Microinjected DNA from the X Chromosome Affects Sex Determination in *Caenorhabditis elegans*

WILLIAM K. MCCOUBREY, KIM D. NORDSTROM, PHILIP M. MENEELY

The signal for sex determination in the nematode *Caenorhabditis elegans* is the ratio of the number of X chromosomes to the number of sets of autosomes (X/A ratio). By previous genetic tests, elements that feminized chromosomal males appeared to be widespread on the X chromosome, but the nature of these elements was not determined. In experiments to define a feminizing element molecularly, cloned sequences were added to chromosomally male embryos by microinjection into the mother. Three different X-chromosome clones, including part of an actin gene, part of a myosin heavy chain gene, and all of two myosin light chain genes, feminize chromosomal males. Both somatic and germline aspects of sex determination are affected. In contrast, about 40 kilobases of nematode autosomal DNA, phage lambda DNA, and plasmid pBR322 DNA do not affect sex determination. A feminizing region was localized to a maximum of 131 base pairs within an intron of the X-linked actin gene; a part of the gene that does not have this region is not feminizing. The results suggest that short, discrete elements found associated with many X-linked genes may act as signals for sex determination in *C. elegans*.

SEXUAL DIMORPHISM IS CORRELATED WITH THE CHROMOsome number in many animals; that is, the presence, absence, or number of sex chromosomes determines sex (*1*). However, the means by which a sex chromosome controls sexual morphology is largely unknown. In mammals, the presence of a Y chromosome results in testis development, regardless of the number of X chromosomes present (*2, 3*). A putative male-determining region on the Y chromosome of mammals has been located, and a candidate for the testis-determining gene has been identified (*3*).

In other animals, the Y chromosome is not male-determining, and other signals for sex determination are used (*4*). For example, in the nematode *Caenorhabditis elegans* there is no Y chromosome. Diploid males have a single X chromosome and are XO. Diploid hermaphrodites are XX. Thus, the difference between male and hermaphrodite development must depend on the number or amount of an element present in both sexes rather than on genetic material present in only one sex. In other words, the animal must "count" the amount of X-chromosome material present (*5*).

The authors are in the Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.

Genetic experiments with polyploids have shown that the signal for sex determination is not simply the number of X chromosomes, but the balance between the number of X chromosomes (X) and the number of sets of autosomes (A), termed the X/A ratio (*6*). An X/A ratio was first recognized as the signal for sex determination in *Drosophila melanogaster*, in which it has been extensively studied genetically (*7*). For *C. elegans*, if the X/A ratio is 0.67 (triploids with two X chromosomes, symbolized 3A;2X) or lower, the animal develops as a male. If the X/A ratio is 0.75 (4A;3X) or higher, the animal develops as a hermaphrodite.

Our experiments are directed toward identifying X-linked elements that are part of the X/A ratio. Such elements are predicted to have a simple property: they should have dosage-sensitive effects on sex determination. Thus, additional copies of an X-linked element used in assessing the X/A ratio should feminize animals with a male chromosome constitution. We microinjected cloned X-chromosome DNA into the mother and examined the chromosomally male progeny. In view of the very small difference in the X/A ratio that controls male or hermaphrodite development, we used males known to be very sensitive to small changes in the X-chromosome dose (*6*), and we now show that three different X-chromosome clones (representing four X-linked genes) feminize chromosomal males. There is no effect on sex determination when phage lambda or vector DNA is injected or, more strikingly, when about 40 kilobases (kb) of nematode autosomal DNA is injected. For one of the X-linked genes, a feminizing element is located within a 131–base pair (bp) region of an intron. This X-linked sequence may play a key role in the chromosomal signal for sex determination in *C. elegans*.

Sex determination in *C. elegans*. In *C. elegans* there are two sexes, hermaphrodites and males. Hermaphrodites can be thought of as somatic females that make some sperm; true females do not occur naturally (*5*). Hermaphrodites can reproduce by either internal self-fertilization or cross-fertilization by males. Males and hermaphrodites differ extensively in sexual morphology (Fig. 1). Sexual dimorphism in the somatic tissues is controlled by a few key genes that have been ordered in a regulatory cascade (*5*). Most of these genes also affect the germline, and additional regulatory genes appear to affect only the germline. The X/A ratio is postulated to control the expression of these genes in some unknown way.

In addition to the normal male and hermaphrodite sexes, there are intersexual animals, which we now describe. Intersexes are not natural forms of sexual development but are produced, in our experiments, by feminizing chromosomal males. Intersexes have a sexual morphology with characteristics of both male and hermaphrodite development.

Identifying feminizing elements by microinjection. Cloned X-chromosomal sequences were microinjected into hermaphrodites.

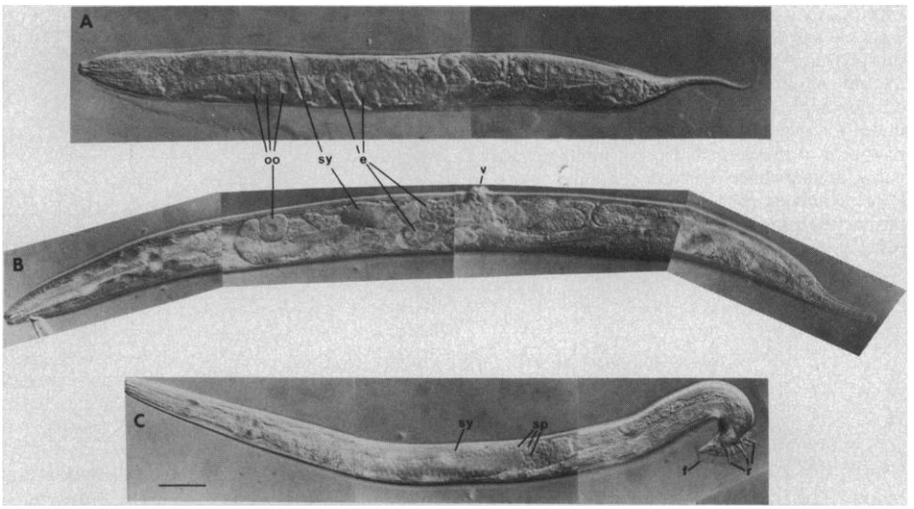

Fig. 1. Sexually normal hermaphrodites (**A** and **B**) and male (**C**). Hermaphrodites have a two-armed gonad containing sperm, oocytes, and usually embryos. In the absence of males, the embryos arise from internal self-fertilization. Oocytes are block-shaped and have a large flat nucleus and prominent nucleolus; embryos are ovoid and have multiple cells and an eggshell. There is a vulva in the middle of hermaphrodites. Males make only sperm in a one-armed gonad that exits near the tail. The most distinctive characteristic of males is the tail. The male tail has a fan, nine pairs of sensory rays, and a pair of spicules. (The spicules are not visible in the photographs, although they were routinely assayed for their presence as a male characteristic.) By contrast, the hermaphrodite tail terminates in a simple whip-like tail spike. Photographed with Nomarski optics. Labels: oo, oocytes; sy, syncytial gonad; e, embryo; v, vulva; sp, sperm including spermatocytes; f, tail fan; and r, rays. The scale bar is 50 μm.

Clones containing a feminizing element should cause intersexual development of the chromosomal male progeny and clones that did not contain a feminizing element should not affect sexual development of the male progeny. Analogous genetic experiments were done by Madl and Herman (*6*), who looked for feminizing elements by constructing strains that were chromosomally male (3A;2X) but with an X-chromosome duplication. They tested four regions of the X chromosome and found that all regions feminized 3A;2X males (*6*). In subsequent experiments, two additional duplications tested by the same method also affected sex determination in 3A;2X males (*8*). When the X-chromosome duplication is large, many animals having 3A;2X + X duplication are hermaphrodites (*6*). When the X-chromosome duplication is smaller, some of the 3A;2X + duplication animals are intersexual. For the smallest duplications tested, no effect on sex determination was observed; however, a larger duplication of the same region results in intersexual or hermaphrodite development. These results have been interpreted to indicate that the X-linked component of the X/A ratio is a feminizing element widespread on the chromosome (*5, 6*).

Microinjecting cloned DNA provides a way to define a feminizing element more precisely than genetic experiments could. For our protocol (*9*), genetically marked tetraploid hermaphrodites were microinjected (*10*) and immediately mated with diploid males carrying the duplication *mnDp8* (Fig. 2). The duplication has a region of the X chromosome stably attached to an autosome; *mnDp8* has no effect alone on sex determination in this cross (*6*) but serves to render the males more sensitive to small changes in X-chromosome dose. The progeny of the mating will be 3A;3X hermaphrodites with or without *mnDp8* (distinguished by genetic markers) and 3A;2X males with or without *mnDp8* (*9*). In the absence of injected DNA, no intersexes are seen (*6, 11*). From previous work (*12*), we expect that injected DNA will be inherited by some of the progeny. An effect of the injected DNA on sex determination will be seen as either intersexual F_1 animals or a progeny sex ratio skewed in favor of hermaphrodites (*6*), depending on whether some 3A;2X chromosomal males develop as intersexes or as hermaphrodites. Broods were assayed for the presence of intersexual progeny, defined to be superficially male animals with hermaphrodite characteristics such as oocytes, embryos, gonadal transformations, or a vulva.

A pooled set of X-chromosomal clones affects sex determination. Since the molecular distribution of X-linked feminizing elements was unknown, a set of seven clones representing about 60 kb of X-chromosome DNA from *C. elegans* was pooled and injected (*13*). Most injected worms produced only sexually normal cross-progeny. However, two (of 32) injected animals had intersexual progeny; that is, some progeny were superficially male but were found on closer examination to have morphological characteristics of hermaphrodites. The intersexes represented 17 percent (4 of 23) of the male progeny of the two injected worms. The intersexual progeny indicated that injection of the pool of X-chromosome DNA affected sex determination.

In addition to the two animals that produced intersexual progeny, six (of 32) injected animals produced broods with biased sex ratios: more than 75 percent of the cross-progeny were hermaphrodites (*14*). The abnormal sex ratio may be the result of some 3A;2X progeny developing as hermaphrodites rather than males, by analogy with the results with large X-chromosome duplications (*6*). We do not know why the progeny of these six injected animals developed as hermaphrodites while the progeny of two other injected animals included intersexes. The difference may be due to the amount of DNA injected or its stability. It may also be that the pool contains multiple kinds of feminizing elements with different or synergistic effects. However, we do not know whether these are direct effects on sex determination or are due, for example, to embryonic death or abnormal chromosome segregation arising from the amount of DNA injected.

The intersexual phenotype. The intersexual animals arising from injections with the pool of DNA or from individual clones have similar phenotypes; the intersexual phenotype and the experiments

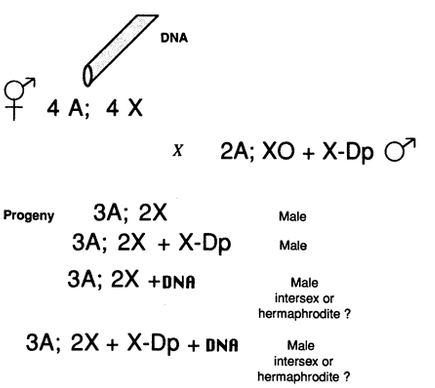

Fig. 2. Experimental strategy to test X-chromosome DNA in 3A;2X males (*9*). DNA was microinjected into the tetraploid mother before she was mated. She was expected to make two classes of ova: some with the injected DNA and some without. The male is diploid and carries the X-chromosome duplication *mnDp8*. Only the four classes of 3A;2X chromosomal males are shown. The classes are those with or without *mnDp8* (from the sperm) and those with and without the injected DNA (from the ova). The inheritance of the DNA is inferred (*12*) but proved in only a few cases. If the injected DNA has no effect on sex determination, all 3A;2X progeny are expected to be normal males since *mnDp8* alone has no effect on sex determination when inherited through sperm (*6*). If the DNA affects sex determination, some progeny are expected to be intersexes or hermaphrodites.

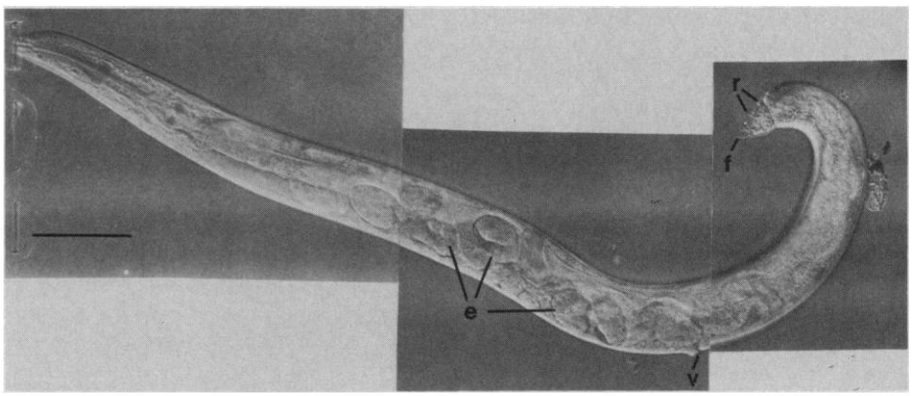

Fig. 3. An intersex resulting from injecting the *act-4* clone pCeA4. The tail fan and rays are distinctive for male development, and the embryos and vulva are distinctive for hermaphrodite development. In this animal, the tail fan is reduced somewhat and the vulva is posterior to the midpoint of the animal. Not all intersexes had a vulva; among those that did, the vulva varied in location and morphology. Labels and photography are the same as for Fig. 1. The scale bar is 100 μm.

to identify an element giving rise to this phenotype are described below. The intersexes pictured arose from injections with individual clones.

The most obvious characteristics of normal sexual dimorphism—the tail, the shape of the gonad, the germline, and the presence or absence of a vulva—were scored (Fig. 1). By our definition, an intersex has characteristics of both sexes. For example, an intersex may exhibit a male tail with fan, rays, and spicules, and a male-like one-armed gonad, as well as hermaphrodite characteristics such as a vulva and embryos (Fig. 3). The embryos probably arise from internal self-fertilization in the intersexes; the embryos are normal since they often hatched inside the parent to form viable larvae. Other intersexual animals had a two-armed gonad (as do hermaphrodites) or an abnormal or poorly developed gonad. Some intersexes had oocytes, but no embryos; the number of oocytes and embryos in an animal ranged from one to more than ten. Tail morphology also varied (Fig. 4), with some animals displaying nearly normal male tails and others missing some rays or the spicules. One unusual tail morphology (Fig. 4C), seen in two unrelated animals, had both male rays and a hermaphrodite tail spike (*15*). This morphology shows that different cells in the same tail can go through different developmental programs.

Two lines of evidence indicate that these intersexes have defects in basic aspects of sex determination. First, all aspects of sexual morphology that we examined are affected. Second, the sexual phenotype of an intersex resembles a mosaic of male and hermaphrodite development, with one structure having nearly normal male morphology and another having nearly normal hermaphrodite morphology. A mosaic-type developmental pattern indicates that hermaphrodite development is occurring in a chromosomal male rather than that male development is simply abnormal and suggests that the signal for sex determination is altered (*16*).

Defining smaller clones with feminizing elements. Since the pool of X-chromosome DNA gave intersexual progeny when injected into the mother, individual clones were injected in order to identify a feminizing element. The first individual clone tested was the plasmid pCeA4 containing part of the X-linked actin gene *act-4* (*17*). All injected animals had about 50 percent male cross-progeny. However, many of the males in fact were intersexual when examined more closely. For pCeA4, 27 percent of the injected tetraploid worms produced intersexual progeny (Table 1). The intersexes were about 9 percent of the total male progeny that inherited *mnDp8*, and 18 percent of the male progeny in broods with intersexes. (All males that did not inherit *mnDp8* were sexually normal in injections with individual clones.) Thus, pCeA4 affects sex determination in 3A;2X + *mnDp8* animals.

The plasmid pCeA4 has 1214 bp of *act-4* sequence (Fig. 5), 975 bp from exons and 239 from introns, but no part of either the 5′ or 3′ untranslated regions (*17, 18*). In order to locate a feminizing element within pCeA4, restriction fragments (*19*) were subcloned and tested (Table 1 and Fig. 5). A feminizing element is located in a fragment composed of 130 bp at the 3′ end of the first intron and 1 bp of the second exon. Plasmid pCeA4.4 does not have this region of the *act-4* gene and does not affect sex determination (Table 1). The frequency of injected worms giving intersexual progeny did not change much as smaller pieces were tested, except possibly for the

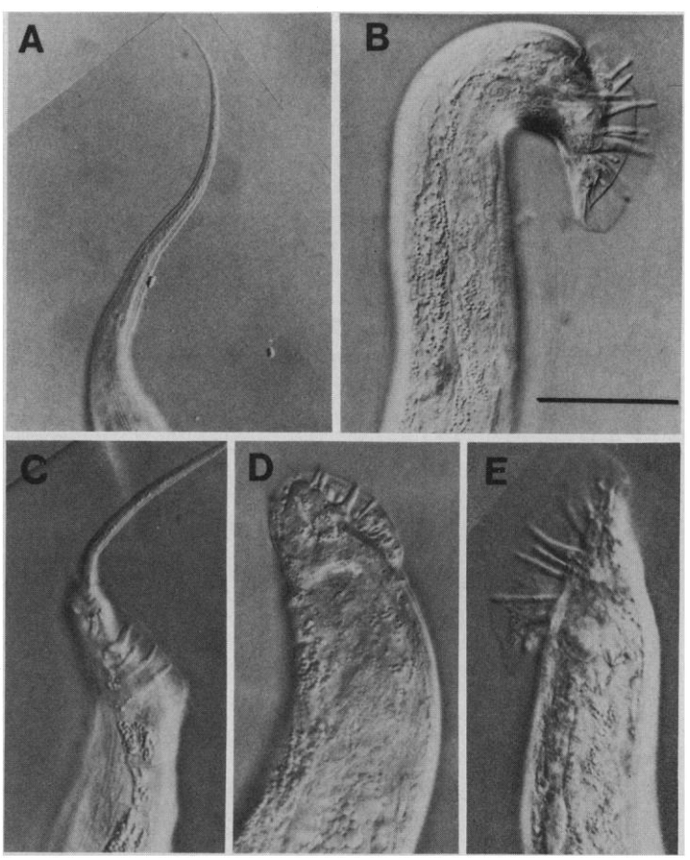

Fig. 4. Normal and intersexual tails. Photographed under Nomarski optics. All views are lateral. The scale bar is 50 μm. (**A** and **B**) Normal diploid hermaphrodite (A) and male (B) tails included for comparison. The hermaphrodite has a tail spike, the male has a tail consisting of a fan and rays. The tails of the three intersexes are shown in (C), (D), and (E). (**C**) Both rays and a tail spike are present. The fan is reduced. (**D**) A reduced male tail. The rays are shorter, the fan is smaller, and some rays may be absent. (**E**) A nearly normal male tail from an intersexual animal. This animal was classified as intersexual because it had embryos and oocytes.

injections with pCeA130 which may be simply due to better technique with later injections. The injected animals that had intersexual progeny had about the same percentage of intersexual progeny, 22 percent overall (Table 1). Thus, in the case of *act-4*, we have localized a feminizing element to a small region that is almost entirely intronic.

One explanation for these results is that almost any DNA has sequences with nonspecific effects on sex determination. An advantage of using *act-4* is that one of the autosomal actin genes can serve as a control. There are three autosomal actin genes in *C. elegans* in addition to the X-linked *act-4* (*17, 18*); more than 85 percent of the nucleotide sequence within coding regions is the same among the four genes (*18*). A plasmid containing about 4.5 kb of DNA including the entire autosomal actin gene *act-3* (*18*) was tested. None of the injected worms produced intersexual progeny (Table 1). On the basis of the frequencies seen with *act-4*, we would expect about six injected worms to produce intersexual progeny, yielding at least ten intersexual animals. The result with *act-3* is consistent with the feminizing effect being localized to the *act-4* intron, since the introns of the two genes are completely different. A cosmid containing about 35 kb of nematode DNA including the autosomal gene *lin-12* (*20*) was also tested. No intersexes were seen (Table 1). Phage lambda DNA and pBR322 DNA also do not give intersexes when injected (Table 1). Injection of the vector pIC20H resulted in a single intersexual animal. Since only one intersex was seen and the frequency was much lower than for the positive clone pCeA130 (Table 1), we regard this as a nonspecific effect. To show that a feminizing element appears to reside specifically in the *act-4* intron, the 131-bp fragment from the X-linked *act-4* gene was cloned into the autosomal gene *act-3* (*21*) and the clone was tested. This clone does give intersexes at a high frequency (Table 1). We conclude that the feminization is due to *act-4* sequences.

The second clone tested from the 60-kb pool was a plasmid containing coding region from the X-linked myosin heavy chain gene *myo-2* (*22*). With this plasmid, none of 11 injected worms had any intersexes among 78 male progeny. However, a phage (*23*) containing a larger portion of the *myo-2* gene did give intersexes; that is, 3 of 13 injected animals produced intersexual progeny. The frequency of intersexes in the broods of these three injected animals was 22 percent (5 of 23), similar to the frequency seen with *act-4* injections. Thus, injection of a larger region of the *myo-2* gene resulted in intersexes but the smaller piece did not, suggesting that, as was seen with *act-4*, only certain parts of X-linked genes affect sex determination. The feminizing *myo-2* phage contains about two-thirds of the coding portion of the gene as well as the 3′ untranslated region (*23*).

A plasmid containing two myosin light chain genes, *mlc-1* and *mlc-2*, and 2.6 kb of sequence separating them (*24*) was also tested. This plasmid, not in the pool assayed originally, has about 5 kb of DNA from a third region of the X chromosome (*24*). Three of 12 worms injected with the *mlc* plasmid had intersexual progeny. The intersexes comprised 15 percent of the male progeny of all injected worms and 35 percent (9 of 26) of the male progeny in broods with intersexes. Again, the frequencies are similar to those seen with positive clones in the *act-4* and *myo-2* injections.

In summary, clones representing three different regions of the X chromosome alter male sex determination when injected into the mother. Furthermore, the frequency of injected animals giving intersexes and the fraction of intersexual animals among their progeny are about the same for the three genes. These results lead us to conclude that X-linked sequences affecting male sex determination are common.

Sequence comparison among the clones producing intersexes and inheritance of DNA. The *act-4*, *myo-2*, and *mlc* genes have been sequenced (*17, 18, 22, 24*). The sequence of the smallest feminizing clone, 131 bp from the *act-4* intron (Fig. 6), is 66 percent A-T, but no distinctive features were obvious. We looked for a conserved sequence by searching for the longest stretch of identity in pairwise comparisons between the 131-bp sequence and either the *myo-2* gene or the plasmid with both *mlc-1* and *mlc-2* (*25*). There is a 9-bp match between the *act-4* intron and the 3′ untranslated region of *myo-2*. The *myo-2* clone that had no feminizing effect lacks this sequence, but the feminizing clone contains it. The longest identical stretch between the *act-4* intron and *mlc* sequences is also a 9-bp match, with the opposite strand of the 3′ untranslated region of *mlc-1*. These two separate 9-bp matches to the *act-4* intron overlap for 8 bp (Fig. 6). The common 8-bp sequence is not found in *act-3*, *lin-12*, or phage lambda, but is found in pBR322. The common sequence is intriguing, but given the length, composition, and presence in pBR322, it is possible that the important feature of the *act-4* intron is more complex than a simple conserved sequence.

We infer that the injected DNA is inherited as a large extrachromosomal array that is quasi-stable both meiotically and mitotically (*12*). To test the inheritance and state of the injected DNA, we performed dot blot experiments with single worms (*26*). Control experiments with a transposable element as a probe indicate that sequences present at about 30 copies per haploid genome can be detected if present in all cells, but less frequent sequences probably cannot. Two different groups of animals were tested with the labeled vector as a probe: (i) intersexes and their normal male siblings arising from injections with pCeA4; and (ii) normal males arising from injections with pCeA4.4. From pCeA4-injected animals, 9 of 20 normal F_1 males and 8 of 14 F_1 intersexes had the injected DNA. From pCeA4.4-injected animals, 9 of 16 normal males had the injected DNA. These results show that some normal males and some intersexes inherit the DNA in high copy number.

However, the presence of the DNA does not appear to correlate with sexual transformation. There are several possible explanations for this. The lack of apparent injected DNA in some intersexual animals can be trivially explained if the DNA were present in amounts below the limit of detection or in a form inaccessible to

Table 1. The frequency of intersexual animals from injections with the X-linked actin gene *act-4* and controls. The location of each *act-4* subclone is shown in Fig. 5. Only injected hermaphrodites that were fertile and gave cross-progeny are included. For males, only the male progeny that inherited *mnDp8* are included. All males that did not inherit *mnDp8* were sexually normal. One hermaphrodite arising from an animal injected with T26E12 had an abnormal gonad and lacked a vulva, but did not have clear-cut male characteristics.

Gene	Clone	Size (kb)	Injected hermaphrodites with intersexual progeny	Intersexual animals among	
				Total male progeny	Male progeny of broods with intersexes
		X-linked			
act-4	pCeA4	1.2	3/11	6/68	6/33
	pCeA4.4	0.43	0/28	0/110	
	pCeA4.8	0.78	7/27	11/66	11/33
	pCeA130	0.13	9/21	12/95	12/66
		Controls			
act-3	pCeA13	4.5	0/21	0/115	
lin-12	T26E12	35	0/13	0/75	
λ+pBR322		55	0/9	0/95	
pIC20H		2.7	1/28	1/154	1/8
act-4/act-3	pCeA13.130	4.7	5/11	8/96	8/46

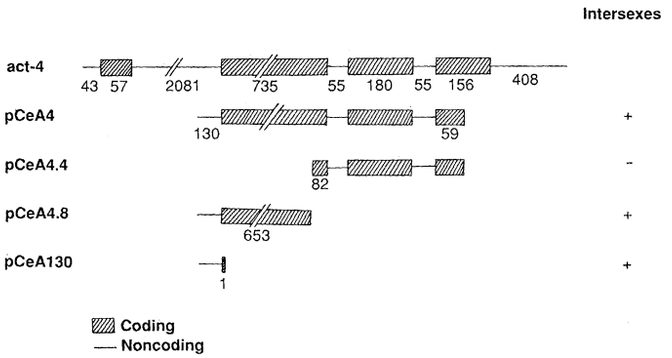

Fig. 5. A schematic of the *act-4* gene and subclones tested (*17, 18*). Coding regions are indicated by hatched boxes and introns and untranslated regions are indicated by straight lines. A box or line interrupted by the double slash indicates that the drawing is not to scale for that region. The size of each region in base pairs is given below the box or line. The result of injecting each subclone is shown on the right; +, intersexual progeny resulted; −, no intersexes were seen. Complete data are shown in Table 1. The entire *act-4* gene has not been tested.

Fig. 6. The sequence of the insert of the smallest *act-4* subclone tested that gives intersexual progeny (*18*). The italicized bases show the sequence that is identical among the different cloned X-linked genes.

```
5'AATTCTCGCC GCAACCTTCT CTCTTTGCAA     30
TGTAAATATA ATGGACATTC TTGAAAGACC       60
TAACGAGAGT ATTCATTTCT TATCTAGGGG       90
TCATCATGGG ATATATTGAA ACAAAAATTG      120
ATAATTTCAGG 3'                        131
```

hybridization. More fundamentally, it is not known when the X/A ratio is assessed in nematode development or which cells assess the ratio. Thus, it is possible that some animals, either normal or intersexual, are mosaic for the DNA; the dot blots measure the injected DNA in the entire animal but not necessarily in the cell lineages that show sexual dimorphism. This explanation might suggest that hermaphrodite lineages arise in cells with injected DNA and male lineages in cells without it. Alternatively, the mosaic character of the intersexes may indicate that the X/A ratio is ambiguous even in those cells that have the DNA, and the decision between male and hermaphrodite development is more stochastic (*6, 7*). Yet another explanation for the lack of correlation between the injected DNA and feminization is that the DNA may act maternally rather than zygotically. We cannot discriminate among these possibilities unless we can assay individual cells for the injected DNA after scoring their sexual development or can stabilize the DNA so that it is not lost meiotically or mitotically.

Implications concerning feminizing elements. We have shown that sex determination in triploid males of *C. elegans* can be altered by microinjecting X-chromosome DNA into the mother. Three different X-linked clones, part of an actin gene, part of a myosin heavy chain gene, and all of two myosin light chain genes, produce intersexual progeny when microinjected. None of the products of these X-linked genes is likely to have any role in sex determination. Thus, feminizing elements probably lie in noncoding regions. In the case of the X-linked actin gene *act-4*, a feminizing element has been defined to within a region of 131 bp in an intron. A subclone of the *act-4* gene that does not have this 131-bp region has no effect on sex determination, and addition of this region to an autosomal actin gene confers feminizing activity on the autosomal gene. An apparently similar result is seen with *myo-2* since a clone from the coding region has no effect, whereas a clone with additional introns, the 3′ untranslated region, and adjoining DNA produced intersexes. These results indicate that specific sequences or regions are responsible for feminization. We postulate that such elements are common on the X chromosome since all three X-linked regions tested produced intersexes.

In contrast to the results with X-chromosome DNA, a plasmid containing the autosomal actin gene *act-3* and a cosmid with the autosomal region including *lin-12* had no effect on sex determination, suggesting that the necessary element is less common on the autosomes than on the X chromosome. More X-linked and autosomal genes must be tested to substantiate this suggestion. Our results indicate that there are at least three feminizing elements in 16 kb of X-chromosome DNA and none in 40 kb of autosomal DNA.

We suggest that the intersexes arise because the injected sequences compete for a gene product involved in assessing the X/A ratio. In effect, these sequences are feminizing because they elevate the X component of the X/A ratio. Detailed models of the X/A ratio based on binding sites have been proposed (*27*). These models are consistent with our data, although there are other explanations for the effect of the injected DNA on sex determination. Whatever the mechanism, specific and relatively short DNA sequences can alter sex determination in *C. elegans*. The sequences do not appear to be rare on the X chromosome, but they do appear to be discrete. More examples are needed to determine the nature, distribution, and function of feminizing elements but, given our initial results, these problems should be tractable to molecular analysis.

REFERENCES AND NOTES

1. For review of earliest work, see E. B. Wilson, *The Cell in Development and Heredity* (Macmillan, New York, ed. 3, 1928), chap. 10.
2. P. N. Goodfellow and S. M. Darling, *Development* **102**, 251 (1988).
3. D. C. Page *et al.*, *Cell* **51**, 1091 (1987).
4. See, for example, J. J. Bull, in *Evolution of Sex-Determining Mechanisms* (Benjamin, Menlo Park, CA, 1983).
5. J. Hodgkin, *Annu. Rev. Genet.* **21**, 133 (1987); *The Nematode Caenorhabditis elegans*, W. B. Wood, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 243–280.
6. J. E. Madl and R. K. Herman, *Genetics* **93**, 393 (1979).
7. B. S. Baker and J. M. Belote, *Annu. Rev. Genet.* **17**, 345 (1983); T. W. Cline, *Genetics* **119**, 829 (1988).
8. P. Meneely, unpublished data.
9. Hermaphrodites of the tetraploid strain SP344 (*dpy-11 V; unc-3 X*) (*6*) were immobilized on dried agarose pads under oil and injected (*10*). Surviving hermaphrodites were individually mated to males heterozygous for the X-chromosome duplication *mnDp8*, which carries *unc-3+*. The male makes four classes of sperm: with or without the duplication and with or without an X chromosome. The mating worms were transferred to fresh plates daily, and the progeny were scored after incubation at 20°C. Each plate of progeny was screened under the dissecting microscope, and the numbers of three classes of cross-progeny were recorded: non-Dpy non-Unc hermaphrodites, non-Dpy non-Unc males, and non-Dpy Unc males. The two male classes differ in that the former carry the duplication and the latter do not. This can be used to determine whether *mnDp8* is necessary for the injected DNA to affect sex determination. For single clones, only non-Unc males were intersexual, implying that *mnDp8* is required for feminization. Males that exhibited any hermaphrodite-like or other unusual characteristics were further examined with Nomarski differential interference contrast microscopy. If no unusual males were observed in the dissecting microscope, about half of the normal males were examined with Nomarski optics to ensure that subtle sexual transformations were not overlooked.
10. For general injection procedures, see A. Fire, *EMBO J.* **5**, 2673 (1986). DNA (1 to 10 pl) of DNA (0.5 mg/ml plus fluorescein isothiocyanate–dextran at 1 mg/ml) was injected into the distal arm of each half of the gonad of SP344 hermaphrodites; we estimate that we injected 10^5 to 10^6 DNA molecules. Injections were monitored with epifluorescence and the injected animals were rehydrated with a high viscosity recovery buffer and M9 salts as described by A. Fire (see above).
11. W. McCoubrey, K. Nordstrom, P. Meneely, unpublished results. The negative results with control DNA and certain X-linked clones also confirm that *mnDp8* alone does not affect sex determination in this cross.
12. D. T. Stinchcomb, J. E. Shaw, S. H. Carr, D. Hirsh, *Mol. Cell. Biol.* **5**, 3484 (1985).
13. The pool of X-linked sequences consisted of four clones in λEMBL4 and three pBR322-derived plasmids. The lambda clones were isolated by L. Donahue [L. M. Donahue, B. A. Quarentillo, W. B. Wood, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7600 (1987)] during a chromosome walk in the region of *act-4*, and provided by her. The plasmids were: pGBA2.2 (provided by R. Waterston and G. Benian), which contains a portion of *myo-2*; pRW98 (provided by R. Waterston), which contains the transfer RNA gene *sup-7*; and pCeA4 (provided by M. Krause), which contains a portion of *act-4*.

14. The size of the brood for these six animals ranged from 60 to more than 120 cross-progeny. A chi-square test indicated the probability ($P = <0.005$) that the sex ratio obtained was not due to chance deviation from a normal sex ratio. The X-chromosome duplication *mnDp8* was not essential for altering the sex ratio in these animals since there was an excess of hermaphrodites among progeny without *mnDp8* as well as among those with the duplication.
15. In normal development, the rays arise from three cells (V5p, V6p, and T) that divide postembryonically in the male [J. E. Sulston and H. R. Horvitz, *Dev. Biol.* **56**, 110 (1977); J. E. Sulston, D. G. Albertson, J. N. Thomson, *ibid.* **78**, 542 (1980)]. In the hermaphrodite, these three cells form part of the hypodermis, and do not make distinctive structures. Likewise, the tail spike arises postembryonically in hermaphrodites from a cell that forms part of the hypodermis in the male. In the tail shown in Fig. 4C, the rays that arise from V6p are present, but the single pair that arises from V5p and the three pairs that arise from T are absent. A tail spike is present. The lineages of the cells that make these rays and the tail spike diverge early, before the 100-cell stage, suggesting that either the sex determination program for the tail is not specified at this time or that it can change after this time.
16. This point is discussed for mosaic intersexes in *Drosophila* [T. W. Cline, *Dev. Biol.* **95**, 260 (1983); *Genetics* **107**, 231 (1984)].
17. J. G. Files, S. H. Carr, D. Hirsh, *J. Mol. Biol.* **164**, 355 (1982); C. P. Landel, M. Krause, R. H. Waterston, D. Hirsh, *ibid.* **180**, 497 (1984).
18. M. Krause and D. Hirsh, personal communication.
19. pCeA4 consists of a 1214-bp Eco RI–Bam HI fragment from *act-4* cloned into pBR325. The insert has a single Xho I site 783 bp from the Eco RI site. Plasmid pCeA4.4 was constructed by deleting the Eco RI–Xho I fragment from pCeA4; plasmid pCeA4.8 was made by deleting the 431-bp Xho I–Bam HI interval; pCeA130 contains the first 131 bp of the pCeA4 insert cloned as an Eco RI–Hae II fragment in pIC20H [J. L. Marsh, M. Erfle, E. J. Wykes, *Gene* **32**, 481 (1984)] by replacing the small Eco RI to Sma I fragment from the polylinker.
20. J. Priess, personal communication.
21. A 242-bp Hind III fragment with the insert of pCeA130 was cloned into the Hind III site of the *act-3* plasmid pCeA13 to make pCeA13.130.
22. J. Karn, N. J. Dibb, D. M. Miller, *Cell Muscle Motility* **6**, 185 (1985); R. Waterston and G. Benian, personal communication.
23. The phage FH#MY5 was picked from a genomic library (obtained from C. Link) made in λEMBL4 with the insert from pGBA2.2 as a probe, and shown by restriction mapping to have an insert of about 10 kb including the 3′ end of *myo-2* and about 5 kb of DNA adjoining the 3′ side.
24. C. Cummins and P. Anderson, personal communication.
25. The sequence of the 131-bp *act-4* intron was compared with the sequence of *myo-2*, and the *mlc* plasmid, as well as with the sequence of *act-3*, *lin-12*, pBR322, and phage lambda with the software GENEPRO [S. Henikoff and J. C. Wallace, *Nucleic Acids Res.* **16**, 6191 (1988)]. Between the *act-4* intron and *myo-2*, the longest identical sequence consists of the eight italicized bases in Fig. 6 and the next C on the 3′ side; this sequence is in the 3′ untranslated region of *myo-2*. Clone FH#MY5 has about 5 kb of unsequenced DNA, so it is possible that the feminizing region is not in the sequenced portion. The longest identical sequence between either *mlc-1* or *mlc-2* and the *act-4* intron is also 9 bp, including the italicized sequence in Fig. 6 and the next A on the 5′ side. The sequence is found on the opposite strand of the 3′ untranslated region of *mlc-1*.
26. Individual worms were placed in an ordered arrangement onto GeneScreen Plus (New England Nuclear) on top of several sheets of Whatman 3MM paper saturated with fresh 0.4*N* NaOH containing 1 percent SDS. Filters were incubated in a humid chamber at 37°C for 30 minutes to facilitate lysis of the animals. The filter was immersed for 30 seconds in 0.4*N* NaOH after incubation, neutralized in 0.2*M* tris-HCl (*p*H 7.5), 2× standard saline citrate, and air dried. The filter was hybridized and washed according to the instructions for Southern hybridizations from New England Nuclear. A control to estimate the sensitivity used the transposable element Tc1, which was readily visible in tetraploids and weakly visible in diploids. Since there are about 30 copies of Tc1 in the haploid genome and about 1000 cells in the animal, we estimate that, in order to be visible by this method, the DNA must be present in about 60,000 copies per animal.
27. H. S. Chandra, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1165 (1985); W. B. Wood, C. Trent, P. Meneely, J. Manser, S. Burgess, in *Genetic Regulation of Development*, 45th Symposium of the Society of Developmental Biology, W. Loomis, Ed. (Liss, New York, 1987), pp. 191–199.
28. We thank M. Krause and J. Priess who provided us with clones, DNA, advice on techniques, and comments and criticisms on both the work and the manuscript; D. Hirsh, R. Waterston, G. Benian, C. Cummins, and P. Anderson for clones and permission to cite data about the *act-4*, *myo-2*, *mlc-1*, and *mlc-2* genes; C. Link for his library; and L. Donahue and B. Quarentillo for other clones. We thank M. Groudine, S. Henikoff, E. Jaffe, C. Laird, R. Levis, and H. Weintraub for discussion of the results and reviewing the manuscript and J. Shaw who several years ago suggested experiments like these (to P.M.M.). Supported by NSF grants (P.M.M.), the March of Dimes Basil O'Connor Starter Program (P.M.M.), and NIH postdoctoral training grant 5T32CA09437-06 (W.K.M.).

11 May 1988; accepted 3 October 1988