). However, the S1 product corresponding to exon 1 was found to be longer than predicted from cDNA sequences, indicating that the cDNAs are incomplete (data not shown). To determine the 5′ end, we performed primer extension analysis. Using an oligonucleotide complementary to a region of exon 1 (JA10, see Materials and methods), we obtained a primer extension product which is 20 nt longer than the S1 product (Figure 7A, cf. lanes 1 and 2). This indicates the existence of an additional small exon at the 5′ end. By direct sequencing of RNA, we determined that *fem-3* RNA contains the *trans*-spliced leader SL1 at its 5′ end (Figure 7B). SL1 is *trans*-spliced to ~10% of *C.elegans* RNAs (Krause and Hirsh, 1987; Bektesh *et al.*, 1988). The deduced sequence of the major *fem-3* RNA, including SL1, is 1690 nt long which is in good agreement with our estimate of 1.7 kb from Northern analysis.

The 1.7 kb *fem-3* RNA has a 1164 nt open reading frame (see below) flanked by a 258 nt 5′ untranslated region (5′UTR), which includes the sequence of SL1, and a 269 nt 3′ untranslated region (3′UTR). Previous work showed that the *fem-3* 3′UTR is critical for regulation of *fem-3* activity (Ahringer and Kimble, 1991). Here we note that the 5′UTR contains a nearly perfect 17 nt inverted repeat, which could form a stem–loop 100 nt from the 5′ end of the RNA (Figure 6; delta G = −18 kcal/mol).

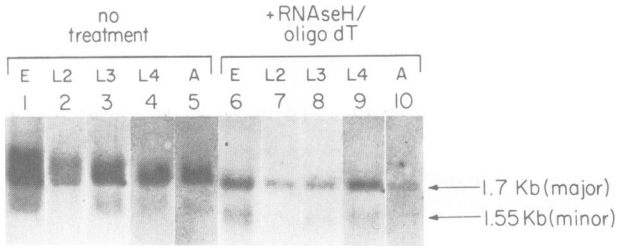

Fig. 4. Polyadenylation of *fem-3* RNA is regulated during development. Northern blot of poly(A)$^+$ RNA from staged animals either untreated, lanes 1–5, or treated with RNase H and oligo(dT) to remove poly(A) tails, lanes 6–10. RNase H cleaves any RNA in an RNA–DNA hybrid. E, embryo; L2, L3 and L4 are second, third and fourth larval stages; A, adult. Autoradiograms of different exposure were spliced together for this figure.

fem-3 encodes a novel protein

The *fem-3* cDNA sequence has one long open reading frame that could encode a protein of 388 amino acids (Figure 6). The first methionine codon in this open reading frame is located within a suitable translation initiation context (Kozak, 1987). No significant homology of the *fem-3* protein with other proteins has been detected in database searches (NBRF, EMBL, Swissprot and GenBank). There is a short stretch of basic amino acids near the N terminus (boxed), which has features of a nuclear localization signal (NLS). Although there is no strong consensus for a NLS, all those so far identified are extremely basic (reviewed in Silver, 1991). Despite this N-terminal stretch of basic amino acids, the predicted *fem-3* protein is acidic, with an isolectric point of 5.41. Sites of potential phosphorylation are noted in Figure 6. In addition, brackets mark three sets of four amino acids, each of which occurs twice in the putative *fem-3* protein.

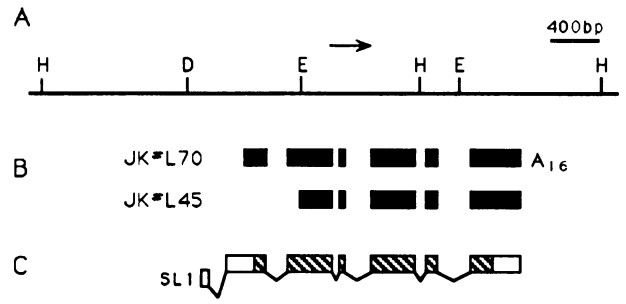

Fig. 5. Structure of the 1.7 kb *fem-3* RNA. **A**, restriction map of *fem-3* genomic region. Positions of restriction sites are basd on numbering of the genomic sequence submitted to GenBank; H, *Hin*dIII (nt 200, nt 3040 and nt 4810); D, *Dra*I (nt 1225); E, *Eco*RI (nt 1905 and nt 3405). Arrow indicates the direction of transcription (Rosenquist and Kimble, 1988). **B**, alignment of cDNAs to the genomic region, JK no. L70 was isolated from a library kindly provided by S.Kim and JK no. L45 from a library constructed by us. **C**, structure of the major *fem-3* RNA, based on cDNA sequence, analysis by primer extension, S1 protection, and direct RNA sequencing (sequence is in Figure 6). SL1 denotes a 22 nt *trans*-spliced leader RNA. The 1.55 kb *fem-3* RNA is likely to be colinear with the 1.7 kb RNA except at the 5′ end, based on RNase H/oligo cleavage and Northern analysis (Ahringer, 1991). The 5′ end of the 1.55 k *fem-3* message has not been established.

Because there is no signal sequence or significant hydrophobic stretch typical of a transmembrane domain, we predict that *fem-3* is an intracellular soluble protein.

Loss of function point mutations alter the putative fem-3 protein

To determine the molecular lesions associated with a loss of *fem-3* function, we sequenced a group of *fem-3* loss of function mutations (Table III). Three of four putative null alleles, including the canonical null, *fem-3(e1996)*, are nonsense mutations that presumably truncate the *fem-3* protein (Table III). This molecular evidence supports genetic arguments that a lack of *fem-3* activity leads to feminization (Hodgkin, 1986). The fourth putative null allele sequenced, *fem-3(e2063)*, is a threonine to isoleucine change in one of the four amino acid blocks, ITRF, that occurs twice in *fem-3*. This change eliminates a polar, hydroxyl group and replaces it with a larger hydrophobic ethyl side chain. Three weaker, temperature sensitive mutations are also non-conservative missense changes. Therefore, null *fem-3* mutants are usually associated with nonsense mutations, whereas weaker *fem-3* mutants are associated with missense mutations in the proposed *fem-3* coding region. These results support our proposal that this open reading frame encodes the *fem-3* protein. The finding of a null class missense mutation, *fem-3(e2063)*, in the repeated sequence ITRF suggests that this motif may be critical for *fem-3* activity.

Discussion

Regulation of fem-3 RNA

fem-3 RNA in early embryos derives solely from the maternal genome and has a substantially longer poly(A) tail than *fem-3* RNA made in adult hermaphrodites. This longer poly(A) tail probably reflects increased polyadenylation of adult, maternally contributed *fem-3* RNA. In other species, the lengthening of poly(A) tails on maternal RNAs can cause an increase in the translational activity of those RNAs (McGrew *et al.*, 1989; Vassalli *et al.*, 1989; reviewed in Jackson and Standart, 1990; Wickens, 1990). Previously, we showed that in *fem-3(gf)* adults, inappropriate *fem-3* activity was correlated with an abnormally long poly(A) tail (Ahringer and Kimble, 1991). Putting together the findings with maternal RNAs in other species and our work on *fem-3* poly(A) tail lengths and activity, we propose that quiescent maternal *fem-3* RNA is translationally activated in the early embryo. During hermaphrodite larval development, the *fem-3* poly(A) tail is of intermediate length. This poly(A) tail length is similar to that observed in *fem-3(gf)* adults, in which *fem-3* is inappropriately activated (Ahringer and Kimble, 1991). Therefore, we speculate that *fem-3* RNA may be translated in hermaphrodites from embryogenesis through the fourth larval stage. This time period roughly corresponds with that postulated to require *fem-3* activity for hermaphrodite spermatogenesis (Hodgkin, 1986; Barton *et al.*, 1987). In adult hermaphrodites, where *fem-3* activity is probably not required, poly(A) tails are shortest.

Maternal *fem-3* RNA appears to be degraded during early embryogenesis. Similarly, the level of early embryonic *glp-1* RNA decreases dramatically shortly after fertilization. Based on the maternal requirement for *glp-1* (Austin and Kimble, 1987; Priess *et al.*, 1987), this early embryonic *glp-1* RNA is likely to be derived from the maternal genome. Since both of these messages decrease in abundance 10-fold by the 8-cell stage, their translation probably occurs before this time. Although it is not known at what time maternal *fem-3* activity is required, the embryonic temperature sensitive period for maternal *glp-1* activity is between the 4- and 28-cell stages (Austin and Kimble, 1987; Priess *et al.*, 1987). Translation of *glp-1* RNA before the 8-cell stage is therefore consistent with this temperature sensitive period. These studies with *fem-3* and *glp-1* suggest that some maternal RNAs in *C.elegans* may be translated and then degraded very early in embryogenesis.

The maternal contribution of *fem-3* influences male development in both somatic and germline tissues of XO animals but only in the germline of XX animals (Hodgkin, 1986; Barton *et al.*, 1987). Because the maternal endowment of *fem-3* RNA appears to be equivalent in XX and XO embryos, at least in level and probably in degree of polyadenylation (in Figure 3, the heterogeneity in size of *fem-3* RNA is similar in XX and XX + XO embryos), we suggest that maternally contributed *fem-3* activity is post-transcriptionally regulated to achieve its sex-specific effects. For example, in XX embryos, *fem-3* protein (or RNA) may be localized to the embryonic germline precursor cell P_4, whereas in XO embryos, it may be present in all precursor cells. Alternatively, *fem-3* protein (or RNA) may be distributed identically in XX and XO embryos and its activity spatially regulated either by post-translational modification of by its interaction with factors that are themselves sex-specifically regulated.

Possible regulatory role for the fem-3 5′UTR

We find an inverted repeat in the predicted *fem-3* 5′UTR. Because a stem−loop in the 5′UTR of messages in other organisms inhibits their translation (Kozak, 1986; Casey *et al.*, 1988), we speculate that the stem−loop in the *fem-3* 5′UTR may similarly influence *fem-3* translation. Two precedents for 5′UTR regulatory elements may be relevant

to *fem-3*. First, in *Xenopus*, a 5′UTR stem−loop inhibits translation of a synthetic RNA in oocytes, but not in fertilized eggs, where helicases are postulated to unwind RNA helices (Fu *et al.*, 1991). Since *fem-3* produces maternal RNA that is likely to be activated during embryogenesis, a similar mechanism may control its expression. Second, in the mammalian ornithine decarboxylase (ODC) RNA, a 5′UTR stem−loop blocks translation, but this block is relieved by the ODC 3′UTR (Grens and Scheffler, 1990). Given the known regulatory element in the *fem-3* 3′UTR (Ahringer and Kimble, 1991), a similar relationship between the 5′ and 3′UTRs may control expression of *fem-3* during development. We are currently exploring the possibilities of a regulatory role for the *fem-3* 5′UTR inverted repeat and for a functional relationship between the 5′UTR inverted repeat and 3′UTR of *fem-3* RNA.

Speculation on the function of fem-3

Genetic analyses have led to the idea that *fem-3* acts as a negative regulator of *tra-1* in the soma and as a positive

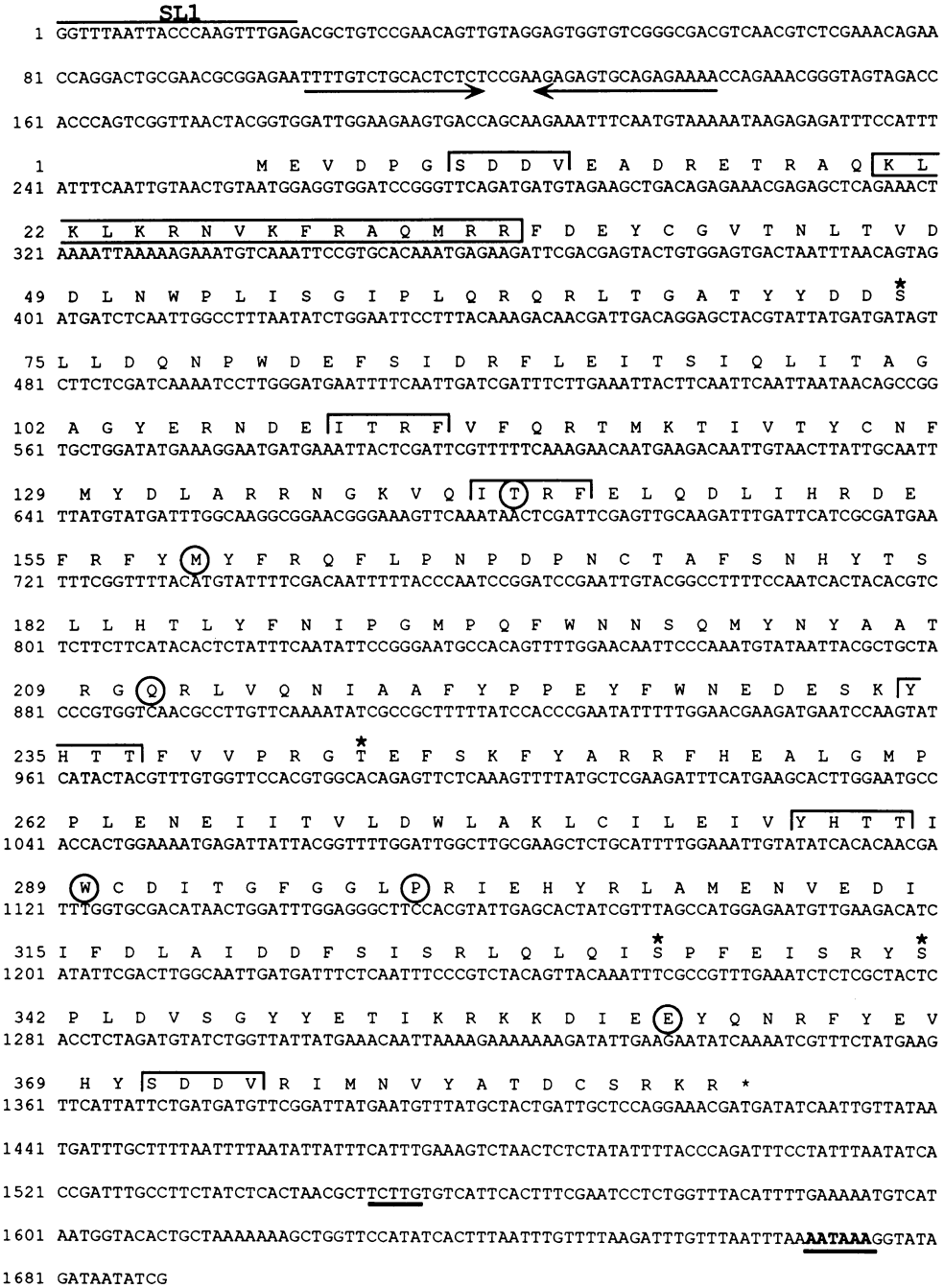

Fig. 6. Sequence of the 1.7 kb *fem-3* RNA. This corresponds to the structure shown in Figure 5C; it is based on the sequence of cDNAs, S1 and primer extension analyses, and direct RNA sequencing. Number one is the first nucleotide in the spliced leader, SL1. Arrows indicate the inverted repeat in the 5′UTR; in bold and underlined is the polyadenylation signal, AATAAA; underlined is the region of 5 nt where *fem-3(gf)* point mutations lie (Ahringer and Kimble, 1991). The predicted *fem-3* protein sequence is shown above the nucleic acid sequence. Boxed is the potential nuclear localization signal. The positions of *fem-3(lf)* mutations presented in Table III are circled. Brackets mark the three sets of four amino acids repeated twice in the protein; stars mark potential sites of phosphorylation: serine or threonine residues that are potential sites for cAMP-dependent kinase (R-X-S/T) and casein kinase II (S-X-X-D/E). 5′ and 3′ splice sites fit the *C.elegans* consensus (Emmons, 1988).

regulator of spermatogenesis in the germline (Hodgkin, 1986). Although the molecular functions of *fem-3* and *tra-1* are unknown, the sequence similarity of *tra-1* to known transcription factors (D.Zarkower and J.Hodgkin, submitted) is consistent with a function of *tra-1* in transcriptional control. Therefore, it is not unreasonable to speculate that the *fem-3* protein, with its possible nuclear localization signal and its overall acidity, may be involved in the regulation of transcription factors. Perhaps the *fem-3* protein inhibits the activity of the presumed *tra-1* transcription factor, but activates an as yet undetermined transcriptional regulator of spermatogenesis. A candidate for the latter is *fog-1*, which has a germline activity essential for spermatogenesis in both sexes (Barton and Kimble, 1990).

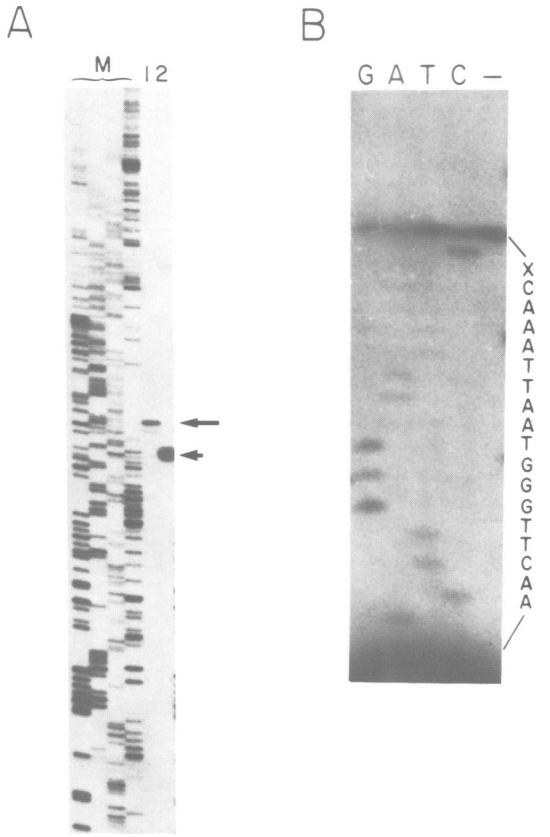

Fig. 7. 5′ end analysis of the major *fem-3* RNA. **A**, primer extension, lane 1; S1 analysis, lane 2. Both primer extension and S1 products begin with oligonucleotide JA10, which hybridizes to exon 1 (see Materials and methods for sequence). Marker lanes, M, are oligonucleotide JA10 sequencing products. **B**, the *fem-3* RNA sequence obtained using oligonucleotide TR17 is that of SL1, a common *trans*-spliced leader in *C.elegans* (Krause and Hirsh, 1987; Bektesh, 1988). Lane G, A, T and C, sequencing reactions; dash, primer extension product. Adult poly(A)$^+$ RNA was used.

Materials and methods

Isolation of animals

Gravid hermaphrodites were gently washed off Petri dishes, leaving laid eggs. Embryos *in utero* were isolated by hypochlorite treatment (Emmons *et al.*, 1979). In Tables I and II embryos were allowed to develop in M9 buffer with shaking at ~23°C. An equivalent volume was removed for each time point. Larval and adult stage animals were obtained by allowing embryos (isolated as above) to grow to the desired stage on Petri dishes.

Nuclei counts

Eggs were incubated in ethanol containing 1 μg/ml DAPI for ≥10 min at room temperature, washed in dH_2O, mounted and viewed under fluorescence (E.Lambie, personal communication). The nuclei from ~100 embryos were counted for each time point.

Northern blots and quantification

Between 1 and 5 μg poly(A) RNA was used per lane except for Figure 1 where total RNA was used; preparation of poly(A) RNA, formaldehyde–agarose electrophoresis, blotting and hybridization were as described (Rosenquist and Kimble, 1988), except that Nytran (S&S) was used instead of nitrocellulose. All probes were [^{32}P]RNAs made *in vitro* (Melton *et al.*, 1984). Unless otherwise noted, *fem-3* was detected using an antisense [^{32}P]RNA probe transcribed from *Stu*I digested pJK50, the *fem-3* cDNA insert from lambda phage JK # L45 cloned in pIBI76 (IBI). *glp-1* and *act-1* were detected using ^{32}P-labelled RNA probes made from pJK131 (a *glp-1* genomic fragment) and pT3/T7-18-103, an *act-1* specific clone (kindly provided by M.Krause) respectively. Northern blot signals were quantified using a beta scanner (Betagen); the total counts obtained were normalized for easier analysis.

RNase H/oligo analyses

One microgram poly(A)$^+$ RNA plus 0.3 μg oligo(dT)$_{12-18}$ were boiled for 1 min in 10 μl dH_2O and allowed to cool to room temperature. Ten microlitres 100 mM KCl was added to the RNA/oligo mixture, incubated for 10 min at room temperature, and then 20 μl of 2 × RNase H digestion buffer (56 mM $MgCl_2$, 40 mM Tris–Cl, pH 7.4, 1 mM EDTA) and 1.6 units RNase H (Pharmacia) was added and incubated for 40 min at 37°C. The products were phenol/$CHCl_3$ extracted, precipitated with carrier (glycogen, Boehringer Mannheim) and analysed on Northern blots.

S1 and primer extension analyses and RNA sequencing

Primer extension and S1 protection procedures are from Calzone *et al.* (1987). For primer extension, end-labelled oligo JA10 (5′-CTGTCAGCT-TCTAC-3′) was used. S1 nuclease probes were generated by hybridizing oligo JA10 to genomic fragment A followed by extension with Klenow DNA polymerase and [^{32}P]dCTP. The product was cleaved with *Dra*I and the appropriate fragment purified from a denaturing polyacrylamide gel. The primer extension product is 20 nt longer than the S1 product and not 22 nt

Table III. Molecular changes associated with *fem-3(lf)* mutations

fem-3 allele	Phenotypic class	Codon change	Amino acid change[d]
e2063[a]	null	ACU to AUU	Thr 142 to Ile
e1996[a]	null	CAA to UAA	Gln 211 to ochre
e2037[a]	null	CAA to UAA	Gln 211 to ochre
e2068[a,b]	null	UGG to UAG	Trp 289 to amber
e2006[a]	ts	AUG to AUA	Met 159 to Ile
e2143[a]	ts	GAA to AAA	Glu 360 to Lys
q77[c]	ts; *fem-3(gf)* revertant	CCA to UCA	Pro 299 to Ser

[a]Hodgkin (1986).
[b]*fem-3(e2068)* is not amber suppressible for the XX phenotype (Hodgkin, 1986), but shows partial suppression of XO phenotypes (J.Hodgkin, personal communication).
[c]Barton *et al.* (1987). q77 was isolated as an intragenic suppressor of *fem-2(q20gf)*; *fem-3(q20q77)* is female at 25°C and is fertile at 15°C (unpublished observation).
[d]Amino acids bearing *fem-3(lf)* mutations are circled in Figure 6.

(the length of SL1) because of a 2 nt overlap in sequence between SL1 and the *fem-3* genomic sequence. For RNA sequencing, the primer extension protocol was modified to include 1.2 mM dNTP and 0.8 mM dideoxy NTP for each of the four bases.

fem-3 genomic and cDNA sequence determination

fem-3 genomic clones were isolated from a *C.elegans* genomic library provided by C.Cummins. λgt10 cDNA clones were isolated from our mixed stage library and from a library provided by S.Kim. Genomic and cDNA fragments were subcloned into vector pIBI76 (IBI). Sequencing subclones were generated by exonuclease III deletion (Henikoff, 1984) and sequencing was done by the dideoxy termination method (Sanger *et al.*, 1977) using Sequenase (USB) or Klenow DNA polymerase. Sequence analysis was performed on the Physical Sciences Laboratory Vax computers with the University of Wisconsin Genetics Computer Group software package (Devereaux *et al.*, 1984). The FOLD program (Zuker and Stiegler, 1981) was used to calculate the delta G of the 5'UTR stem−loop.

Identification of fem-3(lf) mutations

For mutants that could be grown as homozygotes, genomic DNA was isolated from *fem-3(lf)* stocks as described (Emmons and Yesner, 1984). For balanced stocks, about 10 *fem-3(lf)* homozygotes were handpicked into PCR lysis buffer (50 mM KCl, 10 mM Tris, pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.1 mg/ml gelatin, 60 μg/ml proteinase K), incubated at 68°C for 1 h and boiled for 15 min (method from Bob Barstead, personal communication). *fem-3* genomic DNA was amplified by PCR (Saiki *et al.*, 1988) using *Taq* DNA polymerase (Perkin−Elmer Cetus or Amersham) and primers to *fem-3*; products were cloned into the vector pBluescript II KS (Stratagene). Sequencing was performed using Sequenase 2.0 (US Biochemicals) according to the manufacturer's specifications. Genomic DNA corresponding to the major *fem-3* transcript was sequenced, and all mutations were detected in clones from two independent PCR reactions.

Oligonucleotides used

DL1: 5'-CGTTCTTTGCGCGAGCTTC-3' (nt 987−1005); DL2: 5'-TTATGACCATCTCGGTAAAGTA-3' (nt 4695−4674); JA6: 5'-GGATTCATCTTCGTTC-3' (nt 2845−2830); JA9: 5'-CGCCTTGCCAAATCAT-3' (nt 2137−2122); JA10: 5'-CTCTGTCAGCTTCTAC-3' (nt 1678−1663); JA15: 5'-CTTGCAACTCGAATCGAG-3' (nt 2220−2203); TR2: 5'-TAGACGGGAAATTGAG-3' (nt 3597−3582); TR3: 5'-GTCTTTGTAAAGGAATTCC-3' (nt 1920−1902); TR19: 5'-CGTGGAAGCCCTCCAAATCC-3' (nt 3130−3111); and TR17: 5'-CAACTGTT CGGACAGCGTCT-3' (nt 1417−1398).

Acknowledgements

We thank Claudia Cummins for providing the *C.elegans* genomic library, Stuart Kim for a *C.elegans* cDNA library and Mike Krause for the plasmid pT3/T7-18-103. We are grateful to Tom Evans, Jonathan Hodgkin, Marv Wickens and Dave Zarkower for critical comments on the manuscript, and also to Dave Zarkower and Jonathan Hodgkin for sharing unpublished results. This research was supported by US Public Health Grants GM31816 and HD24663 and Research Career Development Award HD00630 to J.K. J.A. and D.N.L. were NSF predoctoral trainees; J.A. was also an NIH trainee of Grant GM 07133. T.A.R. was an NIH trainee of Grant GM07215-11.

References

Ahringer,J. (1991) Ph.D. Thesis, University of Wisconsin.
Ahringer,J. and Kimble,J. (1991) *Nature*, **349**, 346−348.
Austin,J. and Kimble,J. (1987) *Cell*, **51**, 589−599.
Austin,J. and Kimble,J. (1989) *Cell*, **58**, 565−571.
Barton,M.K. and Kimble,J. (1990) *Genetics*, **125**, 29−39.
Barton,M.K., Schedl,T.B. and Kimble,J. (1987) *Genetics*, **115**, 107−119.
Bektesh,S., Van Doren,K. and Hirsh,D. (1988) *Genes Dev.*, **2**, 1277−1283.
Brenner,S. (1974) *Genetics*, **77**, 71−94.
Calzone,F.J., Britten,R.J. and Davidson,E.H. (1987) *Methods Enzymol.*, **152**, 611−632.
Casey,J.L., Hentze,M.W., Koeller,D.M., Coughman,S.W., Touault,T.A., Klausner,R.D. and Harford,J.B. (1988) *Science*, **240**, 924−928.
Devereaux,J.R., Haeverli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387−395.
Doniach,T. and Hodgkin,J. (1984) *Dev. Biol.*, **106**, 223−235.
Emmons,S.W. (1988) In Wood,W.B. (ed.), *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 58.
Emmons,S.W. and Yesner,L. (1984) *Cell*, **36**, 599−605.
Emmons,S.W., Klass,M.R. and Hirsh,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1333−1337.
Files,J.G., Carr,S. and Hirsh,D. (1983) *J. Mol. Biol.*, **164**, 355−375.
Fu,L., Ye,R., Browder,L.W. and Johnston,R.N. (1991) *Science*, **251**, 807−810.
Grens,A. and Scheffler,I.E. (1990) *J. Biol. Chem.*, **265**, 11810−11816.
Haack,H. and Hodgkin,J. (1991) *Mol. Gen. Genet.*, **228**, 482−485.
Henikoff,S. (1984) *Gene*, **28**, 351−359.
Hodgkin,J. (1980) *Genetics*, **96**, 649−664.
Hodgkin,J. (1986) *Genetics*, **114**, 15−52.
Hodgkin,J. (1987) *Genes Dev.*, **1**, 731−745.
Hodgkin,J. (1990) *Nature*, **344**, 721−728.
Jackson,R.J. and Standart,N. (1990) *Cell*, **62**, 15−24.
Kimble,J., Edgar,L. and Hirsh,D. (1984) *Dev. Biol.*, **105**, 234−239.
Kozak,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2850−2854.
Kozak,M. (1987) *Nucleic Acids Res.*, **15**, 8125−8148.
Krause,M. and Hirsh,D. (1987) *Cell*, **49**, 753−761.
L'Hernault,S.W., Shakes,D.C. and Ward,S. (1988) *Genetics*, **120**, 435−452.
Madl,J.E. and Herman,R.K. (1979) *Genetics*, **93**, 393−402.
McGrew,L.L., Dworkin-Rastl,E., Dworkin,M.B. and Richter,J.D. (1989) *Genes Dev.*, **3**, 803−815.
Melton,D.A., Krieg,P., Rebagliati,M., Maniatis,T., Zinn,K. and Green,M. (1984) *Nucleic Acids Res.*, **12**, 7035−7056.
Priess,J.R., Schnabel,H. and Schnabel,R. (1987) *Cell*, **51**, 601−611.
Rosenquist,T.A. (1989) Ph.D. Thesis, University of Wisconsin.
Rosenquist,T.A. and Kimble,J. (1988) *Genes Dev.*, **2**, 606−616.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487−491.
Sanger,R., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Schedl,T. and Kimble,J. (1988) *Genetics*, **119**, 43−61.
Schedl,T., Graham,P.L., Barton,M.K. and Kimble,J. (1989) *Genetics*, **123**, 755−769.
Silver,P. (1991) *Cell*, **64**, 489−497.
Vassalli,J.-D., Huarte,J., Belin,D., Gubler,P., Vassalli,A., O'Connell,M.L., Parton,L.A., Rickles,R.J. and Strickland,S. (1989) *Genes Dev.*, **3**, 2163−2171.
Wickens,M. (1990) *Trends Biochem. Sci.*, **15**, 320−324.
Zuker,M. and Stiegler,P. (1981) *Nucleic Acids Res.*, **9**, 133.

Received on December 31, 1991; revised on March 2, 1992

Note added in proof

The nucleotode sequence data reported here will appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession number X64962 (*C.elegans fem3* mRNA) and X64963 (*C.elegans fem-3* gene).