Identification of a candidate primary sex determination locus, fox-1, on the X chromosome of Caenorhabditis elegans

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SUMMARY

Sex in Caenorhabditis elegans (XX hermaphrodite, XO male) is determined by the X:A ratio, which is the ratio of X chromosome number to autosomal set number. Recent genetic results with X chromosome duplications have suggested that there may be only a small number of major numerator sites on the X chromosome that contribute to this ratio. Mapping of duplication endpoints delimited a region of less than 300 kb, likely to contain one such element. Cosmid clones from this region were tested for numerator activity by constructing transgenic lines carrying extra copies of each tested cosmid. Most cosmid arrays have no effect on the viability of either XX or XO animals. One cosmid array was found to be viable in XX animals, but lethal and feminizing in XO animals, consistent with it containing a major numerator element. Further experiments defined a region of 12-30 kb with apparent numerator activity, which is designated fox-1, ‘Feminizing locus On X’. A cDNA clone hybridizing across part of this region encodes a predicted RNA-binding protein.

Key words: nematode, Caenorhabditis elegans, sex determination, X:A ratio, numerator elements

INTRODUCTION

Sex chromosome counting mechanisms are widespread in the animal kingdom. In many groups, differences in X chromosome dosage are responsible for sex determination. Animals with different X chromosome numbers in the two sexes also usually compensate for the resulting difference in gene dosage, by a process of X chromosome dosage compensation. Proper dosage compensation must also depend on correctly counting X chromosomes. In two well-studied systems, Drosophila melanogaster and Caenorhabditis elegans, both sex determination and dosage compensation are controlled coordinately, by a primary mechanism that measures X chromosome dosage (for recent reviews, see Cline, 1993; Parkhurst and Meneely, 1994). In most mammals, sex determination does not depend on X chromosome dosage, but dosage compensation (X inactivation) does, so here also some kind of counting mechanism must be involved (Grant and Chapman, 1988). The need to assess chromosome number correctly is therefore widespread in the animal kingdom, and represents a challenging general problem: how is the organism able to distinguish between one and two X chromosomes?

The two natural sexes of Caenorhabditis elegans are the self-fertilizing hermaphrodite (essentially a modified female), and the male. Both are diploid, with two sets of five autosomes, but animals with two X chromosomes (XX) develop into hermaphrodites, while animals with one X chromosome (XO) develop into males. The two sexes differ extensively in development, final anatomy and behaviour (for review, see Hodgkin, 1988). They also differ in dosage compensation: XX hermaphrodites down-regulate the expression of most sex-linked genes, equalizing activity with XO males, as assayed both phenotypically and by transcript level (Meyer and Casson, 1986; Meneely and Wood, 1987).

Much effort has been devoted to elucidating the genes involved in sex determination, sexual differentiation and dosage compensation, with the result that a detailed pathway of interacting regulatory genes has been worked out, and is now being subjected to analysis at the molecular level (Hodgkin, 1990; Villeneuve and Meyer, 1990; Kuwabara and Kimble, 1992). Fig. 1 provides an overview of the regulatory genes involved in sex determination and in dosage compensation. The part of the regulatory hierarchy that is least understood is the very first step, the assessment of X chromosome dosage. This paper provides a molecular step forward in understanding how C. elegans is able to tell the difference between XX and XO karyotypes.

Madl and Herman (1979) demonstrated that the primary signal is not the absolute number of X chromosomes, but is instead the ratio of X chromosome number to autosomal set number, the X:A ratio. This ratio is 0.5 in diploid XO males (1X:2A), and 1.0 in diploid XX hermaphrodites (2X:2A). Tetraploid animals with two X chromosomes (2X:4A) also have an X:A ratio of 0.5 and develop into males. Tetraploids having three X chromosomes (3X:4A, ratio 0.75) develop into hermaphrodites, while triploids with two X chromosomes (2X:3A, ratio 0.67) develop into males. The system therefore appears comparable to that found in Drosophila, in which X:A...
ratio also determines sex (Cline, 1993). By analogy with *Drosophila*, it seemed likely that *C. elegans* might also have sex-linked numerator sites and autosomal denominator sites (Parkhurst and Ish-Horowicz, 1992), which together contribute to determine the X:A ratio.

This formal model does not make any prediction about how many numerators and denominators are present. Madl and Herman (1979) carried out experiments with partial X chromosome duplications, testing them for the ability to feminize triploid 2X:3A animals. Their results suggested that there may be multiple sites on the X chromosome, which contribute additively to the X:A ratio. More recently, McCoubrey et al. (1988) embarked on a molecular search for possible numerator sites, by testing microinjected DNA clones for the ability to feminize 2X:3A animals. They found that many pieces of X chromosome DNA had this property, apparently as the result of an octomeric sequence which is found more frequently on the X chromosome than on autosomes. However, none of these sequences has any effect when multiple additional copies are introduced into diploid XO individuals, which remain male (Meneely, 1994). It is possible, therefore, that the experiments on triploid animals are misleading, because 2X:3A animals have an X:A ratio (0.67) which is very close to an intersexual value (0.7), and consequently factors unrelated to the normal sex determination process may become involved. Dosage compensation effects may also confuse the issue (for further discussion see Meneely and Nordstrom, 1988; Hodgkin, 1990; DeLong et al., 1993; Meneely, 1994).

Many X chromosome duplications have been generated for *C. elegans*, so that most of the X chromosome has now been covered. None of these duplications has any effect on the sexual fate of XO animals carrying them: they remain male. Combinations of duplications covering up to two thirds of the X chromosome also fail to feminize XO animals (Meneely, 1994). Almost all of these duplications were selected on the basis of male viability and fertility, so it is possible that some of them might have lost numerator activity when generated. That is, some of these duplications may have small interstitial gaps, in which numerators might be found. There also remain a few regions of the X chromosome for which duplications have not been obtained and it is possible that numerators might be located in these regions.

The problem can also be approached in a different way: what is the proximate effect of the X:A ratio? Genetic and molecular analyses have shown that four genes, *xol-1* and the three *sdc* genes, act at an early point in the pathways, and control both sex determination and dosage compensation (Fig. 1). These two gene classes (*xol*: XO Lethal and *sdc*: Sex and Dosage Compensation) have opposite properties. Mutations in *xol-1* have no effect on XX animals, but lead to the death and feminization of XO animals (Miller et al., 1988). The duplications *mnDp73* and *mnDp57* (Herman and Kari, 1989) were kindly made available by Claire Kari and the Caenorhabditis Genetics Center. The mutation *tra-2(q276)* is an unusual mutation of *tra-2*, which causes transformation of XX animals to a fertile male phenotype (T. B. Schedl, unpublished). Standard genetic nomenclature is used, with one modification: for clarity, karyotypes are written as X chromosome number: autosomal set number (for example, 2X:2A for a normal diploid XX karyotype).

**Fluorescent in situ hybridization (FISH)**

Mapping of chromosomal duplications was carried out as described by Albertson (1993).

**Methods for nucleic acid manipulation**

Standard methods were employed to manipulate cosmid and plasmid DNAs (Sambrook et al., 1989). Cosmid clones were generously and patiently provided by Ratna Shownkeen and Alan Coulson. A northern blot of poly(A)+ RNA from mixed stage hermaphrodites, prepared as described by Chirgwin et al. (1979), was graciously loaned by Patricia Kuwabara.

**cDNA library screening, DNA sequencing and analysis**

A cDNA library constructed from embryonic poly(A)+ RNA, and cloned in Agt11 was kindly made available by Peter Okkema (Okkema and Fire, 1994). This was screened with a probe derived from plasmid CB#H4C9. The insert from one positive clone was cloned into the EcoRV site of pBSKII(+) (Stratagene), to yield plasmid CB#H4E1. DNA sequencing (Sanger et al., 1977) was performed using Sequenase 2.0 (United States Biochemicals) as directed by the manufacturer. Nested deletions were generated using Exonuclease III (Henikoff, 1987). Sequence similarities were detected by BLAST (Altschul et al., 1990).
RESULTS

The starting point for this work was the analysis of a duplication of the left end of the C. elegans X chromosome, eDp26. This duplication was isolated in the course of a search for new dominant feminizing mutations of this nematode (Hodgkin and Albertson, unpublished data). In contrast to all previously isolated duplications of the X chromosome (Meneely, 1994), the eDp26 duplication has a dominant phenotype resembling that of rol-8 mutations. It is homozygous viable in XX individuals, but almost wholly lethal to XO animals. Crosses of eDp26 XX hermaphrodites with wild-type males yielded viable XO male progeny at a frequency of less than 0.1%. Further analysis (to be presented elsewhere) demonstrated that it is both lethal and feminizing to XO animals and acts at a very early point in the sex determination pathway. This suggested that eDp26 might carry one or more additional numerator sites for the assessment of X:A ratio, and therefore cause XO animals to adopt the hermaphroditic modes of sex determination and dosage compensation. Map data indicated that eDp26 is only slightly larger in extent than mnDp73, a duplication of the left end of the X chromosome which is neither lethal nor feminizing to XO individuals (Herman and Kari, 1989). Both duplications cover the left end of the X, and extend rightward for about one eighth of the length of the X chromosome (5.5 map units for mnDp73, 6.5 map units for eDp26).

In order to determine the physical size of the region covered by eDp26 but not by mnDp73, the end points of these duplications, as well as those of another XO viable duplication, mnDp57, were mapped more precisely in relation to the physical map of the C. elegans genome (Coulson et al., 1991), using fluorescent in situ hybridization (FISH: Albertson, 1993). The mapping (Fig. 2) showed that a region of approximately 300 kb, located between the right end of mnDp73 and the left end of mnDp57, is uniquely duplicated by eDp26. The location of eDp26 is unusual, because it is attached in reverse orientation to the left end of the X chromosome, but in other respects the FISH analysis indicates that it is a simple duplication. Therefore the radically different properties of eDp26 and mnDp73 can tentatively be ascribed to the duplication of this 300 kb region.

We reasoned that if a small part of this region were responsible for the XO lethality, then simply introducing extra copies of the relevant stretch of DNA, by transformation, might mimic the effect produced by the whole eDp26 duplication. The transformation system commonly used for C. elegans is particularly suitable for this approach (Fire, 1986; Mello et al., 1991). Most injected DNA tends to form an extrachromosomal array of multiple copies, which then gets transmitted unstably to the next generation. We set out to test individual cosmids from the region uniquely duplicated by eDp26. We constructed transgenic lines using the host strain him-8, which segregates a high frequency of XO male self-progeny as a result of meiotic nondisjunction (Hodgkin et al., 1979). Cosmid arrays were marked by coinjection with pRF4, which encodes a mutant collagen gene and leads to a conspicuous dominant Rol (roller) phenotype (Mello et al., 1991). Most cosmids tested have no effect on XO progeny, and consequently a high frequency of both Rol hermaphrodites and Rol males are found among the self-progeny of transformed him-8 lines. The extrachromosomal arrays exacerbate the Him phenotype, so the frequency of males among Rol progeny is higher than the 37% usually seen with him-8, in the range 40-60% (Table 1, lines 1-3).

Fig. 1. An overview of the sex determination pathway in C. elegans. The upper part of the figure shows genes that have been implicated in this process, and the regulatory interactions between them, as inferred from genetic analysis. Arrows signify positive interactions, barred arrows signify negative interactions. Gene names are xol (XO Lethal), sdc (Sex and Dosage Compensation), dpy (DumpY), her (HERnaphroditization), tra (sexual TRAnsformation), and fem (FEMinization). The lower part of the figure shows predicted gene activity states in somatic tissues of the two sexes, in the pathway leading to the final somatic regulator, tra-1, which encodes a predicted transcription factor. This is believed to dictate female development in somatic cells by promoting female differentiation (F) and repressing male differentiation (M). The her-1 gene is regulated at a transcriptional level, perhaps by the products of the sdc genes, two of which are zinc finger proteins. The genes her-1, tra-2, fem-1 and fem-3 are predicted to encode components of a cell-cell communication system. This figure omits events in germ-line sex determination, which involves the same genes, plus additional germ-line-specific genes and interactions. Some of these are needed to modulate sex determination in the XX germ line, permitting hermaphrodite rather than female development. The figure also omits some predicted minor interactions and feedbacks. For further reviews and references, see Hodgkin, 1988, 1990; Villeneuve and Meyer, 1990; Kuwabara and Kimble, 1992; Klein and Meyer, 1993; Nonet and Meyer, 1991; Trent et al., 1991; Zarkower and Hodgkin, 1992.
One cosmid, R04B3, gave a quite different result. Coinjection of R04B3 with pRF4 led to the establishment of a heritably transmitting line, carrying the unstable array designated eEx28 (using standard *C. elegans* nomenclature, in which extrachromosomal transgene arrays are designated by the two letters *Ex*). The eEx28 array, in contrast to those described above, appeared to have a strong lethal effect on XO progeny. Instead of a Rol male percentage of 40-60%, as seen with control cosmids, only very rare Rol males were seen, and these were small and thin (Table 1). In addition, the line carrying eEx28 segregated a greatly increased number of unshelled eggs and dead first-stage larvae, some of which resembled the dead XO larvae seen with *xol-1* or eDp26. The frequency of inviable progeny is 20.9% for the eEx28 line, as compared to 3.3% for the line carrying the control array eEx26.

For further analysis, the eEx28 array was crossed into a wild-type background. The resulting strain segregated only wild-type and Rol hermaphrodites, some dead eggs and larvae, and no males, as expected for a standard XX strain (total counts for 6 broods: 891 wildtype, 288 Rol, 61 inviable). The inviable progeny frequency is higher than with a normal XX hermaphrodite (4.9% as compared to 0.7%), but a comparable low level of lethality is also caused by arrays of control cosmids.

When crossed with wild-type males, these eEx28 hermaphrodites produced abundant wild-type progeny of both sexes, many Rol hermaphrodites and no Rol males (Rol sex ratio 75:0, for five crosses). Thus, the apparent XO lethality is not due to the presence of *him-8*. The lethality is also not due to maleness per se. This was tested using recessive mutations in the genes *tra-1* and *tra-2*, either of which leads to male development in XX individuals (Hodgkin and Brenner, 1977). Sexual transformation of Rol XX animals by either of the mutations *tra-1(e1099)* or *tra-2(q726)* leads to the production of viable Rol males. This was shown by constructing hermaphrodites of genotype *tra-1/+; eEx28* and *tra-2/+; eEx28*. Both of these segregated Rol self-progeny of both sexes, in approximate 3:1 ratio, as expected (Rol sex ratio 29:12 and 18:7, respectively).

The fertility of the *tra-1; eEx28* XX males was tested by crossing them with marked XX hermaphrodites. Rol cross-progeny were obtained, demonstrating that the eEx28 array can be transmitted through an XX male germ line. The *tra-1; eEx28* XX males were also crossed with marked *him-8* XX hermaphrodites. Such hermaphrodites generate many nullo-X oocytes (Hodgkin et al., 1979) and consequently can be used in crosses to generate patroclinous XO progeny – that is, individuals that have inherited their single X chromosome from the male parent. In crosses of *him-8* hermaphrodites with *tra-1; eEx28* XX males, no viable patroclinous Rol XO progeny were produced. This demonstrates that the eEx28 array can act zygotically, exerting an XO lethal effect whether supplied maternally or paternally.

The lethality caused by the eEx28 array therefore appeared to be due to XO karyotype, comparable to that seen with *xol-*.

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**Fig. 2.** Correlation of physical and genetic maps. The top of the figure shows part of the physical map of *C. elegans*, using standard representation (Coulson et al., 1991). The long lines at the top are YAC clones, and the shorter lines in the middle are cosmid clones, plus a few additional smaller clones. Asterisks signify the presence of additional clones. Double vertical lines, ||, mark gaps in the otherwise continuous coverage by cosmids. The continuous dark bar below is the aligned genetic map, with the position of two markers, *lin-32* and *meP5*, which have been located on both the genetic and physical maps. At the bottom, the lighter bars show the extents and approximate endpoints for three duplications, as deduced from FISH analysis using labelled YAC probes. The distance between the right ends of *mnDp73* and *eDp26* is approximately 300 kb. Black dots mark cosmids that have been tested for Xol (XO lethal) activity. Only those cosmids for which a transmitting line has been obtained are marked. Several other cosmids were injected, but failed to give rise to a transmitting line.

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**Table 1.** Summary of sex ratios for various transgenic arrays. The sex ratio is calculated as the number of males divided by the number of observed hermaphrodites. The crosses were performed with wild-type males and females.

<table>
<thead>
<tr>
<th>Array</th>
<th>Sex Ratio</th>
<th>Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>eEx28</td>
<td>75:0</td>
<td>High</td>
</tr>
<tr>
<td>eEx26</td>
<td>3:2:1</td>
<td>Low</td>
</tr>
<tr>
<td>eDp26</td>
<td>1:2:1</td>
<td>Low</td>
</tr>
</tbody>
</table>
1, and probably the result of inappropriate dosage compensation. To test this possibility, the eEx28 array was crossed into a dpy-26(n199) background. This mutation is XX lethal, XO viable, and representative of the set of genes required for the hermaphrodite mode of dosage compensation (Hodgkin, 1983; Plenefisch et al., 1989; Hsu and Meyer, 1994). In the absence of any of these genes, XX animals are inviable or abnormal, presumably as a result of over-expression of sex-linked genes, while XO animals are viable. As expected, dpy-26; eEx28 XO animals are viable. Furthermore, most of these individuals are either partly or wholly transformed into self-fertile hermaphrodites. Consequently, an XO strain of this genotype can be propagated indefinitely, in contrast to a normal dpy-26 brood, in which the only viable animals are phenotypically male. At each generation, the dpy-26; eEx28 hermaphrodites continue to segregate non-Rol progeny, which have lost the array and are exclusively male in phenotype, and Rol progeny consisting of a mixture of hermaphrodites, intersexes and males (Table 2). The eEx28 array therefore can cause both XO lethality and XO sexual transformation if the lethality is suppressed.

Sexual transformation was also demonstrated in a different way, by examining the effect of eEx28 on the sex of 2X:3A triploids. These were generated by crossing unc-4;lon-2; eEx28 hermaphrodites with 2X:4A tetraploid males (as in Hodgkin, 1987). Almost all progeny from such a cross should be 2X:3A, and therefore male. From one such cross, 98/101 non-Rol progeny were male, as expected. Of Rol progeny, 1/24 was male, 4 were intersexual and 19 were hermaphrodites. Thus, eEx28 transforms 2X:3A animals from male into hermaphrodite.

Similar, and slightly stronger effects, were found with a different transgenic array generated from an overlapping cosmid, eEx36. No Rol male progeny have ever been seen in the him-8; eEx36 strain (Table 1), or in numerous crosses generating eEx36 XO progeny. A dpy-26 strain was constructed for this array also, and stronger, but still variable, sexual transformation of XO animals was observed. The sex ratio among the Rol progeny is comparable to that seen for an XO strain dpy-26; eDp26 (Table 2, lines 2 and 4), consistent with the possibility that the eDp26 Xol effect might be due to increase in the dosage of this small region. Somewhat stronger, but still incomplete, feminization is seen among dpy-26; xol-1 XO progeny (Table 2, line 5; also Miller et al., 1988). The effects seen with these arrays are very similar to those seen with mutations of xol-1, suggesting they act at a very early point in the sex determination pathway. If this is so, then both the lethality and the transformation should be suppressed.

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### Table 1. Self-progeny broods of him-8; eEx hermaphrodites

<table>
<thead>
<tr>
<th>Cosmid</th>
<th>Array</th>
<th>Wild type</th>
<th>Roller</th>
<th>Wild type</th>
<th>Roller</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H:M</td>
<td>H:M %</td>
<td>% males</td>
<td>% males</td>
</tr>
<tr>
<td>K0E210</td>
<td>eEx26</td>
<td>614:450</td>
<td>81:100</td>
<td>42.3</td>
<td>55.2</td>
</tr>
<tr>
<td>D2056</td>
<td>eEx27</td>
<td>ND</td>
<td>76:105</td>
<td>ND</td>
<td>58.0</td>
</tr>
<tr>
<td>FH1</td>
<td>eEx41</td>
<td>ND</td>
<td>195:188</td>
<td>ND</td>
<td>49.1</td>
</tr>
<tr>
<td>R04B3</td>
<td>eEx28</td>
<td>758:388</td>
<td>101:1</td>
<td>33.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>eEx44</td>
<td>581:169</td>
<td>141:0</td>
<td>22.5</td>
<td>0.0</td>
</tr>
<tr>
<td>F07B5</td>
<td>eEx30</td>
<td>722:379</td>
<td>80:1</td>
<td>34.4</td>
<td>1.2</td>
</tr>
<tr>
<td>F1E1E1</td>
<td>eEx26</td>
<td>127:46</td>
<td>127:0</td>
<td>26.6</td>
<td>0.0</td>
</tr>
<tr>
<td>F3E3H6</td>
<td>eEx22</td>
<td>430:227</td>
<td>293:114</td>
<td>34.6</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>eEx42</td>
<td>429:172</td>
<td>309:90</td>
<td>28.6</td>
<td>22.6</td>
</tr>
</tbody>
</table>

For most of these entries, complete viable self-progeny broods were counted for 6 hermaphrodites. 3X hermaphrodites have been omitted from the scores, because the Rol marker is hard to score reliably in a 3X hermaphrodite. Inviable zygote counts have also been omitted, and were not always carried out.

H:M, total hermaphrodite progeny : total male progeny.
ND, not determined.

### Table 2. Self-progeny broods of dpy-26 XO hermaphrodites

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non-Roller</th>
<th>Roller</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>herm.</td>
<td>intersex</td>
</tr>
<tr>
<td>dpy-26; eEx28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dpy-26; eEx36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dpy-26; eEx42</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dpy-26; eDp26</td>
<td>72</td>
<td>41</td>
</tr>
<tr>
<td>dpy-26; xol-1</td>
<td>127</td>
<td>29</td>
</tr>
</tbody>
</table>

Each line records total adult progeny from six hermaphrodites. Numerous unhatched eggs and inviable larvae were also produced, as is usual with XO hermaphrodite parents, but these were not scored. NA, not applicable.

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Fig. 3. Partial restriction map, and arrangement of cosmids and subclones, in the fox-1 region. The restriction map at the top shows HindIII sites above the line and EcoRI sites below. Precise endpoints have not yet been obtained for all cosmids. Most of these clones have been tested for Xol activity by the establishment of one or more transmitting lines: (+) indicates a strong Xol effect, (+) indicates a weak Xol effect, and (−) indicates no effect. Asterisks mark HindIII fragments that hybridize to the cDNA clone, CB4H4E1.
pressured by a mutation in sdc-2 (Introduction, and Fig. 1). Interactions with sdc-2 were tested using eEx36: a hermaphrodite of genotype sdc-2[y15]/+; eEx36 was constructed and crossed with wild-type males. Numerous Rol males of apparently normal viability and male phenotype were produced, indicating that sdc-2 is entirely epistatic to the Xol genes.

Further molecular definition of the region causing the Xol effect was carried out by testing all of the available cosmids in this region of the physical map (Fig. 3), and subsequently by constructing and testing various subclones from this region. For most of these cosmids, at least two transmitting lines were established in independent experiments, and scored for male production, with consistent results (Table 1, plus additional data). The three overlapping cosmids R04B3, F53B5 and F16E1 all exhibit a strong consistent Xol effect, and the partly overlapping cosmids T07D1 and D08C3 showed no effect at all (eEx35 in Table 1, plus four other lines). The overlaps between positive cosmids R04B3 and F53B5, and the negative cosmid T07D1, suggest that the Xol effect is caused by a region of more than 12 kb but less than 30 kb, located toward the left end of R04B3. A more complicated explanation is that partly redundant elements are located on both sides of an essential core. The behaviour of these cosmids suggests that a single locus is responsible for the Xol effect, which we designate fox-1, for 'Feminizing locus On X'.

Attention was further focused on the left end of R04B3 by the properties of arrays carrying cosmids F33H6, which is slightly shorter or rearranged at its left end, compared to R04B3, but longer than F53B5 at its right end. Two independently generated arrays carrying this cosmid each segregated substantially numbers of Rol males, but significantly fewer than control cosmids (Table 1). Also, most of the Rol males grew slowly and were thin, small and pale, or otherwise abnormal. It seemed likely that this was due to a weakened Xol effect. Consistent with this, when one of these arrays (eEx42) was crossed into a dpy-26 background, it exhibited feminizing properties (Table 2), though markedly weaker than eEx28 or eEx36. Each fertile XO hermaphrodite of genotype dpy-26; eEx42 produces fewer than one fertile hermaphrodite daughter, so the strain cannot be sustained.

This result suggested that F33H6 is missing important, but not essential, sequences at its left end. A coinjection experiment, injecting a mixture of T07D1 and F33H6, was therefore performed. Overlapping cosmids are believed to recombine promiscuously when coinjected, so it was hoped that a strong

Fig. 4. Sequence of most of the CDNA clone, CB#H4E1, and the only long open reading frame in it. The ATG at the beginning of the sequence is minimally truncated at the 5′ end, and may be transspliced to SL1 (Krause and Hirsh, 1987). The 3′ end of the sequence may also be truncated, because this clone lacks a poly(A) tail or a convincing polyadenylation signal. The amino acid sequence shown in Fig. 5 is predicted by a mutation in sdc-2 (Introduction, and Fig. 1). Interactions with sdc-2 were tested using eEx36: a hermaphrodite of genotype sdc-2[y15]/+; eEx36 was constructed and crossed with wild-type males. Numerous Rol males of apparently normal viability and male phenotype were produced, indicating that sdc-2 is entirely epistatic to the Xol genes.

Further molecular definition of the region causing the Xol effect was carried out by testing all of the available cosmids in this region of the physical map (Fig. 3), and subsequently by constructing and testing various subclones from this region. For most of these cosmids, at least two transmitting lines were established in independent experiments, and scored for male production, with consistent results (Table 1, plus additional data). The three overlapping cosmids R04B3, F53B5 and F16E1 all exhibit a strong consistent Xol effect, and the partly overlapping cosmids T07D1 and D08C3 showed no effect at all (eEx35 in Table 1, plus four other lines). The overlaps between positive cosmids R04B3 and F53B5, and the negative cosmid T07D1, suggest that the Xol effect is caused by a region of more than 12 kb but less than 30 kb, located toward the left end of R04B3. A more complicated explanation is that partly redundant elements are located on both sides of an essential core. The behaviour of these cosmids suggests that a single locus is responsible for the Xol effect, which we designate fox-1, for 'Feminizing locus On X'.

Attention was further focused on the left end of R04B3 by the properties of arrays carrying cosmid F33H6, which is slightly shorter or rearranged at its left end, compared to R04B3, but longer than F53B5 at its right end. Two independently generated arrays carrying this cosmid each segregated substantially numbers of Rol males, but significantly fewer than control cosmids (Table 1). Also, most of the Rol males grew slowly and were thin, small and pale, or otherwise abnormal. It seemed likely that this was due to a weakened Xol effect. Consistent with this, when one of these arrays (eEx42) was crossed into a dpy-26 background, it exhibited feminizing properties (Table 2), though markedly weaker than eEx28 or eEx36. Each fertile XO hermaphrodite of genotype dpy-26; eEx42 produces fewer than one fertile hermaphrodite daughter, so the strain cannot be sustained.

This result suggested that F33H6 is missing important, but not essential, sequences at its left end. A coinjection experiment, injecting a mixture of T07D1 and F33H6, was therefore performed. Overlapping cosmids are believed to recombine promiscuously when coinjected, so it was hoped that a strong
Xol effect might be recreated. This was found, with array eExd3 (Table 1).

The above results suggested that an essential part of fox-1 lies close to the left end of R04B3, and extends rightward beyond the right end of T07D1. A 6.7 kb EcoRI fragment from this region, CB#H4C9, was used in a preliminary search for transcripts. A northern blot, of poly(A)+ mRNA derived from mixed-stage XX hermaphrodites, was probed, and one RNA species of approximately 1.8 kb was detected. A cDNA library, made from embryonic RNA, was also probed. One phage clone recovered contains an insert of 1.7 kb, likely to correspond to the species detected on the northern blot. When hybridized back to cosmid and genomic DNA cut with a variety of restriction enzymes, this insert hybridizes to at least 3 HindIII fragments across the putative fox-1 region. A subclone from the 3′ end of this cDNA hybridizes to only to rightward fragments. Moreover, it hybridizes to some fragments that are incomplete or missing in T07D1. Therefore, this RNA is transcribed from left to right, and probably extends beyond the right end of T07D1.

The cDNA insert was sequenced, and found to contain an open reading frame of 415 amino acids (Fig. 4), encoding a predicted glutamine-rich protein of relative molecular mass 44.3\(\times\)10^3. The most notable feature of this sequence is a stretch of 90 amino acids, with an unambiguous match to the best characterized RNA-binding domain, the RNP sequence (for review, see Burd and Dreyfuss, 1994). Of 23 strongly conserved sites in the RNP consensus, this sequence contains 19, with appropriate spacing (Fig. 5). Three of the four variant residues can all be found in one or another member of this family of proteins.

**DISCUSSION**

This is the first demonstration of sex reversal in diploid C. elegans caused simply by increased dosage of a small piece of X chromosome DNA. The results are very different from those found in previous attempts to influence sex determination by injecting additional X chromosome DNA (McCoubrey et al., 1988; Meneely, 1994). In those experiments, some (usually incomplete) feminization of 2X:3A triploid animals was achieved, but the clones tested never showed any effect on diploid XO animals. In the present study, we find complete XO lethality, and much stronger feminization, caused by extra copies of a specific sequence, fox-1.

The results show that increased dosage of fox-1 alone is sufficient to direct XO animals into the hermaphrodite mode of dosage compensation and sex determination. XO animals carrying the fox-1 array eEx36 invariably die, but survive if the dosage compensation system is inactivated, showing that 100% of XO animals are directed into the wrong mode of dosage compensation. Moreover, most of the survivors are diverted into the hermaphrodite mode of development and mature as self-fertile hermaphrodites.

We define this element, which appears to be less than 30 kb across, as ‘fox-1’, standing for ‘Feminizing locus On X’. At present, its properties indicate that it is a unitary locus, consisting largely or completely of unique sequence DNA. The genetic data are consistent with action as a numerator element for assessment of X:A ratio. It is located in a region of the X chromosome that has not been duplicated previously, and the duplication that includes this region, eDp26, confers an XO lethal phenotype. Its effects are similar to those of xol-1 mutations, but dominant, and it acts at an early point in the sex determination pathway, upstream of sdc-2. Finally, its effects appear to be strictly zygotic, as expected for a numerator element.

The XO lethality caused by eEx36 is 100%, comparable to that caused by loss-of-function mutations in xol-1. The sexual transformation, however, is significantly weaker than that caused by xol-1. This fact alone implies the existence of additional feminizing sites on the X chromosome, but they may be less potent than fox-1, which could be the most important of a very small number of sex-linked counting elements. Superficially, it might seem that fox-1 is entirely responsible for the lethality and transformation caused by eDp26, because the effects of eEx36 are so similar to those of eDp26, and so different from those of mmDp73. However, an alternative interpretation is that eDp26 carries a single extra dose of fox-1, together with other fox loci located at the left end of the X. The strong effect caused by eEx36 could be due to multiple copies of fox-1, which together render unnecessary the contributions from the other fox loci. It is not possible to determine what the effective copy number of fox-1 in eEx36 might be, because expression from extrachromosomal transgene arrays is likely to be different from endogenous chromosomal DNA (Fire, 1986). However, it is noticeable that all of the independent arrays generated from RO4B3, F16E1 and F53B5 exhibit 99–100% XO lethality, suggesting that any increase in fox-1 dosage is likely to have some effect.

It is important to appreciate that the numerator system may be redundant. Our experiments indicate that fox-1 is sufficient to act as a numerator, but it alone may not be necessary. There may be other strong fox loci, duplication of any one of which would have a Xol effect. If fox-1 is the only major numerator, then a decrease in fox-1 dosage caused by a mutation at this locus would be expected to have a strong dominant lethal Sdc phenotype in XX animals. If the numerators are redundant, then fox-1 mutations might have a weaker effect. Akerib and Meyer (1994) have isolated additional duplications and deficiencies of the left end of X. Large deficiencies, which are likely to include fox-1, do indeed exhibit a dominant Sdc effect, but it is weaker than might be expected. We have obtained similar results (J.H., unpublished data). More precise investigation of the role of fox-1 should now be feasible, by creating mutations in this gene by a reverse genetic approach (Zwaal et al., 1993). Redundancy of numerators also means that they may only be detectable by an approach such as we have used.

Additional strong numerators may exist, but they are likely to be rare. As noted in the Introduction, most of the X chromosome has now been covered with duplications that have no effect on XO animals. Also, transgenic arrays carrying many different pieces of X chromosome DNA have been constructed, none of which has hitherto exhibited any kind of Xol effect.

A second, more speculative component of this research is the cDNA, CB#H4E1, derived from the fox-1 region. The case that fox-1 exerts its effects via this gene product is at present circumstantial, but suggestive. The transcript in question is present in embryos and is transcribed from across a substantial stretch of the essential fox-1 region. The strong Xol cosmids all carry...
an intact copy of this gene, while the overlapping negative cosmid T07D1 probably lacks the 3’ end of the gene. The weak Xol cosmid F33H6 is defective in sequences close to the 5’ end of the transcript, which might well impair its expression. All of this is consistent with the hypothesis. However, it remains possible that the real fox activity is not due to this transcript, but to some other sequence or sequences located in the same region, for example in the introns of this gene. Direct tests of the role of the CBH4AE1 transcript should be feasible, for example by forcing overexpression of the cDNA in XO embryos. Further work should also define what other transcripts may be present, in both XX and XO animals.

A separate property suggesting that this mRNA is indeed a fox-1 product is that it encodes a possible regulatory protein, because the predicted sequence contains an unmistakable match to an RNA-binding consensus. As an RNA-binding protein, it could exert a regulatory effect on transcripts of a target gene or genes in a variety of ways. It could affect splicing, stability, localization, transport, translation, or some other process. In Drosophila, regulation of alternative splicing plays a major role in sex determination (Baker, 1989). In the nematode sex determination pathway, evidence has been found for the inhibition of tra-2 translation by RNA-binding proteins (Goodwin et al., 1993). The most obvious possible target gene for fox-1 action is xol-1, and direct regulation by protein-RNA interactions of some kind is certainly conceivable.

If indeed fox-1 does encode an RNA-binding protein, and it is the dosage of this gene that acts as a major primary sex determinant, then an interesting contrast with Drosophila becomes apparent. In D. melanogaster, the primary sex determinants on the X are a small number of DNA-binding proteins that regulate expression of RNA-binding proteins, products of the Sxl gene. In C. elegans, the primary determinants on the X may instead be RNA-binding proteins that regulate expression of DNA-binding proteins, products of the sdc genes.

The discovery of fox-1 as a defined locus on the X chromosome, which may encode an RNA-binding protein, provides new insights and a valuable tool for investigating one of the remaining mysteries of C. elegans sex determination – the simple but profound distinction between one and two X chromosomes.

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