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Specification of Male Development in *Caenorhabditis elegans*: The *fem* Genes

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Mutation of the gene *fem-2* causes *feminization* of both sexes: hermaphrodites make no sperm, and males produce oocytes in an intersexual somatic gonad. A double mutant harboring *ts* alleles of both *fem-1* (formerly named *isx-1*; G. A. Nelson, K. K. Lew, and S. Ward, 1978, *Dev. Biol.* **66**, 386–409) and *fem-2* causes transformation of XO animals (normally male) into spermless hermaphrodites at restrictive temperature. The phenotypes, temperature-sensitive periods, and maternal effects observed in mutants of each *fem* gene are found to be similar. It is suggested that the *fem* genes are centrally involved in specification of male development in *Caenorhabditis elegans*—both in the germ line of hermaphrodites and in somatic and germ line tissues of males.

INTRODUCTION

Caenorhabditis elegans can develop as either of two sexes: a self-fertilizing hermaphrodite or a male. Determination of sex is normally controlled by the X:autosome ratio (Madl and Herman, 1979). In the typical diploid animal, hermaphrodites are XX, and males are XO. Several genes have been identified that are central to this control. A mutation in any of three *tra* genes causes XX animals to develop along the male pathway (Hodgkin and Brenner, 1977), whereas a mutation in the *her-1* gene results in the development of XO animals as hermaphrodites (Hodgkin, 1980). These genes have been placed in a regulatory pathway according to their pattern of epistasis (Hodgkin, 1980). In this model, *her-1* acts first in response to the X/A ratio, *tra-2* and *tra-3* act next, and *tra-1* serves last as the master gene which controls the switch between male and hermaphrodite development.

Here we describe two additional genes, *fem-1(IV)* and *fem-2(III)*, that affect sex determination in *C. elegans*. Mutation of either *fem-1* or *fem-2* feminizes both XX and XO animals. A temperature-sensitive allele of one of these genes, *fem-1*, has previously been described under the name *isx-1* (Nelson *et al.*, 1978). However, the role of *fem-1* in sex determination has been difficult to understand for two reasons. First, unlike mutations of either the *tra* or *her* genes, the effect of the *fem* allele described is restricted to the somatic gonad and germ line (Nelson *et al.*, 1978). Second, the action of *fem-1* cannot be neatly fitted into the regulatory pathway (Nelson *et al.*, 1978). In the soma, the mutant phenotype of *tra-1* is epistatic to that of *fem-1*, but in the germ line, *fem-1* is epistatic to *tra-1*. In this paper, we describe a temperature-sensitive allele of a second gene, *fem-2*, that displays nearly the same properties as *fem-1*. In addition, we show an effect of the *fem* genes on the sex of nongonadal structures as well as that on the sex of the gonadal structures previously known. This global effect of the *fem* genes shows that both are centrally involved in the sex determination switch.

MATERIALS AND METHODS

Maintenance. *C. elegans* was maintained on agar-filled petri dishes with *Escherichia coli* as described in Brenner (1974). Animals were grown at either 16 (permissive temperature) or 25°C (restrictive temperature) as detailed in the text.

Strains. The nomenclature used here conforms to published guidelines (Horvitz *et al.*, 1979). Mutations used were as follows: *X*, *lon-2(e678)*; *I*, *unc-13(e51)*; *III*, *mec-12(e1605)*, *fem-2(b245)*, *unc-45(e286)*, *dpy-1(e1)*; *IV*, *fem-1(hc17)*, *him-8(e1489)*. Examination of males has been facilitated in this study by use of a *him* mutation; *him* mutants cause a *h*igh *i*ncidence of *m*ales by increasing the frequency of nondisjunction of the X chromosome. Many strains discussed in this paper contain *him-8(e1489)*. *him-8(e1489)* has no effect on sexual differentiation (Table 1).

Mapping. The approximate map position of *fem-2* has been determined by two two-factor crosses and two

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three-factor crosses. Two-factor crosses: Segregation from *mec-12(e1605) fem-2(b245)/++* hermaphrodites gave 196 Mec Fem and 45 Mec nonFem progeny. Segregation from *dpy-1(e1) fem-2(b245)/++* hermaphrodites gave 352 Dpy Fem and 59 Dpy nonFem progeny. Thus, *fem-2* is about 9 map units from *mec-12* and about 7 map units from *dpy-1*. Two three-factor crosses were attempted to determine the precise position of *fem-2* with respect to *unc-45* which lies between *mec-12* and *dpy-1* on chromosome III. From *unc-45(e286) dpy-1(e1)/ fem-2(b245)* hermaphrodites 120/120 Dpy recombinants and 0/140 Unc recombinants carried *fem-2*. From *mec-12(e1605) unc-45(e286)/fem-2(b245)* hermaphrodites, 112/112 Mec recombinants carried *fem-2*. These data place *fem-2* between *mec-12* and *dpy-1* very close to *unc-45* on chromosome III. (The sterile phenotype of *fem-2* was scored in the progeny of the segregants in all cases due to its maternal effect.)

Detection of XO pseudofemales. To detect XO animals, an X-linked marker, *lon-2(e678)X*, was used. Lon hermaphrodites were crossed to nonLon males. Lon cross progeny are expected to be XO, bearing the maternal X chromosome of the Lon hermaphrodite. Since all crosses were performed in a *him* strain, production of diplo-X and haplo-X ova must be considered. Thus, a *him* mother can generate Lon XX progeny if a diplo-X ovum is fertilized by a nullo-X sperm, or nonLon XO progeny if a nullo-X ovum is fertilized by a haplo-X sperm. To determine the fraction of diplo-X ova, *lon-2 unc-15 him-8* hermaphrodites were crossed to *him-8* males. The Lon cross progeny included 135 males and 5 hermaphrodites: less than 4% of the Lon animals were obtained from diplo-X ova. Most Lon cross progeny (96%) are XO. In contrast, the fraction of nullo-X ova is 37% (Hodgkin *et al.*, 1979) so that nonLon progeny include significant numbers of XX and XO animals. Only Lon cross progeny were scored therefore to detect XO pseudofemales.

RESULTS AND DISCUSSION

The fem-2 Phenotype

The temperature-sensitive *(ts)* mutation, *b245*, identifies the *fem-2* locus. This gene maps between *mec-12* and *dpy-1* very close to *unc-45* on chromosome III (see Materials and Methods). At the restrictive temperature, the phenotype of *fem-2(b245)* involves (1) elimination of sperm formation in hermaphrodites (creating a functional female), (2) reduction in the number of sperm produced and induction of oogenesis in males, and (3) intersexual development of the somatic gonad of males (Table 1). Animals bearing a *ts* allele of another gene, *fem-1(hc17)IV* (formerly *isx-1*, Nelson *et al.*, 1978), have essentially the same mutant phenotype (Table 1).

Normally, the hermaphrodite germ line begins sperm formation in late L4 from a set of sperm precursor cells set aside in each half of the gonad (Hirsh *et al.*, 1976). After a brief period of spermatogenesis, the germ line of each half gonad switches from sperm to oocyte production. In *fem-2* (and in *fem-1*, Nelson *et al.*, 1978) hermaphrodites, no sperm precursor cells have been observed. Instead, germ cells differentiate directly as oocytes and simply continue oogenesis. The time of development at which oogenesis begins in *fem-2* females is the same time as that at which spermatogenesis would normally begin. Thus, there appears to be a signal to differentiate which is interpreted by the germ line cells according to the activity of either the *fem-1* or *fem-2* gene. Development of hermaphrodite somatic structures is not affected in the *fem-2* mutant.

Male development in the *fem-2* mutant is altered in essentially the same way as that of the *fem-1* mutant already described (Nelson *et al.*, 1978). In the soma, the effect is restricted to feminization of the somatic gonad. This can best be observed by induction of a vulva in some animals. Vulva formation is dependent on a somatic regulatory cell in the hermaphrodite gonad—the anchor cell (Kimble, 1981). The primary difference between the male somatic phenotype of *fem-1* and that of *fem-2* is the lower penetrance in *fem-2* (Table 1). In the male germ line, some sperm cells are made and then oogenesis begins. The onset of oogenesis in both *fem-1* and *fem-2* males is accompanied by the production of the hermaphrodite specific yolk proteins (Kimble and Sharrock, 1983).

Sexual Transformation of XO Animals

Animals harboring temperature-sensitive alleles of both *fem-1* and *fem-2* have a more extreme mutant phenotype than that of either *fem* mutant alone (Table 1). Even at the permissive temperature of 16°C, a significant fraction of the *fem-1 fem-2* hermaphrodites are sterile due to lack of sperm. More dramatically, if a brood is raised at 25°C, no male animals are observed among the progeny. Because self progeny produced at 16°C include 36% males, the absence of males in the 25°C brood indicates either XO lethality or XO transformation. Little difference is observed in lethality of embryos at 16 and 25°C: 1310/1357 eggs hatched at 16°C (96%) and 1570/1753 eggs hatched at 25°C (90%). The following cross was performed to demonstrate that *fem-1 fem-2* XO animals are transformed into hermaphrodites at 25°C: *fem-1 fem-2 lon-2 unc-13 him-8* hermaphrodites were mated to *fem-1 fem-2 him-8* males. The autosomal marker, *unc-13(e51)*, was used to distinguish cross from self progeny. The X-linked marker *lon-2(e678)X* was used to detect XO pseudofemales (see Materials and Methods).

TABLE 1
MUTANT PHENOTYPES OF *fem* GENES

Mutant phenotype	*fem-2(b245) him-8(e1489)* 16°C	*fem-2(b245) him-8(e1489)* 25°C	*fem-1(hc17) him-8(e1489)* 16°C	*fem-1(hc17) him-8(e1489)* 25°C	*fem-2(b245) fem-1(hc17) him-8(e1489)* 16°C	*fem-2(b245) fem-1(hc17) him-8(e1489)* 25°C	*him-8(e1489)* 25°C
Percentage spermless hermaphrodites[a] (n = 100)	2	100	4	100	68	100	0
Percentage males with oocytes[b] (n = 100)	0	83	0	ND	2	(No males)	0
Percentage males with intersexual gonads[c] (n = 100)	1	33	9	91	4	(No males)	0
Percentage male progeny in self-brood[d] (n > 1000)	32	35	45	39	36	0	37[e]

Note. Comparison of phenotypes of *fem-2, fem-1,* and *fem-1fem-2* mutant strains raised at 16 and 25°C. All animals examined were self progeny of parents homozygous for the mutations indicated at the top of each column.

[a] L4 hermaphrodite animals raised at 16 or 25°C were separated onto individual petri plates. If animals were spermless, no progeny were produced. Sterile animals that made oocytes were therefore scored as spermless; this phenotype has been confirmed by Nomarski optics.

[b] Adult males raised at 16 or 25°C were scored for presence of oocytes using Nomarski optics. Germ cells that differentiated as large, granular cells typical of oocytes were scored as oocytes; some of these oocyte-like cells had the appropriate size and shape of oocytes. Abnormally cleaving eggs were sometimes observed in the male gonad suggesting that the oocyte-like cells could be fertilized and could support some embryogenesis.

[c] The morphology of gonads of adult males raised at 16 or 25°C was examined with Normarski optics. Feminization of the male somatic gonad caused variable morphogenetic defects of the gonad, and sometimes caused vulva formation. Induction of the vulva depends on a hermaphrodite cell in the somatic gonad, the anchor cell (Kimble, 1981). Nongonadal tissues appeared to be unaffected.

[d] Hermaphrodite parents were either raised at 16 or shifted to 25°C as L4s, and the percentage of male progeny in the total brood was counted.

[e] 37% at 20°C (Hodgkin *et al.*, 1979).

Most nonUncLon progeny from the cross are expected to be XO males with the X chromosome derived from the maternal parent. However, no progeny of this cross were male. Instead, they included approximately equal numbers of Lon and nonLon spermless hermaphrodites (Fig. 1). When mated to wild-type males, the Lon her-

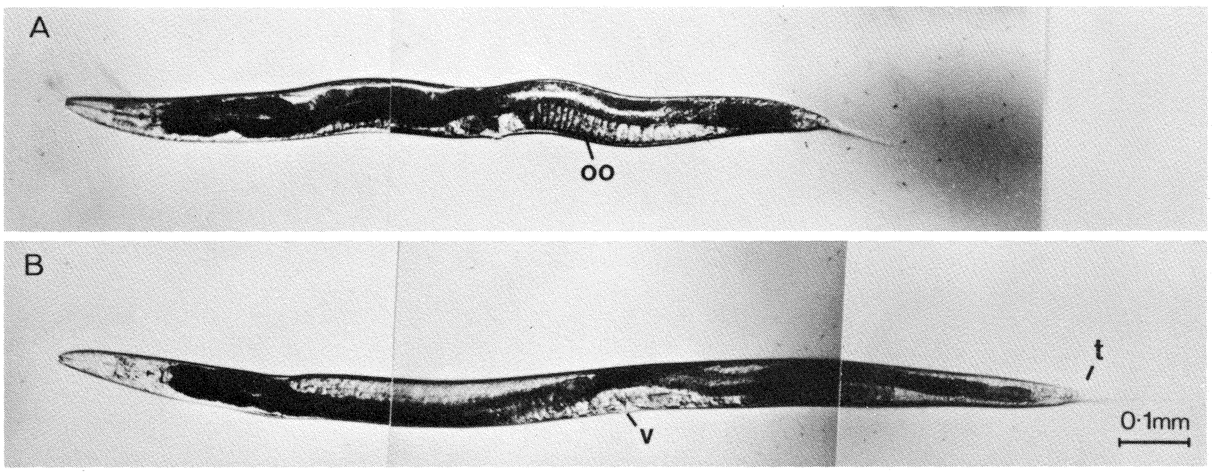

FIG. 1. Mutant phenotype of *fem-1 fem-2* animals, Nomarski micrographs. Pseudofemale XO animals are detected by use of an X-linked morphological marker, *lon-2* (see Materials and Methods). The two sexes are distinguished by several criteria: Hermaphrodites possess a vulva for egg-laying, a tapered tail, and a symmetrical gonad; males have a specialized tail for copulation, no vulva, and an asymmetrical gonad. (A) nonLon *fem-1 fem-2* animals are all spermless hermaphrodites at 25°C. Oocytes (oo) remain unfertilized. (B) Lon *fem-1 fem-2* animals are also all spermless hermaphrodites if raised at 25°C. These animals are mostly XO animals since *him-8* females produce very few diplo-X ova. Vulva (v); tapering tail (t). The gonad, observed at higher magnification, is a typical hermaphrodite two-armed gonad.

maphrodites were observed to function as females and to produce viable progeny by cross-fertilization.

This experiment shows that both somatic and germ line tissues of XO animals are sexually transformed from male to female in the strain harboring both *fem* mutations. It is striking that mutation of either *fem-1* or *fem-2* is sufficient to transform the germ line completely and the somatic gonad partially, but that neither is sufficient alone to affect the nongonadal somatic structures. This tissue-specific difference may reflect a difference in threshold of gene product required, in stability of the gene product, or in the function of the gene products in the various tissues examined.

Temperature-Sensitive Periods of fem-2 Effect in Germ Line and Somatic Gonad

The temperature-sensitive period (TSP) of the *fem-2* effect on the switch from sperm to oocyte differentiation has been examined in both sexes. In the simplest cases, where temperature-sensitive mutant genes produce thermally unstable products, TSPs represent either periods of synthesis or periods of function of the gene product. The *fem-2* TSP in the hermaphrodite germ line is brief, beginning in L1 (first larval stage) and ending in L2 (Fig. 2A). In contrast, the *fem-2* TSP in the male germ line begins during L2 and extends through adulthood (Fig. 2B). These TSPs are essentially the same as those observed for *fem-1(hc17)* (Nelson *et al.*, 1978; Edgar and Kimble, unpublished data). Activity of both *fem* genes is therefore required in the germ line only briefly for the transient period of sperm production in the L4 hermaphrodite, but it is necessary continuously for the uninterrupted sperm production typical of males. The short TSP in hermaphrodites means that the action of the *fem* genes must be fixed at a time of development when a total of only about 16 germ line cells exist in the hermaphrodite. Since the approximate 300 sperm made must be the product of about 75 precursor cells, this determination step occurs before the birth of the final sperm precursor cells. Yet, no evidence for a divergence in the lineage of sperm and oocytes during L1 has emerged. Hodgkin (1974) has shown genetically that sperm and oocytes develop from common precursors, and that their lineages diverge later than L1. Furthermore, laser ablation experiments have not been able to identify any particular sperm precursor cells since the same number of sperm is produced despite ablation of various germ cells in early gonadogenesis (Kimble, unpublished results) and since all germ cells present in L1 and L2 gonads are capable of spermatogenesis (Kimble and White, 1981).

The TSP of the *fem-2* effect on the XO somatic gonad is early in larval development (0–12 hr after hatching, 25°C; data not shown). This TSP is basically the same as the TSP of *fem-1* in the XO somatic gonad (Nelson *et al.*, 1978) and of *tra-2* in the XX somatic gonad (Klass *et al.*, 1976). The determination of sex in the somatic gonad thus seems to be coincident with the first morphological indication of sex in that organ (Kimble and Hirsh, 1979).

Temperature-Sensitive Period of "fem" Genes on Nongonadal Somatic Structures

The TSP for sex determination by the *fem* genes in the nongonadal somatic structures was analyzed in the *fem-1 fem-2* double mutant. The X-linked marker *lon-2* was used again in this experiment to detect XO pseudofemales. The shift-up and shift-down curves in this experiment are reversed (Fig. 2C). Possible interpretations of such reversals have been discussed elsewhere (Wood *et al.*, 1980). One plausible explanation is that sufficient product has been made by mid-L1 in XO animals to allow male development and that product made during L2 can rescue XO animals from sexual transformation into pseudofemales. The two curves therefore suggest a broad period of gene activity spanning at least the first two larval stages. Such a TSP corresponds well to the time of temperature sensitivity observed for control of sex in the tail of XX animals by *tra-2* (Klass *et al.*, 1976). Recently, it has been shown that the first sign of sexual dimorphism occurs midway during embryogenesis in both XX and XO animals (Sulston *et al.*, 1983). At this time, four male-specific neurons die in XX animals, and two hermaphrodite-specific neurons die in XO animals. The effect of mutation in *tra-2, fem-1,* or *fem-2* on these deaths is not known.

Maternal Effect of fem-2

The *fem-2* phenotype is observed among the homozygous *fem-2* progeny of homozygous *fem-2* parents (shifted to restrictive temperature (25°C) as L4 animals). However, two mutant effects of *fem-2* can be maternally rescued by a wild-type gene in the maternal genome. Homozygous *fem-2* progeny of a heterozygous *fem-2*/+ mother do not show the complete mutant phenotype, even though both parents and progeny were raised at 25°C. Instead, XX animals make sperm (98/100, 98%) and XO animals do not show an intersexual phenotype of the somatic gonad (68/68, 100%). XO animals, however, do make oocytes (24/68, 35%). The two effects that are completely rescued by the presence of a wild-type gene in the maternal genome are the same two phenotypes that have early larval TSPs. Similar maternal effects have been observed for *fem-1* (Doniach and Hodgkin, submitted for publication) and for the *fem-1 fem-2* double mutant (Kimble, unpublished data).

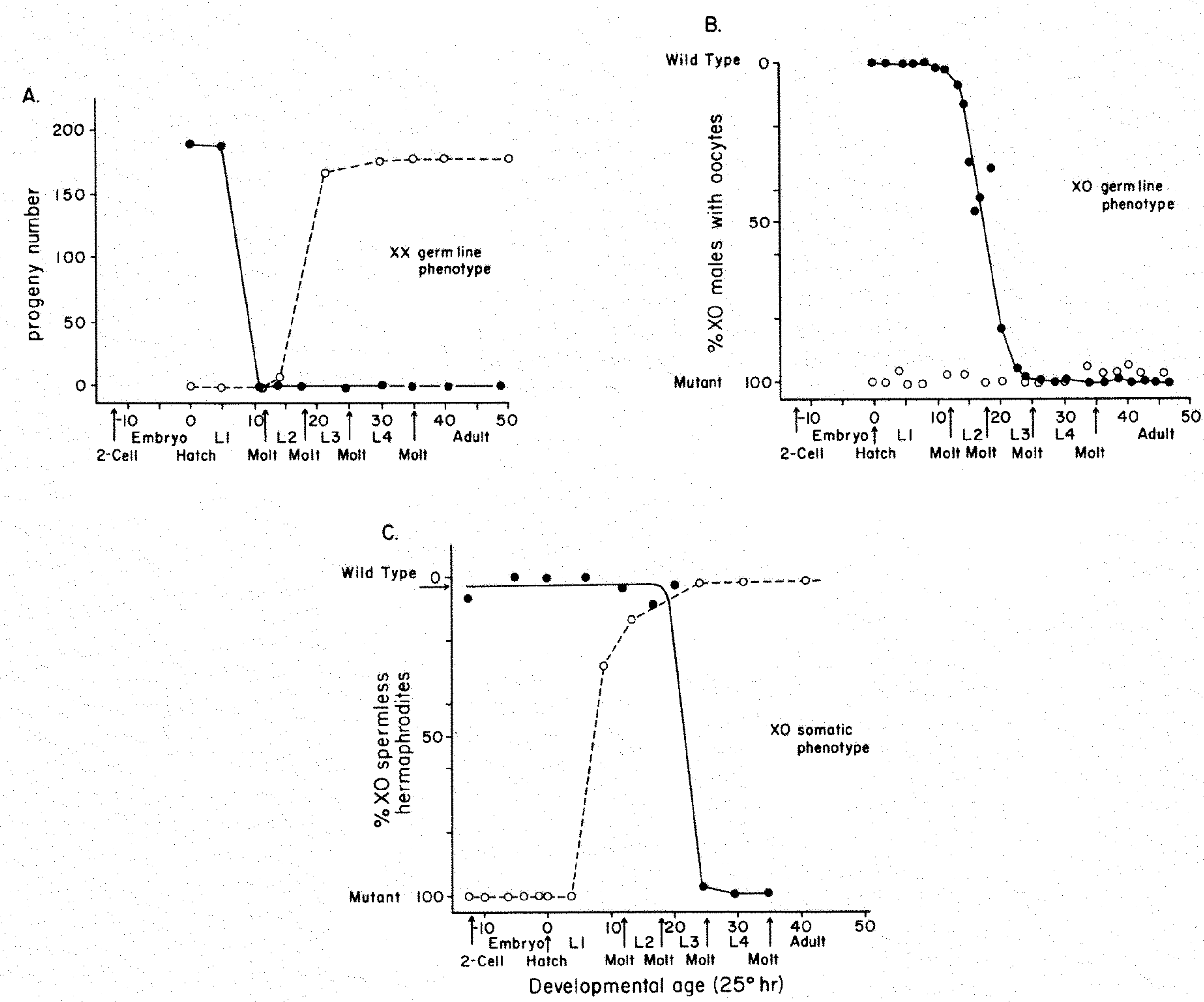

FIG. 2. Temperature-sensitive periods (TSPs) of the *fem-2* and *fem-1 fem-2* phenotypes. In each graph, the abscissa represents developmental time and the ordinate the mutant phenotype. Staged animals were shifted from permissive temperature (16°C) to restrictive temperature (25°C), or vice versa in a series of reciprocal shift experiments (Hirsh and Vanderslice, 1976). ●, Shifts down; ○, shifts up. In (A) and (B), *fem-2* animals were synchronized at hatching. In (C), *fem-1 fem-2* animals were synchronized at the 2-cell stage of embryogenesis. (A) TSP of spermless phenotype in XX *fem-2* hermaphrodites. Number of sperm produced is scored by counting the self brood size. Since each sperm is utilized to fertilize one egg in *C. elegans*, the number of self progeny equals the number of sperm made by the hermaphrodite. Each point is an average of brood sizes of 5–10 animals. The spermless phenotype has been corroborated by observation with Nomarski optics and with Feulgen-stained preparations. No unusual migration or death of germ cells has been observed that might indicate that one population of cells has been physically replaced by another. (B) TSP of oocyte phenotype in XO *fem-2* males. Each point represents 50–100 males scored by Feulgen for presence of larger endomitotic cells typical of oocytes. In Nomarski, large granular germ cells similar to oocytes are observed in the male gonad. No end of the TSP could be found in shifts up: males of all developmental stages (including adult) shifted to restrictive temperature make oocyte-like germ cells. (C) TSP of sexual transformation in the somatic tissues of XO *fem-1 fem-2* animals. Sexually transformed XO progeny were again detected by using an X-linked marker *lon-2*. The percentage of XO spermless hermaphrodites, scored in Lon progeny, equals the number of Lon spermless hermaphrodites/number of total Lon animals. Animals were scored as transformed only if they lacked *any* sign of a male tail and possessed a spermless hermaphrodite gonad.

Thus, the maternal contribution of wild-type *fem-2* gene product is sufficient to rescue the *fem-2* phenotype even though its TSP is during early larval development. It should be noted that neither *ts fem* mutant is rescued by mutant maternal gene product even though it was produced at permissive temperature.

Speculations on the Role of fem Genes in Sex Determination

The role of *fem-1* and *fem-2* in regulation of sex determination is not clear. However, the phenotype of a dominant mutant in another gene in the sex determination pathway, *tra-1* may provide a clue. Animals carrying *tra-1(dom)* (Hodgkin, 1983) and those carrying recessive mutations in both *fem* genes (this paper) have identical mutant phenotypes—transformation of both XX and XO animals into spermless hermaphrodites. A *tra-1(null)* mutation, in contrast, transforms XX animals into males (Hodgkin and Brenner, 1977). If the *tra-1(dom)* allele causes constitutive expression of the *tra-1* gene product, as argued by Hodgkin (1983), and if the singular phenotype observed in *tra-1(dom)* and in *fem-1 fem-2* results from the same underlying defect, then the *fem* gene products might function as negative regulators of *tra-1*. The phenotype of *tra-1(null) fem* double mutants should test this idea. If the *fem* mutant phenotype is caused by a lack of negative regulation of *tra-1*, a *tra-1(null) fem* double mutant should exhibit a *tra-1(null)* rather than a *fem* mutant phenotype. This prediction holds true in the somatic tissues—XX and XO animals are morphologically male rather than hermaphrodite—but not in the germ line. Both *tra-1(null)fem-1* (Nelson *et al.*, 1978; Doniach and Hodgkin, submitted for publication) and *tra-1(null)fem-2* (Edgar and Kimble, unpublished data) double mutants make oocyte-like cells in the germ line of both XX and XO animals. This suggests that the role of the *fem* genes in sex determination may be positive regulation of the germ line to produce sperm and negative regulation of *tra-1* in somatic tissues to effect male development.

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