Sex Determination in Polyploids of *Caenorhabditis elegans*

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ABSTRACT

In *Caenorhabditis elegans* triploid animals with two *X* chromosomes (symbolized 3A;2*X*) are males. However, these triploid males can be feminized by making them mutant for recessive dosage compensation mutations, by adding *X* chromosome duplications or by microinjecting particular DNA sequences termed feminizing elements. None of these treatments affects diploid males. This study explores several aspects of these treatments in polyploids. The dosage compensation mutants exhibit a strong maternal effect, such that reduction of any of the dosage compensation gene functions in the mother leads to sex reversal of 3A;2*X* animals. Likewise, all *X* chromosome duplications tested cause both sex reversal and intersexual development of many 3A;2*X* animals. Microinjected feminizing element DNA does not cause extensive sex reversal, but does result in intersexual development in 3A;2*X* animals. Neither *X* chromosome duplications nor microinjected feminizing elements show the extreme maternal effect of the dosage compensation mutants, although there is indirect evidence for a maternal effect of the feminizing elements. In particular, very little feminizing element DNA needs to be microinjected in order to feminize triploid males, far less than what is needed for stable inheritance, implying that feminizing elements can work within the mother's gonad. However, even very high concentrations of microinjected feminizing elements do not affect sex determination in diploid males, suggesting that they are not part of the numerator of the *X*/A ratio. In addition, no pair of *X* chromosome duplications feminizes diploid males, suggesting that none of these duplications contains a numerator of the *X*/A ratio. Instead, I infer that an *X*-linked locus, as yet undefined, must be present in two copies for hermaphrodite development to ensue or that the two *X* chromosomes might interact.

THE nematode *Caenorhabditis elegans* has two sexes, males and hermaphrodites. Because there is no *Y* chromosome, the chromosomal signal for sex determination must involve the ability to count the number of *X* chromosomes. The counting is not absolute, however, since animals with two *X* chromosomes can be either hermaphrodite or male depending on their ploidy. That is, diploids with two *X* chromosomes are hermaphrodites, while triploids or tetraploids with two *X* chromosomes are males (MADL and HERMAN 1979). From this work in polyploids, the signal for sex determination has been inferred to be the *X*/A ratio. Thus, if the *X*/A ratio is at or below 0.67, male development results; these animals could be 2A;1*X*, 3A;2*X* or 4A;2*X*. On the other hand, if the *X*/A ratio is 0.75 or above, hermaphrodite development results; these animals could be 2A;2*X*, 3A;3*X*, 4A;3*X* or 4A;4*X*. These relationships are summarized in Figure 1.

The molecular nature of the *X*/A signal is unknown, and the applicability of the results with polyploids to sex determination in diploids has not been demonstrated. In an initial attempt to define how the *X*/A ratio is implemented in *C. elegans*, MADL and HERMAN (1979) used *X* chromosome duplications to make triploid males (3A;2*X*) trisomic for different *X*-linked regions. They reasoned that a segmental trisomy that caused triploid males to show intersexual or hermaphrodite development must contain a dose-sensitive feminizing element. Their experiment was similar in design to the early work on the *X*/A ratio in *Drosophila melanogaster* (DOBZHANSKY and SCHULTZ 1934) (reviewed in BAKER and BELOTE 1983). As was found initially with Drosophila, MADL and HERMAN (1979) showed that the dose of several different regions of the *X* chromosome affected sex determination in triploids and suggested that dose-sensitive feminizing properties are widespread on the *X* chromosome. It is important to realize that these experiments showed effects only in 3A;2*X* animals; none of the duplications affected normal diploid males. Likewise, the molecular nature of the dose-sensitive feminizing properties was necessarily undefined, as was the mechanism by which they might affect sex determination.

Similar conclusions about feminizing elements on the *X* chromosome have been drawn from microinjection experiments in polyploids (MCCOUBREY *et al.* 1988). DNA from different *X*-linked or autosomal genes was microinjected into tetraploid hermaphrodites, which were then mated with diploid males, and their triploid 3A;2*X* male cross-progeny examined for evidence of intersexual development. Microinjected DNA from several genes on the *X* chromosome has been shown to feminize triploid males, whereas sequences from autosomal genes with similar functions do not feminize

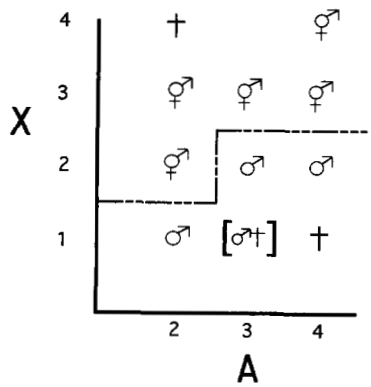

FIGURE 1.—The *X*/A ratio in *C. elegans*. Sexual phenotype is shown as a function of the number of *X* chromosomes on the ordinate and the number of sets of autosomes on the abscissa. Chromosome constitutions below the dashed line result in male development, whereas those above the dashed line result in hermaphrodite development. 2A;4*X* animals are inviable and 3A;1*X* animals are males with low viability; 3A;4*X* animals have not been reported.

triploid males (MCCOUBREY *et al.* 1988; S. ROBERTSON, W. MCCOUBREY and P. MENEELY, manuscript in preparation). The *X*-linked genes with such feminizing elements do not have functions associated with sex determination, and the sequences required to feminize triploid males have been localized to small non-coding regions. These studies suggest again that there is some general property of the *X* chromosome or of *X*-linked genes that affects sex determination in triploids. However, the mechanism by which feminizing elements affect sex determination in polyploids is still unknown.

In addition to experiments with duplications and injected feminizing element DNA, which add more copies of *X* chromosome genes or sequences, the autosomal mutants *dpy-21* and *dpy-28* also have been shown to feminize 3A;2*X* triploids, such that 3A;2*X* animals homozygous for either *dpy-21* or *dpy-28* are intersexual or hermaphrodite (HODGKIN 1987a; PLENEFISCH *et al.* 1989). Neither *dpy-21* nor *dpy-28* mutants affect sex determination in diploid males, either alone or in combination, although each is known to affect *X* chromosome dosage compensation. The ability of these mutants to feminize triploids has led to the idea that dosage compensation and sex determination are connected via a feedback loop (HODGKIN 1987a; PLENEFISCH *et al.* 1989; MENEELY and NORDSTROM 1988; VILLENEUVE and MEYER 1990; DELONG *et al.* 1993). Again, the means by which these mutants cause feminization and the significance of the results in triploids for sex determination in diploids are not known.

In this report, I extend earlier work on sex determination in diploid, triploid and tetraploid males using the dosage compensation mutants, *X* chromosome duplications and microinjected feminizing element DNA. This study shows that these treatments feminize triploid males only and do not feminize diploid males; microinjected feminizing element DNA also does not affect tetraploid males. From this and other results, I infer that two *X* chromosomes are needed or that some region or gene on the *X* chromosome is required in two copies for hermaphrodite development to occur. These results lead to a somewhat different interpretation of how sex determination might be occurring in diploids.

MATERIALS AND METHODS

General methods and strains used: All of the worms were grown at 20° on standard media and handled by routine techniques (SULSTON and HODGKIN 1988). All of the genes and duplications used have been described previously (MENEELY and WOOD 1987; HODGKIN *et al.* 1988; HERMAN and KARI 1989).

Tetraploid strains SP344, SP345 and SP346 are described in MADL and HERMAN (1979). SP345 hermaphrodites are homozygous for the recessive autosomal marker *dpy-11 (e224) V*. SP344 is homozygous for both *dpy-11* and the recessive *X*-linked marker *unc-3 (e151) X*. SP346 is homozygous wild-type. Tetraploid males from SP346 were obtained by picking about 50 individual SP346 hermaphrodites and allowing them to self-fertilize. Several of these produced about 30% tetraploid male self-progeny, indicating that the parent was probably 4A;3*X*. From a plate with males, about 10 individual hermaphrodites were picked separately to maintain the 4A;3*X* strain (MADL and HERMAN 1979). Tetraploid males were also routinely backcrossed to SP346 hermaphrodites to propagate and maintain a tetraploid male strain. A tetraploid strain marked with *dpy-5I* and *unc-3* was constructed by mating tetraploid males with diploid hermaphrodites of genotype *dpy-5 (e61) I; unc-3 (e151) X*. From this mating, many non-Dpy non-Unc hermaphrodites were picked and allowed to self-fertilize. Most of these animals, which are expected to be triploids, were sterile or of low fertility. From among the few progeny, the largest non-Dpy non-Unc hermaphrodites, Dpy hermaphrodites, and Unc hermaphrodites were picked separately and allowed to self-fertilize. Large animals were picked since polyploids are routinely larger than diploids (MADL and HERMAN 1979). From among the progeny of these animals, a single Dpy Unc hermaphrodite was used to initiate a separate strain. By genetic tests, this strain proved to be tetraploid.

This strain and all of the tetraploid strains reverted to diploidy unless care was taken to maintain the large animals. Since reversion to diploidy may indicate meiotic instability which could affect the interpretation of these results, the level of autosomal non-disjunction was measured in both *dpy-11; unc-3* and *dpy-5; unc-3* tetraploid hermaphrodites and in 4A;2*X* tetraploid males. These tests used diploid strains homozygous for *him-6*, a mutant which causes meiotic non-disjunction of all chromosomes in both hermaphrodites and males (HODGKIN *et al.* 1979). In a *him-6* mutant strain, about 3% of the ova are disomic and about 6–10% of the sperm are nullisomic for any one chromosome (HODGKIN *et al.* 1979; HAACK and HODGKIN 1991). To determine if linkage group *V* and linkage group *I* were non-disjoining at high rates in tetraploid hermaphrodites, *him-6 (e1423)* males were mated to *dpy-11 V; unc-3 X* or *dpy-5 I; unc-3 X* tetraploid hermaphrodites and the frequency of Dpy non-Unc cross-progeny was determined. Such Dpy non-Unc cross-progeny could arise from the fertilization of ova trisomic for LG*V* or LG*I* by sperm nullisomic for the same autosome and would be a measure of the spontaneous rate of autosomal non-disjunction in tetraploid hermaphrodites (HODGKIN *et al.* 1979; HAACK and HODGKIN 1991). No Dpy non-Unc progeny were seen among approximately 7000 cross-progeny examined for each

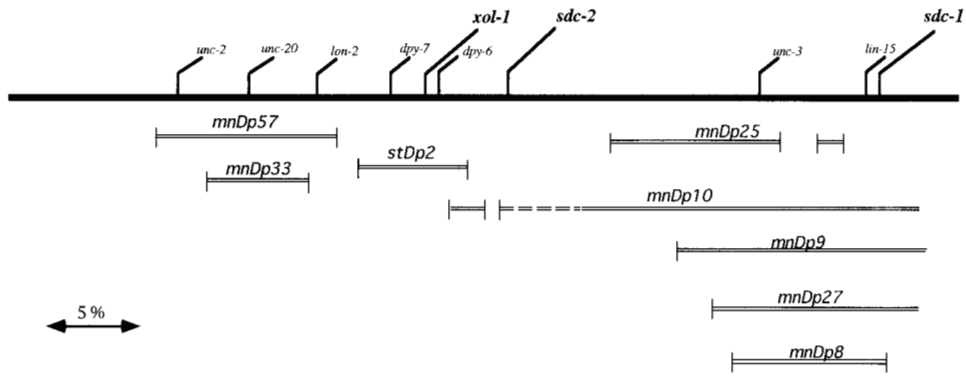

FIGURE 2.—A genetic map of the *X* chromosome showing the duplications and markers used in these experiments. The gene names in boldface and the larger font, *xol-1*, *sdc-2* and *sdc-1*, are the three *X*-linked genes known to affect both sex determination and dosage compensation; their positions are shown for reference, although the genes were not used in these experiments. *mnDp10* probably does not include *sdc-2+* (K. TANNER and W. B. WOOD, personal communication), but its precise genetic endpoint in that region is undefined, as indicated by the dashed double line. Both *mnDp10* and *mnDp25* duplicate non-contiguous blocks of the *X* chromosome.

chromosome, indicating that the spontaneous non-disjunction of LG*I* and LG*V* during oogenesis in these tetraploids does not occur at a high frequency. To test for autosomal non-disjunction during spermatogenesis in tetraploid males, 4A;2*X* males were mated to *dpy-5 I; unc-4 (e120) II; him-6* hermaphrodites and to *him-6 unc-31 (e169) IV; dpy-11 V* hermaphrodites, and the cross-progeny examined for Dpy non-Unc or Unc non-Dpy animals. Again, none were seen among about 5000 cross-progeny examined from each mating. Therefore, the rate of autosomal non-disjunction from these tetraploid males and hermaphrodites is low, so the possible effects of autosomal non-disjunction during meiosis were discounted.

In all matings for which intersexual progeny were possible, two to four males were mated with one hermaphrodite, and the mating was transferred to fresh plates daily for 4 days. All of the cross-progeny were examined using a dissecting microscope and usually counted. All of the male or malelike progeny were examined for intersexual development by Nomarski microscopy (SULSTON and HODGKIN 1988). Animals were photographed using Tri-X-Pan film with a camera attached to a Zeiss Universal microscope.

Experiments with the dosage compensation mutants: The dosage compensation mutants *dpy-21 (e428) V, dpy-26 (n199) IV, dpy-27 (rh18) III, dpy-28 (s939) III,* and *dpy-28 (y1) III* have all been previously described (HODGKIN 1983; MENEELY and WOOD 1987; PLENEFISCH *et al.* 1989). For matings of *dpy* mutant hermaphrodites with 4A;2*X* males, the hermaphrodites were also homozygous for a recessive *unc* marker. For *dpy-28 (s939), unc-32 III* was used as the marker, as the Unc Dpy progeny of the strain *nT1/unc-32 (e189) dpy-28 (s939).* For the other mutants, *unc-3 (e151) X* was used. Since both *dpy-21* and *dpy-28 (y1)* are viable as homozygotes, the strains were *dpy-21; unc-3* or *dpy-28 (y1); unc-3.* The *dpy-26; unc-3* hermaphrodites were the Dpy Unc-3 segregants from a *dpy-26/unc-31 (e169) IV; unc-3* strain, while the *dpy-27; unc-3* hermaphrodites were the Dpy Unc-3 segregrants from a *dpy-27/unc-36 III (e251); unc-3* strain. For matings of *dpy* males to tetraploid hermaphrodites, the spontaneous male self-progeny (which are non-Dpy) from a homozygous *dpy* hermaphrodite were found and backcrossed to the *dpy* hermaphrodite. For *dpy-26* and *dpy-28* (both alleles), these self-progeny males arise at relatively high frequency because the mutants also have defects in meiosis resulting in *X* chromosome non-disjunction; approximately 5% of the eggs laid by these strains give rise to *X*O males. However, this rate of non-disjunction is much lower than could account for the effects of these mutants on sex determination. Self-progeny males were less common for *dpy-21* and *dpy-27* but still easy to find since they tend to be more viable and active than the hermaphrodites. Males were mated to SP345 tetraploid strains, and the non-Dpy-11 cross-progeny were counted. These were assumed to be 3A;2*X* and 3A;3*X* animals (see RESULTS), but this was not directly determined.

Experiments involving *X* chromosome duplications in triploids: For testing the effect of an *X* chromosome duplication, diploid males heterozgyous for the duplication were mated to tetraploid SP344 or SP345 hermaphrodites. All of the non-Dpy hermaphrodite and male cross-progeny were counted and all of the males or malelike animals were examined by Nomarski microscopy. The duplication genotypes used were: *mnDp57 I/+; unc-2 (e55)X* or *mnDp57/+; lon-2 (e678) X, mnDp33 IV/+; unc-20 (e112) X, stDp2 II/+; dpy-6 (e14) X, mnDp25 I/+; unc-3 (e151), mnDp27 II/+; unc-3, mnDp10/+; unc-3, mnDp9 I/+; unc-3* and *mnDp8 I/+; unc-3* (Figure 2). For experiments in which the triploid progeny of a diploid hermaphrodite were examined, tetraploid males from SP346 were mated to *mnDp25; dpy-7 (e88) unc-3* hermaphrodites, *mnDp8; dpy-7 unc-3* hermaphrodites, or *mnDp57; unc-2 dpy-7* hermaphrodites. The *dpy-7* marker is not duplicated by these duplications, and was used to determine that the male was heterozygous for maternal and paternal *X* chromosomes. Because the mother is homozygous for the duplication, all of her progeny are heterozygous for the duplication.

Experiments with *mnDp10* strains gave extremely variable results. For instance, in three separate matings of *mnDp10/+; unc-3/0* males to SP344, the following results were obtained. Mating A produced 2 males, 75 hermaphrodites and 0 intersexes; mating B produced 59 males, 180 hermaphrodites, and 10 intersexes; mating C produced 13 males, 82 hermaphrodites and 0 intersexes. Others have seen variable results with *mnDp10* and have concluded that the duplication appears to change in size (K. TANNER and W. B. WOOD, personal communication; B. MEYER, personal communication). For my experiments, these data with *mnDp10* are pooled and the duplication was not used extensively.

Microinjection methods: The methods used for microinjection have been described (MCCOUBREY *et al.* 1988), except that SP345 hermaphrodites were used instead of the tetraploid *dpy-11; unc-3* hermaphrodites used previously. In addition, we previously mated injected hermaphrodites with males carrying

the duplication *mnDp8* and examined the duplication-bearing progeny. The frequency of intersexes arising from control matings with *mnDp8* was low, as previously shown by MADL and HERMAN (1979). However, more detailed analysis (see RESULTS) showed that *mnDp8* did produce some intersexes and definitely affected the sex ratio of the cross-progeny. This had been overlooked previously, perhaps because neither we nor MADL and HERMAN counted all of the cross-progeny, as was done for this analysis. Therefore, the use of *mnDp8* or any other *X* chromosome duplication was unsuitable, and no duplication was used. The basic results of MCCOUBREY *et al.* (1988) have been repeated and verified without using a duplication and are reported here.

To examine the effects of an injected plasmid on the progeny of tetraploid mothers, the plasmid was injected into SP345 and the hermaphrodite mated with either diploid wild-type males (to produce triploid males) or tetraploid males from SP346 to produce tetraploid males. In each case, the cross-progeny are non-Dpy. Most of the sperm produced by a tetraploid 4A;2*X* male are 2A;1*X* because the *X* chromosomes disjoin from each other (see RESULTS). When 2A;1*X* sperm fertilize 2A;2*X* ova from the tetraploid mother, the zygote is 4A;3*X* and is an hermaphrodite. Thus, these crosses have an excess of hermaphrodites to males simply due to the meiotic segregation in a tetraploid unrelated to any sex determination defects. The males that arise from this cross come from nondisjunction in the male that results in 2A;0*X* sperm; these are about 10–15% of the total cross-progeny.

Diploid hermaphrodites to be injected were marked with either *dpy-5* or *dpy-11*. Injected hermaphrodites were mated with tetraploid males as described above. Animals were injected with p64VZ at either 1 μg/ml or 10 μg/ml. The non-Dpy progeny, both males and hermaphrodites were counted, and all of the male progeny examined by Nomarski microscopy. For testing the effect of the duplications and the feminizing element together in a diploid, *mnDp25; unc-3* hermaphrodites or *mnDp8; unc-3* hermaphrodites were injected with p64VZ at 1 μg/ml or 10 μg/ml. The injected hermaphrodites were mated with tetraploid males, and the male progeny examined.

The vector pVZ1 (HENIKOFF and EGTEDARZADEH 1987) was used for all of the plasmid constructs, which were made by W. MCCOUBREY or S. ROBERTSON in my laboratory. Details of the plasmids and the sequences will be published elsewhere; the insert in p36VZ and in p64VZ were oligonucleotides synthesized by the Fred Hutchinson Cancer Research Center Biotechnology Center to correspond in sequence to part of the *act-4* intron. The plasmids were sequenced to ascertain that the insert was as expected. The oligonucleotides had the following sequences, with cloning sites in lower case letters and *act-4* sequence in capital letters.

p64VZ:

5′cATTCATTTCTTATCTAGGGGGTCATCATGGGAT-
ATATTGAAACAAAAATTGATAAT TTCggatc-3′

p36VZ:

5′-gatccGCAACCTTCTCTCTTTGCAATGTAAATATAg-3′

The titration of p64VZ and many of the other injections were done by blind tests. That is, different concentrations of p64VZ were prepared and coded by S. ROBERTSON and given to me for testing without knowledge of what was being injected. The comparison among p64VZ, p36VZ and pVZ1 was also done using blind injections. To estimate the number of copies of p64VZ injected, an oligonucleotide was labeled to a defined specific activity and injected into SP345 hermaphrodites; 25 injected worms were immediately picked individually into vials and counted in a scintillation counter and the activity was used to estimate the volume and copy number injected. Animals varied by as much as fivefold in the amount injected. Given the variation in sample determination, the estimate of the copy number injected is at best within an order of magnitude.

Construction and testing of stable lines carrying p64VZ: A mixture containing the feminizing plasmid p64VZ and a plasmid with the dominant marker *rol-6* (MELLO *et al.* 1991), at a concentration of 50 μg/ml of each plasmid, was co-injected into wild-type hermaphrodites either by me or by ANNA QUISEL in my laboratory. From the injected animals, Roller F_1 hermaphrodites were picked and propagated. Other stable lines were established from injecting the *rol-6* plasmid alone, without co-injecting p64VZ. MELLO *et al.* (1991) found that this procedure produces extrachromosomal arrays of about 100–200 copies of both *rol-6* and the plasmid of interest. To test these lines for feminization, one stable line initiated from a single Roller hermaphrodite was mated with wild-type males; the resulting Roller male progeny were then mated to SP345 hermaphrodites, and the male progeny were examined for feminization. Relatively few cross-progeny resulted from these crosses, probably because Rol males mate poorly. It should be noted that few of the cross-progeny of these matings showed the obvious Roller phenotype of *rol-6*. A more subtle phenotype of *rol-6*, that is, the animal lifts and twists its head, could be seen, however. Likewise, injecting the *rol-6* plasmid into tetraploids produced no Roller F_1 animals. Presumably, this failure to see the strong Roller phenotype represents a dosage effect of the *rol-6* plasmid in polyploids.

Construction of strains with two different *X* chromosome duplications: In order to construct males with two different *X* chromosome duplications, several multiply marked hermaphrodite strains were first constructed and maintained. For example, in order to test the combination of *mnDp57* and *stDp2*, an hermaphrodite with the genotype *stDp2/+; lon-2 unc-18 (e81)* was used. Both *lon-2* and *unc-18* are recessive *X*-linked markers; *stDp2* includes *unc-18+* but not *lon-2+*, whereas *mnDp57* includes *lon-2+* but not *unc-18+* (Figure 2). Therefore, an *stDp2/+; lon-2 unc-18* hermaphrodite is Lon non-Unc. Males of genotype *mnDp57/+; unc-2* were mated to the Lon non-Unc *stDp2/+; lon-2 unc-18* hermaphrodites. The cross-progeny will include wild-type hermaphrodites of various genotypes, but no Lon or Unc hermaphrodites; and Lon non-Unc males, non-Lon Unc males, Lon Unc males, and non-Lon non-Unc males. These non-Lon non-Unc males carry both duplications, and were counted and examined for alterations in sexual morphology. In the initial experiments, Lon non-Unc and non-Lon Unc males were also counted to determine that the males carrying two duplications were surviving at nearly normal frequency; once the survival of males carrying two duplications was ascertained, the presence of the other classes of males was used for approximate comparisons but they were not counted. The pairs of duplications tested are listed in Table 3, with "Duplication 1" referring to the paternally derived duplication, and "Duplication 2" referring to the maternally derived duplication. When *mnDp57* was introduced paternally, the hermaphrodite's *X* chromosome was doubled marked with *lon-2* and either *unc-18* (for *stDp2*, as described above) or *unc-3* (for *mnDp8*, *mnDp27*, *mnDp25*, *mnDp9* or *mnDp10*). When *stDp2* was derived paternally, the hermaphrodite's *X* chromosome was doubly marked with *dpy-7* and *unc-3*.

RESULTS

Triploids with two *X* chromosomes are usually male (Figure 1), but are feminized by recessive dosage compensation mutants, by *X* chromosome duplications, or by certain microinjected DNA sequences termed femi-

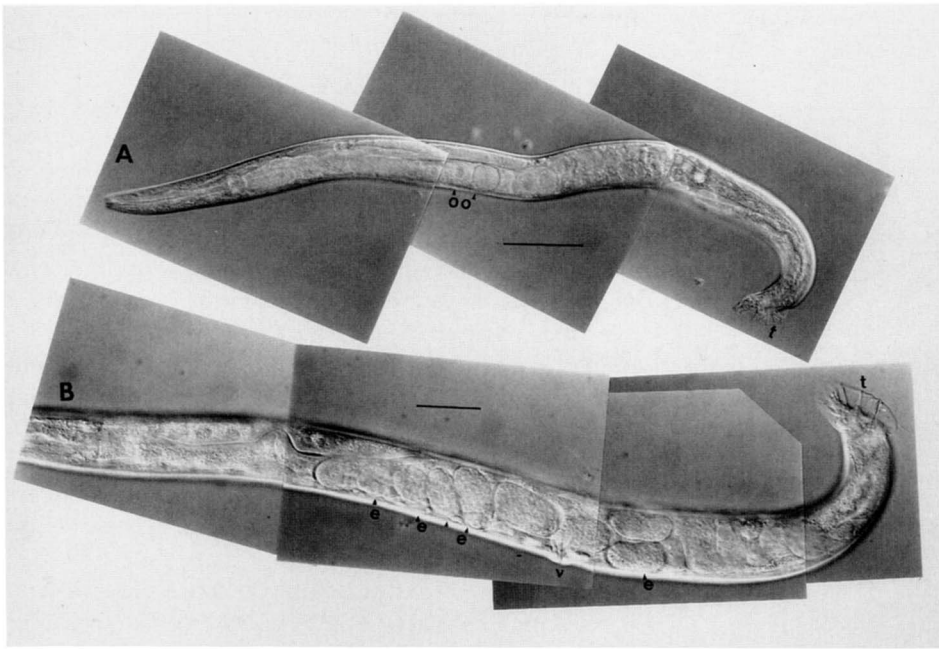

FIGURE 3.—Intersexes arising from the injection of p64VZ. These animals are typical of two of the types of intersexes seen. (A) A somatically normal male with a male gonad, male tail with normal rays and fan (labeled "t") and oocytes (labeled "oo"). Oocytes are easily recognized by the large round nucleus. The scale bar is 100 μm. (B) An intersex with a structures of both hermaphrodite and male development. The animal has a two-armed somatic gonad like a hermaphrodite, and has embryos (labeled "e") and oocytes (not labeled). At the midbody is a vulva (labeled "v"). The tail has the rays and fan characteristic of male development (labeled "t"). The scale bar is 100 μm.

nizing elements (MADL and HERMAN 1979; HODGKIN 1987a; PLENEFISCH *et al.* 1989; MCCOUBREY *et al.* 1988). I explore the effects of all three of these feminizing treatments in more detail in this report.

Feminization is defined to have occurred when a chromosomal male (that is, 2A;1*X*, 3A;2*X* or 4A;2*X*) exhibits aspects of hermaphrodite development. In some cases, there is complete sex reversal such that chromosomal males become sexually normal and semi-fertile hermaphrodites, whereas in other cases chromosomal males are sexually transformed into intersexes that have some normal characteristics of both sexes. Complete sex reversal is recognized by changes in the expected sex ratio, without noticeable embryonic death; that is, the sex ratio of males to hermaphrodites decreases because some chromosomally male embryos develop as hermaphrodites. Sex transformation into intersexes is recognized from the morphology, such as animals with both a male tail and a vulva (a "classic" intersex) or, more subtly, as somatically normal males that make oocytes or oocytes and embryos within the male gonad (a germline intersex). Examples of such animals are shown in Figure 3.

In this study, the 3A;2*X* triploid karyotype is inferred from matings between tetraploid and diploid animals, but is not proved directly. Therefore it is possible that some of the animals examined and counted as 3A;2*X* had a different karyotype or were chromosomal mosaics. However, none of the matings is expected to produce autosomal aneuploids or mitotic chromosome loss even at low frequency (see MATERIALS AND METHODS), let alone at the high frequency at which sex determination defects were observed.

Effect of dosage compensation mutants on 3A;2*X* triploids: *Maternal inheritance:* Mutants in at least four autosomal genes, *dpy-21*, *dpy-26*, *dpy-27* and *dpy-28*, cause elevated *X*-linked gene expression in 2*X* diploid hermaphrodites (VILLENEUVE and MEYER 1990). *dpy-26*, *dpy-27* and *dpy-28 (s939)* mutants exhibit a strong maternal effect and have many inviable progeny; those hermaphrodites that do hatch are extremely Dpy and do not survive or reproduce well. Based on its viability and Dpy phenotypes, *y1* appears to be a weaker allele of *dpy-28* than is *s939* (PLENEFISCH *et al.* 1989). The Dpy and inviability phenes of these mutants apparently arise because of a failure to down-regulate *X*-linked gene expression in *XX* animals (HODGKIN 1987b; VILLENEUVE and MEYER 1990).

Although none of these mutants affects sex determination in diploid males, either alone or in combination, both *dpy-21* and *dpy-28* mutants have been shown to affect sex determination in 3A;2*X* triploid males (HODGKIN 1987a; PLENEFISCH *et al.* 1989). For example, *dpy-21* 3A;2*X* animals are either intersexes or hermaphrodites, and *dpy-28* 3A;2*X* animals are hermaphrodites. I have expanded the earlier results by HODGKIN and PLENEFISCH *et al.* with *dpy-21* and *dpy-28* and included both *dpy-26* and *dpy-27* in this analysis.

To test the effect of these mutants in 3A;2*X* animals, tetraploid males (4A;2*X*) were mated to homozygous *dpy* hermaphrodites for each of the mutants and for the unrelated gene *dpy-5* (Table 1A). The results with *dpy-5* show the control results expected from this mating when no dosage compensation mutant is present and illustrate the segregation of the *X* chromosome in 4A;2*X* males. In the control mating with *dpy-5*, 2A;1*X* sperm result in 3A;2*X* progeny (which are males), while 2A;2*X* sperm result in 3A;3*X* progeny (which are hermaphrodites). Roughly 90% of the progeny of this mating are males (Table 1A), judged to be triploid from their large size and virtual sterility. About 10% of the progeny are hermaphrodites, apparently the result of 2A;2*X* sperm

TABLE 1

Mutants in dosage compensation cause sex reversal of 3A;2X animals

	Hermaphrodites	Males	Intersexes	Sex ratio[a]	Feminized (%)[b]
A. Maternal effects					
Cross: 4A;2X males × *dpy* hermaphrodites					
Mother					
dpy-5	103	953	2	9.25	0.2
dpy-21	608	804	26	1.32	40.0
dpy-26	715	53	5	0.07	93.1
dpy-27	1137	377	33	0.33	75.4
dpy-28(s939)	677	101	5	0.15	86.4
dpy-28(y1)	722	188	7	0.26	78.1
B. Zygotic effects					
Cross: 2A;1X *dpy* males × 4A;4X hermaphrodites					
Father					
dpy+	547	512	0	0.94	
dpy-21	952	832	10	0.87	5.2
dpy-26	284	259	3	0.91	2.9
dpy-27	729	656	10	0.90	4.1
dpy-28 (s939)	603	390	9	0.65	21.3
dpy-28(y1)	1073	714	37	0.67	23.2

[a] Sex ratio is the ratio of males to hermaphrodites.
[b] Feminized % is the estimated percentage of chromosomal males that show either sex reversal or intersexual development.

produced by the tetraploid father. Two animals (of 1056 cross-progeny examined) were somatically normal males that had both sperm and oocytes in the germline, and were therefore classified as intersexes. Intersexes in which only the germline is feminized have been seen at low frequency in nearly all of the polyploid matings, particularly among the last progeny of an hermaphrodite, suggesting that rare 3A;2X animals are germline intersexes. Nonetheless, these intersexual animals are a very small fraction of the progeny, and the sex ratio among sexually normal animals is 9.25 males:1 hermaphrodite. Therefore, the two X chromosomes in the 4A;2X male most often disjoin from each other, but X chromosome non-disjunction is more frequent during spermatogenesis in 4A;2X males than during gametogenesis in 2A;2X hermaphrodites.

The cross between these same 4A;2X tetraploid males and any of the dosage compensation *dpy* mutants gives dramatically different results than are seen with *dpy-5* (Table 1A). When tetraploid males are mated to *dpy-21* hermaphrodites, only 55% of the progeny are males and the sex ratio of normal male to normal hermaphrodite is 1.32. This result suggests that many *dpy-21*/+/+ 3A;2X animals develop as hermaphrodites.

The result with *dpy-21* is actually the least dramatic case of sex reversal among the dosage compensation mutants. For all of the other dosage compensation mutants, the vast majority of the *dpy*/+/+; 2X progeny are apparently developing as hermaphrodites (Table 1A). In the extreme cases, with *dpy-26* and *dpy-28 (s939)*, 90% or more of the progeny are hermaphrodites and the sex ratio is 0.15 or less. These results imply that most of the 3A;2X *dpy-26*/+/+, *dpy-27*/+/+, and *dpy-28*/+/+ animals are developing as hermaphrodites rather than males.

In addition to the apparent sex reversal, about 3% of the apparent 3A;2X male cross-progeny from the *dpy-21* hermaphrodites are intersexual (Table 1A). These intersexes were of two types: in 18 of 26 intersexes only the germline was feminized, while in 8 of 26 intersexes both the germline and soma were feminized. The intersexes of both types have sperm and ova in the germline in the typical hermaphrodite pattern: sperm are made first, then oogenesis begins. These intersexes often have embryos suggesting that these gametes can fertilize each other. Both types of intersexes are also seen among the 3A;2X progeny of the other dosage compensation mutants. Although the numbers are fairly small, there did appear to be differences in the type of intersexes the mutants produced. Both *dpy-26* and *dpy-28 (s939)* gave exclusively intersexes with both the germline and soma transformed (a total of 24 intersexes observed including some animals from experiments not represented in Table 1). For *dpy-27* (28/33) and *dpy-28 (y1)* (5/7), the majority of the intersexes had only the germline transformed. The reason for this apparent difference in frequency of the various types of intersexes is not known.

The putative 3A;2X hermaphrodite progeny are indistinguishable from their triploid 3A;3X hermaphrodite siblings so the conclusion that the skewed sex ratio comes from sex reversal of 3A;2X animals is an inference. However, it is unlikely that the skewing of the sex ratio is due to preferential death of the 3A;2X progeny, since the broods from these matings had more than 100 offspring and no increased embryonic lethality was observed. Since as much as 90% of the progeny are expected to be 3A;2X, the preferential death of these animals would have easily detected. It is also worth noting that the feminization of 3A;2X animals for *dpy-28* alleles follows the pattern seen with the severity of the two *dpy-28* alleles in diploids; the weaker allele *y1* results in fewer sex-reversed progeny than does the more severe allele *s939*. This result is consistent with the interpretation that the diploid phenotypes and the feminization in triploids are due to the absence or reduction of the same function.

Effect of dosage compensation mutants on 3A;2X triploids: *Paternal inheritance:* All of these dosage compensation *dpy* mutants are recessive in their effects on X-linked gene expression and viability, yet the feminization is occuring in *dpy*/+/+ animals. In order to determine if this is due to a partially dominant zygotic effect or to a maternal effect, *dpy*/+/+;2X animals were constructed in which the *dpy* mutant allele was inherited from the father. The results are shown in Table 1B. In the control mating of wild-type N2 males to tetraploid strains, 48% of the progeny were males (presumably 3A;2X) and 52% were hermaphrodite (Table 1B). The

same cross, using the same tetraploid strain for the mother, was done with each of the *dpy* males. If the effect of the *dpy* mutants were due to dominant action in the zygote, the results would be similar to the effect seen in Table 1A, and a preponderance of hermaphrodite progeny would arise. If instead the effect of the *dpy* mutants were due to maternal effects, no distortion of the sex ratio would be expected. The results (Table 1B) indicate that the effect of *dpy-21*, *dpy-27* and *dpy-26* on 3A;2*X* animals is largely the result of a maternal effect; the sex ratio is virtually the same in these matings as in the control cross. However, the mutants do show a small zygotic dominant effect in that a few cross-progeny develop as germline intersexes. *dpy-28* mutants give a somewhat different result, reducing the sex ratio from 0.94 to 0.65–0.67. In addition, intersexes were seen for both mutants. Thus, *dpy-28* mutants do have a slight dominant feminizing effect in triploids, although it is much less severe than the feminization arising from maternal effects. The two alleles of *dpy-28*, which differ in their viability in diploids and in the maternal effect in triploids, do not differ markedly in this dominant zygotic effect.

The phenotypic effects of these mutants in diploids is largely restricted to 2*X* and 3*X* animals (HODGKIN 1983; MENEELY and WOOD 1984; PLENEFISCH *et al.* 1989). These results clearly show that the loss of any of these gene functions in the mother's gonad or ova results in most 3A;2*X* animals developing as hermaphrodites; that is, when the *dpy* gene product is absent maternally, a *dpy*/+/+;2*X* animal develops like a 2A;2*X* or 3A;3*X* animal.

Effect of *X* chromosome duplications on 3A;2*X* triploids: *Paternal inheritance:* MADL and HERMAN (1979) determined that several different *X* chromosome duplications affected sex determination in 3A;2*X* triploids. I have repeated and expanded these results with the duplications shown in Figure 2.

The basic experiment is to mate males heterozygous for an *X* chromosome duplication to tetraploid hermaphrodites, and to examine the male and hermaphrodite cross-progeny for skewing of the sex ratio or for intersexual animals. Since the duplications originate from the *X* chromosome and therefore might affect the sex ratio via meiotic effects on *X* chromosome disjunction, a series of control matings of the duplication-bearing males to *dpy-11; unc-3* diploid hermaphrodites. In all cases, meiotic effects in the *Dp*/+;*XO* male were slight and about half of their cross-progeny are males (MADL and HERMAN 1979; data not shown).

To test the effects of these duplications on 3A;2*X* males, duplication-bearing diploid males (*e.g.*, *mnDp8*/+; *unc-3*/0) were mated to *dpy-11; unc-3* tetraploids or to *dpy-5; unc-3* tetraploids, and the presence of the duplication monitored by the *X*-linked recessive marker *unc-3*. All classes of non-Dpy cross-progeny were counted, and all of the apparent male cross-progeny were examined for evidence of intersexual development. The data are given in Table 2A.

TABLE 2

***X* chromosome duplications cause sex reversal of 3A;2*X* animals**

Duplication	Hermaphrodites	Males	Intersexes	Sex ratio	Feminized (%)
A. Cross: *Dp*/+;*unc-3*/0 males × *dpy;lunc-3* 4N hermaphrodites					
mnDp8	894	588	26	0.66	22.5
mnDp27	467	294	20	0.63	24.0
mnDp9	483	34	9	0.07	86.8
mnDp25	1589	140	22	0.09	84.1
mnDp10	337	74	10	0.22	67.0
B. Cross: *Dp*/+;*XO* males × *dpy-11* 4N hermaphrodites					
mnDp25	261	111	10	0.10	83.2
mnDp57	363	227	24	0.40	52.6
stDp2	182	114	9	0.36	50.0
mnDp33	505	443	1	0.77	13.1
C. Cross: 4A;2*X* males × *Dp/Dp;XX* hermaphrodites					
None	94	608	2	6.47	
mnDp8	185	365	1	1.97	23.5
mnDp25	95	179	1	1.88	24.8
mnDp57	190	245	2	1.29	35.3

Clearly all of the duplications had an excess of hermaphrodite progeny, as indicated by sex ratios of less than 1, and *mnDp9*, *mnDp25* and *mnDp10* produced a great excess of hermaphrodite progeny, as reported previously (MADL and HERMAN 1979). Based on a sample smaller than mine, MADL and HERMAN suggested that *mnDp8* and *mnDp27* had little feminizing effect, although their data for *mnDp27* do show an excess of hermaphrodite cross-progeny. My results suggest that all of these duplications cause sex reversal of some or many 3A;2*X* animals. In addition to effects on the sex ratio, all of the duplications also produced intersexual progeny. This had been previously reported for *mnDp9*, *mnDp10* and *mnDp25*, but intersexes were not seen with *mnDp8* or *mnDp27* previously, again possibly because of the smaller sample size or because not all malelike progeny were examined by Nomarski microscopy.

If the production of intersexes and the skewing of the sex ratio are consequences of the same effect of the duplications (as seems likely), the total feminizing effect of each duplication can be estimated. This estimate assumes that 3A;3*X* progeny and 3A;2*X* progeny from this mating are approximately equally frequent (as indicated by measurements in strains without rearrangements; note Table 1B). Therefore, of the 1508 cross-progeny carrying *mnDp8*, 759 of the 1508 progeny carrying *mnDp8* are expected to be 3A;2*X* animals and 749 are expected to be 3A;3*X*. [This estimate takes into account a slight meiotic effect of *mnDp8* (not shown) and assumes no difference in viability between 3A;2*X* + *mnDp8* and 3A;3*X* + *mnDp8* which is considered below.] Thus, of the expected 759 3A;2*X* animals, 171 (759 minus the observed 588 males), or more than 22%, were feminized by the duplication, with about 3.4% showing intersexual properties and about 19% actually develop-

ing as hermaphrodites. Similar estimates were made for all of these duplications and are tabulated in the rightmost column of Table 2A. Both *mnDp8* and *mnDp27* affect more than 20% of the putative 3A;2*X* progeny, while both *mnDp9* and *mnDp25* affect more than 80% of the 3A;2*X* progeny. Crosses with *mnDp10* gave inconsistent results and were not pursued further (see MATERIALS AND METHODS).

One alternative explanation for the skewed sex ratio is that 3A;2*X* animals carrying an *X* chromosome duplication die more frequently than do 3A;3*X* animals carrying the same duplication. Because polyploid hermaphrodites have a variable number of inviable progeny, this possibility is hard to rule out conclusively. However, if embryonic lethality were common, one expects to see many fewer cross-progeny with the duplication than without the duplication. MADL and HERMAN (1979) presented data that argued against preferential death of duplication-bearing triploids, which I have repeated with similar conclusions. For example, progeny that do not inherit the duplication are Unc; if the duplication reduces the viability of one or both classes of triploid progeny, Unc progeny would outnumber non-Unc progeny. For *mnDp8*, there were 673 viable Unc male progeny and 702 Unc hermaphrodite progeny, or 1375 total viable triploid progeny that did not inherit *mnDp8*, compared to 1508 triploid progeny that inherited *mnDp8*. Clearly *mnDp8* did not reduce the viability of the triploids by this measure; in fact, there was actually a surplus of viable progeny carrying *mnDp8* (consistent with data from MADL and HERMAN). Note also that the sex ratio among animals that did not inherit the duplication (673/702 = 0.96) is about the same as in crosses in which no duplication was used (512/547 = 0.94 in Table 1B), and much higher than among animals with the duplication. A similar analysis with similar results was done for all of the duplications tested in Table 2A. In all cases, the non-Unc cross progeny were as frequent as or more frequent than the Unc progeny from the same cross, indicating that the duplications tested do not significantly reduce the viability of 3A;2*X* animals (other data not shown).

Other *X* chromosome duplications that do not include *unc-3*+ were also tested for their ability to cause feminization, and the results shown in Table 2B. In this case, a tetraploid hermaphrodite homozygous for the autosomal marker *dpy-11 V* but with no *X*-linked marker was used as the mother. This permitted additional regions of the *X* chromosome to be assayed for feminizing effects. Since the inheritance of the duplication in the triploid progeny could not be directly monitored, the segregation of each duplication was measured in diploids and used to calculate the expected results in tetraploids. To show that this approach, though indirect, could be used, control crosses were done with *mnDp25*, and the results compared to those above (Table 2B). When *mnDp25/+; unc-3/0* males were mated to *dpy-11* tetraploid hermaphrodites, 382 cross-progeny were observed. From crosses with *mnDp25* in diploids, 191 of these progeny are predicted to have the duplication and 191 are predicted to lack the duplication. Each class is predicted (again from the segregation in diploids) to include 95 males and 96 hermaphrodites. Thus, of the observed 111 males, 95 were probably 3A;2*X* animals that did not inherit *mnDp25* which means that 16 were probably 3A;2*X* animals that inherited *mnDp25*. Therefore, of the predicted 95 3A;2*X* + *mnDp25* animals, 16 apparently were males, 10 were apparently intersexual, and the remaining 69 were probably hermaphrodites. Thus, 79 of the expected 95 3A;2*X*+ *mnDp25* animals were feminized, or 83.2%. When the chromosomes were more directly monitored in the cross previously, 83.9% of the 3A;2*X* + *mnDp25* animals were feminized; the similar results from the two methods shows that these inferences about the progeny ratios of these matings were appropriate. Similar estimates with *mnDp8* indicated that 28.4% of the 3A;2*X* + *mnDp8* progeny were feminized (data not shown), again a similar estimate to what was seen previously.

The same procedure was used to estimate the feminization caused by three duplications that were not tested previously. For *mnDp57* and *stDp2*, half or more of the predicted 3A;2*X* + *Dp* animals apparently were sex-reversed and for the duplication *mnDp33* about 13% of the 3A;2*X* + *Dp* animals were feminized. These numbers are indirect estimates, but they do suggest that all of the duplications tested cause feminization of 3A;2*X* animals. MADL and HERMAN (1979) concluded that there must be at least four different feminizing sites on the *X* chromosome; my results imply at least two and (if the overlapping duplications *mnDp33* and *mnDp57* are different in their ability to feminize, as the estimates suggest), probably three more feminizing sites. More significantly, all eight duplications tested feminized 3A;2*X* animals.

The ability of these duplications to feminize 3A;2*X* animals is roughly correlated with the genetic size of the duplicated region, but there are exceptions that may be informative. For instance, although *mnDp57* and *stDp2* have similar effects on 3A;2*X* animals, *stDp2* includes a smaller fraction of the *X* chromosome genetic map (Figure 2), suggesting that the correlation between feminization and genetic size does not hold equally well for all regions of the *X* chromosome. Even more noteworthy is the effect of *mnDp25* and *mnDp9*, which are the largest and include a large interval to the right of the gene *unc-9* (Figure 2), cause most 3A;2*X* animals to develop as hermaphrodites.

Effect of *X* chromosome duplications on 3A;2*X* triploids: *Maternal inheritance:* When inherited from the father, all of the duplications feminized some 3A;2*X* animals. Three duplications were also tested for maternal

effects by constructing 3A;2*X* + *Dp* animals which inherited the duplication from the mother. To do this, 4A;2*X* males were mated to diploid hermaphrodites homozygous for the duplication, *i.e.*, to *mnDp8/mnDp8; dpy-7 unc-3* hermaphrodites. Since the mother is homozygous for the duplication, all of her progeny must be heterozygotes. Thus the phenotype of the non-Dpy non-Unc cross-progeny was scored. The results are shown in Table 2C. In the control mating of 4A;2*X* males to *dpy-7 unc-3* hermaphrodites, 704 cross-progeny were counted, of which 94 were hermaphrodites, 2 were intersexes and 608 were males; as before (Table 1A), the hermaphrodites are apparently 3A;3*X* animals that arise from *X* chromosome non-disjunction in the 4A;2*X* father. Thus, based on the control cross, 13.4% of the progeny of the mating are expected to be 3A;3*X* and intersexes are rare. With *mnDp8* inherited from the mother, 551 cross-progeny were observed: 185 hermaphrodites, 365 males, and 1 intersex (Table 2C). Since 13.4% of these cross-progeny (74 progeny total) are expected to be 3A;3*X*, the mating gave (185 − 74 =) 111 hermaphrodites more than predicted. These are presumed to be sexually reversed 3A;2*X* + *mnDp8* animals. Therefore of the predicted total of 477 3A;2*X* + *mnDp8* animals, 365 were males, 1 was an intersex, and 111 probably developed as hermaphrodites; in other words, 112/477 (23.5%) of the presumed 3A;2*X* progeny were feminized. This is similar to the fraction of 3A;2*X* animals that are feminized when *mnDp8* is inherited from the father, and suggests that the duplication does not have a maternal effect. The same experiment was done using *mnDp25; dpy-7 unc-3* hermaphrodites and *mnDp57; unc-2 dpy-7* hermaphrodites and the results compared to inheriting these duplications from the father. Neither *mnDp25* nor *mnDp57* had a maternal effect on the feminization of 3A;2*X* progeny. In fact, many fewer progeny were apparently feminized when *mnDp25* was inherited maternally than when it was inherited paternally. The reason for this difference is not known.

In summary, all eight *X* chromosome duplications tested reduced the expected sex ratio in triploid progeny. In crosses in which the chromosomes could be monitored, this effect is apparently due to sex reversal by the duplications such that some or many 3A;2*X* + *Dp* animals develop as hermaphrodites. Duplications differed in the frequency of feminized progeny. This effect correlates with both the larger size of some duplications and the regions duplicated. There may be a region to the right of the *unc-9* locus (duplicated in both *mnDp25* and *mnDp9*) that causes many progeny to develop as hermaphrodites; the *stDp2* duplication also feminized a greater percentage of the animals than predicted from genetic size alone, suggesting some locus that it duplicates also may have a major effect on sex determination in 3A;2*X* animals. None of three duplications tested showed an obvious maternal effect.

TABLE 3

Combinations of *X* chromosome duplications tested for effects in diploid males

Duplication 1	Duplication 2	Percent *X* map
mnDp57	*stDp2*	35
mnDp57	*mnDp8*	40
mnDp57	*mnDp27*	45
mnDp57	*mnDp25*	50
mnDp57	*mnDp9*	50
mnDp57	*mnDp10*	70
stDp2	*mnDp8*	35
stDp2	*mnDp25*	45

Diploid males are not feminized by combinations of *X* chromosome duplications: Although none of the *X* chromosome duplications alone can affect sex determination in diploid males (HERMAN *et al.* 1979), a combination of these duplications might be predicted to affect diploid males. This was tested by making diploid males with two different *X* chromosome duplications (see MATERIALS AND METHODS). Seven duplications, representing more than 80% of the *X* chromosome genetic map, were tested in eight different combinations (Figure 2 and Table 3). The smallest combination of these duplications adds a third of an *X* chromosome, while the largest pair adds more than two-thirds of an *X* chromosome to the *X*O male. In each case, more than 150 males carrying both duplications were examined. Although occasional animals were small and stunted, perhaps suggesting defects in dosage compensation, all of the animals were sexually normal males. Thus, even a combination of duplications representing more than two-thirds of an *X* chromosome does not feminize diploid males.

Three *X*-linked genes, *xol-1*, *sdc-1* and *sdc-2*, have been implicated in the control of sex determination and dosage compensation (VILLENEUVE and MEYER 1990). None of these loci is dosage-sensitive by itself, as demonstrated by the normal development of hermaphrodites with duplications and deficiencies for the genes, and males with duplications of *xol-1*+ and *sdc-1*+. The experiments with the duplications here also rule out any effects of varying the dose of *xol-1*+ (present on *stDp2*) and *sdc-1*+ (present on *mnDp8*) simultaneously. *sdc-2*+ is apparently not duplicated by *mnDp10* or any of the other duplications (K. TANNER and W. B. WOOD, personal communication; B. MEYER, personal communication), so it is formally possible that duplicating *sdc-2*+ and another of the known *X*-linked sex determination and dosage compensation loci could have effects in diploid males. It is also possible that a high copy number array of these genes could affect sex determination or dosage compensation; this was not seen for *sdc-1* (NONET and MEYER 1991).

TABLE 4

Injected DNA and 3A;2X triploids

Cross: 2A;1X male × injected 4A;4X hermaphrodite			
Injected DNA	A^a	B^b	C^c
None	1/18	1/443	
pVZ1	1/15	1/344	
pCeA130	9/15	39/509	39/398
p64VZ	30/36	107/1135	107/1062
p36VZ	0/10	0/350	

[a] The fraction of injected hermaphrodites with intersexual progeny.

[b] The fraction of intersexes among all of the male progeny of all matings.

[c] The fraction of intersexes among the male progeny with at least one intersex.

Microinjected DNA feminizes triploid males: McCOUBREY *et al.* (1988) used microinjection in an attempt to define feminizing elements on the *X* chromosome, an approach analogous to that with *X* chromosome duplications. Briefly, DNA was injected into the gonad of a genetically marked tetraploid hermaphrodite, and the injected animal was mated with diploid males to produce 3A;2*X* and 3A;3*X* progeny (see MATERIALS AND METHODS) We previously used *mnDp8* males in this mating but, given the effects of *mnDp8* itself on sex determination, wild-type males with no *X* chromosome rearrangement were used instead (see MATERIALS AND METHODS). In these experiments, the appearance of intersexes was taken as an indicator of feminization; effects on the sex ratio are considered below. Uninjected hermaphrodites rarely give intersexes (Table 4, line 1). Hermaphrodites injected with a 131-bp segment (in the plasmid pCeA130) from the first intron of the *X*-linked gene *act-4* produced some feminized progeny (Table 4, line 3) (McCOUBREY *et al.* 1988). The majority of injected hermaphrodites have intersexual progeny; furthermore, in a brood with an intersex, about 10% of the males are intersexual. We also previously recognized an eight base sequence that is present in three different *X*-linked feminizing elements including *act-4* (McCOUBREY *et al.* 1988). Regions of the *act-4* intron smaller than the 131 bp inserted in pCeA130 were tested (Table 4; and P. MENEELY, W. McCOUBREY and S. ROBERTSON, unpublished). The plasmid p64VZ with an insert of 64 bp including the octamer (Figure 4) also results in about 10% of the triploid male progeny developing as intersexes; again, most of the injected animals have intersexual progeny (Table 4, line 4).

Two negative controls show that the sequence inserted in p64VZ is responsible for feminization. pVZ1 is the plasmid vector used in making the subclones, and p36VZ contains a different region of the insert from pCeA130 (Figure 4), and neither is capable of feminizing 3A;2*X* animals (Table 4). Intersexes do arise occasionally in these experiments, typically as a single offspring of an injected hermaphrodite, but this can be readily distinguished from the results with pCeA130 and p64VZ (Table 4). This confirms and extends our previous work on the *X*-linked feminizing element, showing that only 64 of the 131 bp from *act-4* are needed to feminize triploid males.

The injected feminizing element was also used to ask if the paternal chromosomes (and thus the zygotic genome) play any role in sex determination of these polyploid strains. To test the role of the sperm genome, tetraploid hermaphrodites were injected with p64VZ as before; however, instead of mating the injected hermaphrodites with diploid males to produce triploid 3A;2*X* progeny, the injected animals were mated with 4A;2*X* tetraploid males. The resulting male progeny are 4A;2*X*, having arisen from 2A;2*X* ova from the mother and 2A;0*X* sperm from the father. [The more common 2A;1*X* sperm from the tetraploid father will produce 4A;3*X* progeny, which are hermaphrodites.] All of these tetraploid animals (of 205 examined) were sexually normal males. Thus, tetraploid males are not feminized by feminizing element DNA. This confirms that the autosomal genome in the zygote also plays a role in sex determination of polyploid animals.

Although the overall effects of the injected feminizing elements were similar to the effects of duplications and dosage compensation mutants, the sex ratio among animals with injected feminizing elements was not markedly skewed from the expected value in these experiments. For example, for p64VZ-injected animals in Table 4, the broods that had an intersex were comprised of 1210 hermaphrodites and 1062 males. This was not significantly different from the broods among uninjected animals or from the ratio in broods with no intersexes (data not shown). Previously, when multiple clones were injected, skewed sex ratios were seen in some broods, although it was not clear if this was due to embryonic death or to the effects of the feminizing elements (McCOUBREY *et al.* 1988).

Stable extrachromosomal arrays of a feminizing element also feminize 3A;2X triploids: Additional experiments were done by using stable diploid lines that carry a plasmid with the autosomal dominant marker *rol-6* and the feminizing plasmid p64VZ as an extrachromosomal array (see MATERIALS AND METHODS). This was done, in part, to compare more directly the effects of injected feminizing elements with the effect of *X* chromosome duplications. The duplications are introduced paternally and therefore must act in the zygote, whereas the feminizing element is introduced maternally and could act zygotically, maternally, or both. I first note that diploid males with p64VZ as an array are sexually normal and fertile, demonstrating that this array does not affect 2A;1*X* males (see below). When these males were mated with tetraploid hermaphrodites, 16 of 241 (6.6%) of the 3A;2*X* cross-progeny males were intersexual, indicating

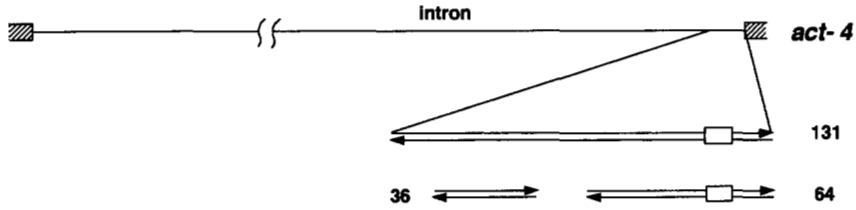

FIGURE 4.—A schematic map of the first intron of the *X*-linked *act-4* gene. The feminizing element lies near the 3′ end of this intron. The open box shows the location of the conserved octamer 5′TTTCAATA 3′. The 131-bp fragment was the insert for pCeA130. Two oligonucleotides corresponding in sequence to the regions shown were synthesized, of 36 and 64 bp, and were used as the insert of p36VZ and p64VZ, respectively.

that the stable arrays can feminize triploids. This mating produced 284 cross-progeny hermaphrodites in addition to the 241 males, showing that the sex ratio is again largely unaffected. Diploid males carrying the *rol-6* plasmid alone do not sire intersexual progeny when crossed with tetraploids (0/360 males), so feminization depends upon sequences present in p64VZ. The presence of intersexual progeny sired by males with a feminizing element as an extrachromosomal array, together with the injection experiments, indicate that the feminizing element affects sex determination in triploids regardless of whether it is introduced maternally or paternally. However, the frequency of intersexes does appear to be lower from mating than from injection. The lower frequency of intersexual progeny may reflect the inherent instability of an extrachromosomal array, or it may indicate that some feminization occurs maternally as well.

Threshold effect of the feminizing element in triploid males: A simple interpretation of the injection results is that the injected DNA is competing with the *X* chromosome for some *X* chromosome-specific component present in limited amounts. This possibility was tested by injecting tetraploid hermaphrodites with p64VZ at a range of concentrations. The results are shown in Table 5. Concentrations of p64VZ ranging from about 1 μg/ml to about 10 pg/ml are equally effective in feminizing triploid males, with each concentration resulting in roughly 15–20% intersexes among those broods with an intersex (Table 5). Most of the injected hermaphrodites had intersexual progeny, and these animals had an average of about four intersexual offspring. This consistent frequency indicates that injecting additional copies of the feminizing element p64VZ above 10 pg/ml does not have cumulative effects, suggesting that the amount of the feminizing element itself is not limiting. However, at plasmid concentrations of 1 pg/ml or less, very few intersexual animals are seen, similar to uninjected control animals. These experiments show that the threshold from a feminizing to a non-feminizing concentration of p64VZ is abrupt. They further show that very small amounts of p64VZ are needed to feminize 3A;2*X* males. From injections of radiolabeled material, I estimate that injecting at a concentration of 10 pg/ml under my conditions is equivalent to 500–5000 additional copies of p64VZ in the mother's gonad (see MATERIALS AND METHODS).

TABLE 5

Titration of the injected feminizing element p64VZ

Concentration injected (per ml)	A^a	B^b	C^c	D^d
1 μg	5/8	29/243	29/178	16.3
100 ng	12/14	31/162	31/156	19.9
10 ng	16/22	62/436	62/431	18.2
100 pg	4/8	16/128	16/83	19.3
10 pg	4/6	17/135	17/108	15.7
1 pg	3/14	3/210	3/126	2.4
0.1 pg	1/9	1/96	1/45	2.5

[a] The fraction of injected hermaphrodites with intersexual progeny.
[b] The fraction of intersexes among all of the male progeny of all matings.
[c] The fraction of intersexes among the male progeny of broods with at least one intersex.
[d] The percent of intersexes in broods with at least one intersex.

Injected DNA does not affect sex determination in diploid males: If an injected feminizing element were a dosage-sensitive numerator element of the *X*/A ratio, it may be expected to show effects when injected at high concentrations in diploids. This postulate was tested by injecting p64VZ into diploids at 10 μg/ml, or six orders of magnitude more than what is needed to feminize a triploid male. The injected diploid animals were mated with wild-type diploid males and the diploid male cross-progeny examined for intersexual development. More than a thousand males were examined, and no intersexes were observed. Therefore, an injected feminizing element does not affect diploid sex determination even at very high concentrations.

This experiment was repeated by injecting p64VZ at either 1 μg/ml or 10 μg/ml into diploid strains carrying the duplications *mnDp25* or *mnDp8*. These injected hermaphrodites were mated with wild-type diploid males and their wild-type male cross-progeny examined. Again, no intersexes were seen among more than a thousand males examined. This indicates that diploid males cannot be feminized even with a combination of injected feminizing elements and an *X* chromosome duplication.

To summarize the microinjection experiments, intersexual progeny were seen whenever a 64-bp feminizing

element was introduced into 3A;2*X* animals either by microinjection or by mating. The production of intersexual progeny clearly depended on the presence of the 64-bp feminizing element. These results demonstrate that the feminization could occur zygotically, although they do not rule out an additional maternal effect. In fact, the very low concentration needed to produce feminized progeny suggests that the effect can occur in the mother's gonad. The feminization in triploid animals had a threshold in that injecting less than 10 pg/ml (an estimated 500–5000 copies of the feminizing element) did not cause feminization whereas any concentration above that produced similar numbers of intersexual animals. Tetraploid males were not feminized, and no feminization was seen with diploid males even when the feminizing element was injected at a very large excess.

DISCUSSION

Triploid animals with two *X* chromosomes are males, but can be feminized to become either intersexes or hermaphrodites by at least three different experimental treatments: dosage compensation *dpy* mutants (HODGKIN 1987a; PLENEFISCH *et al.* 1989); *X* chromosome duplications (MADL and HERMAN 1979); and microinjected "feminizing element" DNA sequences (MCCOUBREY *et al.* 1988). My data on these treatments can be summarized as follows.

Dosage compensation mutants: All of the dosage compensation *dpy* mutants tested, in the genes *dpy-21*, *dpy-26*, *dpy-27* and *dpy-28* (two alleles), caused a maternal effect sex reversal of many 3A;2*X* animals. That is, when the *dpy+* gene function is absent from the diploid mother, many of her 3A;2*X* progeny are hermaphrodite rather than male. Some 3A;2*X* animals are also intersexual, but these are a small minority compared to the sex-reversed animals. The dosage compensation *dpy* mutants, which are recessive in their effects on dosage compensation, show a slight dominance in their effect on sex determination in 3A;2*X* animals; this is most pronounced, but still fairly minor, with the *dpy-28* mutants. The maternal effect of *dpy-26*, *dpy-27* and *dpy-28* mutants on sex determination is not unexpected, since all of these mutants also show maternal effects on dosage compensation and viability in diploids. The frequency of sex reversed triploid animals for each mutant correlates with the severity of the dosage compensation phenotype in diploids, suggesting that these different phenotypes arise from the deficit or absence of a common gene function. None of these mutants affects sex determination in diploid males, even in double mutant combinations (PLENEFISCH *et al.* 1989). Instead, the obvious mutant phenotypes for these genes are seen only in animals with two or more *X* chromosomes.

The sex reversal phenotype of the dosage compensation *dpy* mutants means that triploid *dpy*/+/+; 2*X* animals have the sexual phenotype of diploid +/+;2*X* animals. This might imply that one or more of these genes constitutes a denominator element in the *X*/A ratio. However, in the simplest models for the *X*/A ratio, one expects denominator elements to have dose-sensitive effects in diploids, which these mutants do not have. Instead, it seems likely that the sex determination defect arises from the elevated *X*-linked gene expression characteristic of dosage compensation mutant strains. Exactly how a disruption of dosage compensation might also affect sex determination is unknown, but has been considered by DELONG *et al.* (1993) in their discussion of the mutant *sdc-3*. These authors propose a feedback between the level of *X*-linked gene expression and an early step in the sex determination pathway. Another possibility is that these genes may be involved with the assembly of some *X* chromosome specific chromatin conformation during oogenesis. When that conformation is disrupted, fundamental properties of the *X* chromosome such as dosage compensation and sex determination are also disrupted. The interpretation that the mutant phenotype arises from disruptions in *X*-specific chromosome structure is similar to what has been seen with *X* chromosome structure in both Drosophila (LUCCHESI and MANNING 1987; TURNER *et al.* 1992) and mammals (JEPPESON and TURNER 1993), and what has been inferred from experiments with dosage compensation mutants in Drosophila (KURODA *et al.* 1991; GORMAN *et al.* 1993; PALMER *et al.* 1993) (reviewed by HENIKOFF and MENEELY 1993). However, there is no direct evidence for *X* chromosome-specific structure in *C. elegans* hermaphrodites, and the molecular functions of these genes are not yet known.

***X* chromosome duplications:** The original experiments by MADL and HERMAN (1979) used *X* chromosome duplications to attempt to understand how the *X*/A ratio was read. I have expanded and reinterpreted some of this work as follows. All eight *X* chromosome duplications tested (representing at least six different non-overlapping regions of the *X* chromosome) cause some or most 3A;2*X* animals to develop as hermaphrodites rather than males. The frequency of sex reversal ranges from about 10% of the 3A;2*X* animals becoming hermaphrodite to more than 80% of the animals being sex reversed. At least two effects seem to be at work. First, large duplications (as measured by genetic map distance) cause sex reversal more frequently than small duplications; this effect is consistent with some feminizing property being widespread on the *X* chromosome, as hypothesized by MADL and HERMAN (1979). Second, different regions of the *X* chromosome also differ in the fraction of sex reversed animals: that is, the correlation between sex reversal and genetic size is imperfect. In particular, the region duplicated by *stDp2* results in more sex reversed animals than expected from size alone, and both *mnDp25* and *mnDp9* result in virtually all hermaphrodites. Contrary to expectations, no maternal

effects on sex determination were seen with any of the three duplications tested.

Microinjected feminizing element DNA: McCoubrey *et al.* (1988) showed that certain DNA sequences associated with *X*-linked genes can also cause intersexual development of some 3A;2*X* animals. These sequences have been termed "feminizing elements," without hypothesizing how they might act. The molecular means by which a feminizing element produces intersexes in triploids is not known, although the sequence has been shown to be a protein binding site *in vitro* (S. Robertson, W. McCoubrey and P. Meneely, unpublished). Several additional aspects of the effect of feminizing elements are demonstrated in this work.

First, unlike the effect of the dosage compensation mutants and the duplications, the injected feminizing element causes intersexual development rather than sex reversal. Although occasional broods of injected animals are observed that are predominantly hermaphrodites (McCoubrey *et al.* 1988; P. Meneely, unpublished), the more consistent effect is the production of intersexual animals. The broods that are predominantly hermaphrodites could be the result either of preferential death of male progeny or of sex reversal of the male progeny. However, these broods are not common, and the overall sex ratio is not dramatically different from that in uninjected animals. The reason that the feminizing element causes intersexes while duplications and dosage compensation mutants cause sex reversal is unclear, although it may reflect meiotic and/or mitotic instability of the feminizing element DNA. It may also indicate that the feminizing element DNA can reproduce only part of the effect seen with duplications and dosage compensation mutants or even that the feminizing element affects sexual differentiation via a different pathway altogether.

Second, very little feminizing element DNA needs to be injected in order to produce feminization. The usual DNA concentration for microinjection is 50–100 μg/ml (Mello *et al.* 1991), but this concentration is 10^5 higher than what is needed to produce intersexes. All concentrations greater than 10 pg/ml gave the same frequency of intersexual progeny, indicating that the number of copies of feminizing element is not limiting. However, injections with concentrations lower than 10 pg/ml gave few or no intersexes. It has been suggested that injected DNA at a concentration of less than 10–50 μg/ml is not inherited (Mello *et al.* 1991). If this is so for the feminizing element p64VZ, the effect of the feminizing element at these low concentrations is probably due to effects within the gonad of the mother. Perhaps the DNA is competing for some protein present in the mother's gonad in a limited excess over its binding sites, so that injecting additional copies of the binding sequence inhibits the protein from binding to its preferred genomic sites. If the binding protein has a role in *X* chromosome structure, binding in the mother's gonad may alter some aspect of chromosome structure and/or very early *X*-linked gene expression. However, the feminizing element can also be introduced via male sperm and again causes feminization. This indicates that the feminizing element can work zygotically as well as maternally.

Reexamination of the X/A ratio: 3A;2*X* animals are usually males, but show intersexual or hermaphrodite development when *X* chromosome duplications or microinjected feminizing element DNA are present or when any of the dosage compensation gene products are lacking in the mother. The intersexuality of these triploids has been understood to represent the effect of an ambiguous signal for sex determination (Madl and Herman 1979; McCoubrey *et al.* 1988; Villeneuve and Meyer 1990). *X* chromosome duplications and feminizing element DNA might then be inferred to constitute or affect important components of a quantitative signal for sex determination with a threshold for male *vs.* hermaphrodite sexual development between an *X*/A ratio of 0.67 and 0.75.

However, since duplications and feminizing elements do not affect sex determination in diploids, the signal for sex determination must not be linear. Most strikingly, pairs of duplications, comprising as much as two-thirds of an *X* chromosome do not affect diploid males. Likewise, a vast excess of injected feminizing element DNA also does not affect diploid males, and double mutant combinations with the dosage compensation mutants do not affect sex determination in *X*O diploids (Plenefisch *et al.* 1989). In all cases, feminized animals have only been observed among progeny with two *X* chromosomes. From these results I infer that hermaphrodite development requires two *X* chromosomes or two copies of some dose-sensitive *X*-linked locus or loci. In other words, the *X*/A ratio either is not a linear signal or it only applies when two or more *X* chromosomes are present.

It is not clear what property two *X* chromosomes might possess besides gene dosage that could affect sex determination, although some hypothetical examples come to mind. One suggestion is that two *X* chromosomes may be capable of somatic pairing or interacting in some way analogous to transvection (Tartof and Henikoff 1991). This (hypothetical) interaction then activates hermaphrodite development. In diploids, two *X* chromosomes are present only in zygotes destined to become hermaphrodites; in polyploids, two *X* chromosomes are present not only in the zygote but also throughout meiosis. Thus, hermaphrodite development may be turned on whenever two *X* chromosomes are present but overridden by autosomal masculinizing factors in triploids and tetraploids. Since the interaction is (postulated to be) a property of the *X* chromosome *per se*, it is possible that no duplication could mimic this effect. This "model" has no support either genetically or cytologically, and is simply offered as a formal possibility.

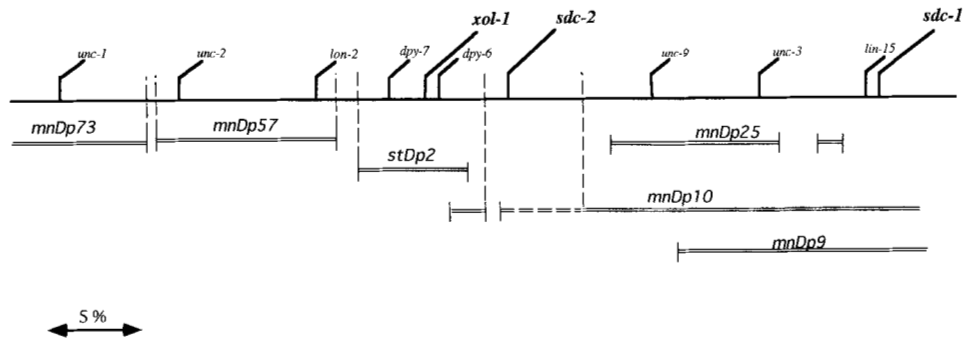

FIGURE 5.—A summary map of the *X* chromosome, showing the duplications that do not affect sex determination in diploid males below the line. Only the largest duplications that cover a particular region are shown. All of these duplications except *mnDp73* have been shown to cause sex reversal of triploid males, and *mnDp73* has not been tested. Regions that are not duplicated are shown with vertical dashed lines, and represent the most likely candidates for regions including dose-sensitive loci. More detailed molecular and cytogenetic analysis of the *X* chromosome and these duplications could reveal other regions that these duplications do not duplicate, which might also contain dose-sensitive loci.

Alternatively, there may be one or more specific but unidentified *X*-linked loci with dose-dependent effects on sex determination. When this locus is present in 2*X* copies, hermaphrodite development can occur; when present in 1*X* copy, only male development can ensue. An *X*-linked locus with dose-sensitive effects on sex determination is a candidate for the numerator of the *X*/A ratio. It is useful to compare my results with what has been seen in Drosophila, where some components of the numerator of the *X*/A ratio are defined. In Drosophila, the *X*-linked dose-sensitive numerator elements *sis-a* and *sis-b* were originally recognized in diploids because duplicating both simultaneously caused male lethality, an effect neither locus had alone (CLINE 1986, 1988). *sis-a* and *sis-b* are positive regulators of the *X*-linked gene *Sxl*, which is essential for female development (CLINE 1984) [reviewed by CLINE (1993) and PARKHURST and MENEELY (1994)]. Duplicating *sis-a* and *sis-b* simultaneously turns on *Sxl* in males, resulting in death (CLINE 1986, 1988).

Since none of these duplications is dose-sensitive in diploids of *C. elegans*, even in combination, the numerator elements must not lie in the regions of the *X* chromosome represented by these duplications. The duplications cover about two-thirds of the known *X* chromosome genetic map so, assuming that a duplication includes a contiguous region of the chromosome without small "holes," the numerator must lie in one or more of the other regions of the *X* chromosome. There are at least two such regions: between the left endpoint of *mnDp10* and the right end of *stDp2*, and distal to the left endpoint of *mnDp57* (Figure 5). This latter region is further subdivided by additional *X* chromosome duplications characterized by HERMAN and KARI (1989). None of these duplications affects sex determination in diploids; in fact, the means by which all of the duplications were isolated would preclude identifying a region with dose-sensitive effects in diploid males (HERMAN *et al.* 1979, 1982; HERMAN and KARI 1989). This argues that the duplications of the region to the left of *unc-2* also do not include any candidates for numerator elements. Thus the regions most likely to contain a numerator element, if one can be identified, are those indicated in Figure 5.

Although the duplications tested here do not appear to include a dose-sensitive locus, they do affect sex determination in triploid males. This may be the result of a general elevation of *X*-linked gene expression in duplication strains (MENEELY and NORDSTROM 1988). A similar explanation could account for the feminizing effect of dosage compensation mutants, which also cause a general elevation of *X*-linked gene expression. Therefore, I postulate that a diploid with one *X* chromosome cannot be feminized by any combination of these duplications because two copies of the (unknown) numerator locus are required. A diploid with two *X* chromosomes is feminized without any other modification of this hypothetical numerator. However, in a triploid with two *X* chromosomes, feminization requires not only two copies of the numerator locus but some other treatment that modifies the *X* chromosome or elevates *X*-linked gene expression such as *X* chromosome duplications, feminizing element DNA, or dosage compensation mutations. This other factor can apparently be supplied either maternally or zygotically.

Although two *X* chromosomes and some modifying factor can feminize triploid males, a feminizing element is not sufficient to feminize tetraploid males. This demonstrates again that there is an autosomal component to the sex determination signal, but it gives no clue as to what this autosomal component might be. One possibility is that the autosomal component (the "denominator" of the *X*/A ratio) is a specific locus. Loss of function mutations at this locus are predicted to feminize (or kill) animals with a male karyotype (PARKHURST and ISH-HOROWICZ 1992; CLINE 1993). No dose-sensitive autosomal locus that affects sex determination has been identified in diploids for *C. elegans* but, as with the *X*

chromosome modifiers, it may be necessary to test the effect in triploids and tetraploids. The autosomal component may also be the dose of a number of different loci jointly, which would be difficult to elucidate. It is also possible that the "autosomal component," which is defined solely by the effect on polyploids, may not be any specific locus but may be some more general effect of polyploidy such as cell or nuclear volume.

In conclusion, I postulate that hermaphrodite development in *C. elegans* requires two copies of the *X* chromosome or of some specific (but undefined) *X*-linked locus. The duplications, dosage compensation mutations and feminizing elements that disrupt sex determination in triploids probably are not themselves direct components of the *X*/A ratio. Instead, these may help to define more general constituents of the *X* chromosome, possibly structural components. Further insight will come from identifying dosage-sensitive regions of the *X* chromosome in diploids and from a molecular understanding of *X* chromosome structure.

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