A novel regulatory mutation in the *C. elegans* sex determination gene *tra-2* defines a candidate ligand/receptor interaction site

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**SUMMARY**

Sex determination in the nematode *C. elegans* is dependent on cell-to-cell communication, which appears to be mediated by the predicted membrane protein TRA-2A and the secreted protein HER-1. In XO males, HER-1 is hypothesised to function as a repressive ligand that inactivates the TRA-2A receptor. In XX animals, HER-1 is absent and TRA-2A promotes hermaphrodite development by negatively regulating the FEM proteins. This paper describes the molecular and genetic characterisation of a novel class of feminising mutations called *tra-2*(eg), for enhanced gain-of-function. In XX animals, mutant *tra-2*(eg) activity promotes entirely normal hermaphrodite development. However, the *tra-2*(eg) mutations generate an XO-specific gain-of-function phenotype, because they transform XO mutants from male into hermaphrodite.

Therefore, the *tra-2*(eg) mutations identify a major regulatory site, which may be the TRA-2A/HER-1 interaction site. All ten *tra-2*(eg) mutations encode identical missense changes in a predicted extracellular domain of TRA-2A, named the EG site. It is proposed that the *tra-2*(eg) mutation encodes a TRA-2A protein that functions constitutively in XO animals, because it is defective in HER-1 binding. Phenotypic characterisation of sexually transformed XO *tra-2*(eg) hermaphrodites reveals that their fertility is strongly affected by dosage compensation mutations, suggesting that dosage compensation plays a role in normal gametogenesis.

Key words: sex determination, *Caenorhabditis elegans*, hermaphrodite, ligand, receptor

**INTRODUCTION**

The nematode *C. elegans* naturally exists as either an XX hermaphrodite or an XO male. The *C. elegans* hermaphrodite is essentially a self-fertile female: her soma is indistinguishable from females of closely related, but exclusively male/female nematodes (Baird et al., 1994), and her germ line produces sperm first, then oocytes. The primary determinant of sex in *C. elegans* is the ratio of X chromosomes to sets of autosomes - the X:A ratio (Madl and Herman, 1979). In response to the X:A ratio, the *xol* and *sdc* genes control both dosage compensation and sex determination (Fig. 1A). Dosage compensation equalises X-linked transcription between XX and XO diploids (for review, see Hsu and Meyer, 1993). An additional set of seven genes, *her-1, tra-1, tra-2, tra-3, fem-1, fem-2* and *fem-3*, control sexual fate, but have no apparent role in dosage compensation (for details of the pathway, refer to legend for Fig. 1) (reviewed in Villeneuve and Meyer, 1990; Kuwabara and Kimble, 1992). The terminal regulator of somatic sexual cell fate is *tra-1*, which promotes XX hermaphrodite somatic development (Hodgkin and Brenner, 1977; Zarkower and Hodgkin, 1992). The *tra-2* gene plays a central role in controlling somatic and germ-line sexual cell fate. XX mutants that lack wild-type *tra-2* have a male soma and a germ line that produces only sperm; XO *tra-2* mutants are normal males. This paper focuses on the regulation of TRA-2A (Kuwabara et al., 1992), the predicted membrane protein encoded by the largest of three *tra-2* mRNAs (4.7 kb, 1.9 kb and 1.8 kb) (Okkema and Kimble, 1991). TRA-2A is the component of the *tra-2* locus that is both necessary and sufficient to promote female somatic development (Kuwabara and Kimble, 1995).

It has been proposed that the membrane protein TRA-2A functions as a receptor that controls sexual cell fate through its participation in various protein-protein interactions (Fig. 1A,B) (Kuwabara et al., 1992; Kuwabara and Kimble, 1995). In XX animals, TRA-2A is likely to function constitutively, because no positively acting ligand has been identified (Kuwabara et al., 1992). Recent evidence indicates that a carboxy terminal domain of TRA-2A inhibits the activity of one or more of the fem gene products, perhaps through sequestration (Fig. 1B) (Kuwabara and Kimble, 1995). As a result of low FEM activity, TRA-1, a zinc finger protein and putative transcriptional regulator, promotes hermaphrodite somatic development (Zarkower and Hodgkin, 1992).

In XO animals, it is postulated that TRA-2A is inactivated by a repressive ligand, named HER-1 (Fig. 1A,B), which is predicted to be secreted (Perry et al., 1993) and to function cell non-autonomously (Hunter and Wood, 1992). This repression is likely to be XO-specific, because *her-1* mRNAs are detected only in XO animals (Trent et al., 1991). Inactivation of TRA-2A, in turn, allows the FEM proteins to promote male development by inhibiting TRA-1 activity. Therefore, cell-to-cell signalling mediated by TRA-2A and HER-1 is a crucial step...
A.

Dosage compensation (dpy-21, dpy-26, dpy-27, dpy-28, dpy-30)

Sex determination

B.

XX HERMAPHRODITE

XO MALE

Fig. 1. (A) Genetic pathway of sex determination and dosage compensation in C. elegans (modified from Hodgkin, 1990; Villeneuve and Meyer, 1990). The X:A ratio is the primary determinant of sex and sets the activity states (designated high or low) of the genes xol-1, sdc-1, -2 and -3, which regulate both sex determination and dosage compensation. In XX animals, the sdc genes are active and negatively regulate her-1, probably at the transcriptional level, because her-1 mRNAs are detected in XX, but not XO animals (Trent et al., 1991). In turn, the tra-2 and tra-3 genes are active and negatively regulate the fem genes. Inhibition of fem gene activity allows tra-1 to promote female somatic development. High sdc gene activity also activates the dosage compensation dpy genes, which function to lower X-linked transcription. In XO animals, high xol-1 activity negatively regulates the sdc genes. In turn, her-1 negatively regulates the activities of tra-2 and tra-3. Inhibition of tra-2 and tra-3 activities allows the fem genes to promote male development by negatively regulating tra-1. Low sdc activity prevents the activation of dosage compensation XO males. (B) Molecular model of somatic sex determination in C. elegans (adapted from Kuwabara et al., 1992). Left, XX hermaphrodite development. TRA-2A is depicted as a membrane protein with an extracellular amino terminus and an intracellular carboxy terminal tail. A carboxy terminal region of TRA-2A is likely to inhibit the activity of the fem gene products, perhaps by sequestration (Kuwabara and Kimble, 1995). In turn, TRA-1 functions in the nucleus to promote hermaphrodite somatic development. Right, XO male development. her-1 mRNAs are detected in XO, but not XX animals (Trent et al., 1991). The HER-1 protein encodes a predicted secreted protein that functions cell autonomously to promote male development (Perry et al., 1993; Hunter and Wood, 1992). It is postulated that HER-1 is a repressive ligand that inactivates TRA-2A. In turn, the FEM proteins are released from inhibition and promote male development by negatively regulating TRA-1.

in controlling sexual cell fate decisions (Kuwabara et al., 1992; Hunter and Wood, 1992). The function of this cell-to-cell signalling event may be to ensure that all cells in the nematode adopt only one of two possible sexual fates (Kuwabara et al., 1992).

TRA-2A is an unusual cell surface receptor in that the HER-1 ligand is proposed to repress TRA-2A rather than to activate it (Kuwabara et al., 1992). In most other signal transduction processes, ligand binding leads to receptor activation (e.g. EGFl action on EGF receptor, for review see Heldin, 1995). However, repressive ligand/receptor interactions may be more widespread than is presently apparent. A similar negative interaction has been proposed to occur between the Drosophila hedgehog and patched proteins during positional signalling (Ingham et al., 1991). The protein sequences of patched and tra-2 share marginal similarity (Kuwabara et al., 1992). Clearly, a molecular understanding of the function and regulation of TRA-2A activity is essential to understand sex determination in C. elegans at the biochemical level and more generally to explore the mechanisms of repressive ligand/receptor interactions.

This paper investigates the regulation of TRA-2A by HER-1 by presenting the molecular and genetic characterisation of a novel class of dominant XO-feminising mutations. These mutations, named tra-2(eg) for enhanced gain-of-function, are shown to encode missense changes in a predicted extracellular domain of TRA-2A. The phenotypic properties of the tra-2(eg) mutation have also been analysed in detail by obtaining an intragenic recombinant that carries only the tra-2(eg) mutation, because all of the tra-2(eg) mutations were originally isolated as tra-2(gf, eg) double mutations (Hodgkin and Albertson, 1995). The recombinant tra-2(eg) mutation results in a dominant transformation of XO animals from male into hermaphrodite, demonstrating the status of tra-2 as a switch gene controlling all aspects of sexual phenotype. Taken together, the molecular and genetic properties of the tra-2(eg) mutations indicate that these mutations are likely to identify a region of direct interaction between TRA-2A and HER-1. The genetic analysis of the tra-2(eg) mutations has also led to the unexpected finding that the fertility of XO tra-2(eg) mutants is dramatically improved by mutations in genes involved in dosage compensation. This observation provokes the speculation that interactions may exist between the sex determination and dosage compensation pathways in the hermaphrodite germ line.

MATERIALS AND METHODS

Nematode culture, strains and general handling methods

General methods for genetic manipulation, culturing and synchronisation of nematodes have been described (Brenner, 1974; Sulston and Hodgkin, 1988). All genetic characterisations were at 20°C unless otherwise indicated, using worms that were not starved or recovering from the dauer state.

Standard nomenclature is used in this paper (Horvitz et al., 1979). The suffix gf designates gain-of-function and eg designates enhanced gain-of-function. Unless otherwise stated, it is implicit that all other alleles are loss-of-function (lf). When the maternal and zygotic genotypes are important the superscripts ° or ‰ refer to the presence or absence of gene activity. The following mutations and chromosomal rearrangements were used. Most mutations in this paper are described elsewhere (Hodgkin et al., 1988).
Linkage group (LG) II: dpy-2(e8), dpy-10(e128), tra-2(e1095), q276, e2046g(f), unc-104(e1265), unc-4(e120), mnC1.

LGIII: dpy-28(y1)

LGIV: unc-24(e138), fem-3(q20gf), him-8(e1489), dpy-20(e1282), dpy-26(n199), tra-3(e1767)

LGV: him-5(e1490), her-1(ri695gfi)

LGX: unc-2(e5)

Molecular cloning methods

General methods for manipulating nucleic acids are described by Sambrook et al. (1989). Nematode DNA was prepared as described by Emmons and Yesner (1984). Nematode RNA was isolated by the guanidinium thiocyanate protocol of Chirgwin et al. (1979). Poly(A)+ mRNA was selected on oligo(dT) cellulose (Pharmacia). Synchronised populations of nematodes were obtained by hypochlorite treatment of gravid adults to obtain eggs, followed by an arrest at the L1 stage by starvation in M9 buffer. Animals were subsequently fed and harvested as gravid adults.

Genomic DNA was amplified by the polymerase chain reaction (PCR), cloned and sequenced. Worm genomic DNA was subjected to 35 cycles of amplification using Taq polymerase (Promega) as directed by the manufacturer using the following parameters: 95°C for 30 seconds, 55°C for 50 seconds, 72°C for 1 minute per kb of final amplified product, followed by a final extension at 72°C for 10 minutes. PCR amplified fragments were made blunt with DNA polymerase I Klenow fragment (Cambia), phosphorylated with T4 kinase (New England Biolabs), gel purified using GeneClean (Bio 101) and ligated into the EcoRV site of pBSKII(+) (Stratagene), which had been treated with calf intestine alkaline phosphatase (Boehringer Mannheim). Double-stranded templates were sequenced with Sequenase (United States Biochemical).

Sequence and location of oligonucleotide primers

Oligonucleotides used to amplify tra-2 gene regions are listed below. The positions of tra-2 oligonucleotides are listed in brackets and are based on the numbering of the 4.7 kb tra-2 cDNA sequence (Kuwabara et al., 1992).

PK21 GTGAGGTGCTAGTGGACAGAT (mx and 3’ UTR) (4613-4632)
PK27 ATTGGAAATAGAAGACGACG (eg) (527-546)
PK59 ATTTGAGAATAGAATACCGAC (eg) (4051-4070)
PK101 ATCTGACATCTTCTATTTGAACT (eg) (869-891)
PK36 TACCGCTGTCCGGGTCT (Northern probe) (3354-3371)
PK27 ATTGGAATAAGAATACCGAC (eg) (527-546)
PK81 TTCTCAAAAGGAGACGACATT (Northern probe) (4465-4487)

Molecular characterisation of tra-2(eg) mutations

To determine the molecular basis of the tra-2(eg) mutations, genomic DNA was isolated from ten homozygous strains of genotype tra-2(eg), eg unc-4; fem-3(qf) dpy-20, carrying ten independent eg alleles (e2531, e2534, e2536, e2538, e2540, e2552, e2555, e2571, e2581, and e2582eg). Regions corresponding to the tra-2 3’ UTR, mx (for mixed character) region and exons were PCR amplified, cloned and sequenced, as described above. To minimise the risk of PCR-induced artefacts, all sequence changes were verified by analysing an independently generated PCR fragment. To control for potential polymorphisms between strains, DNA was cloned and sequenced from the tra-2(eg) parental strain: CB4915 = tra-2(e2046gfi); dpy-28. This strain was placed in cryostorage after the first two tra-2(eg) mutants were obtained and was subsequently thawed for DNA analysis (J. Hodgkin, personal communication). Therefore, any base change present in a tra-2(eg) mutant and absent from the CB4915 parent must have been independently introduced into each mutant as a result of mutagenesis.

Intragenic recombination to separate tra-2(e2531eg) from tra-2(e2046gfi)

The following strategy was used to identify an intragenic recombinant that carries the tra-2(e2531eg) mutation, but not the tra-2(e2046gfi) mutation. Heterozygous + tra-2(e2046gfi, e2531eg) + dpy-2 + unc-104 non-Dpy non-Unc females were mated with dpy-2 + unc-104/mmc1 males and F1 progeny were screened for Dpy non-Unc recombinants. Female recombinants were propagated by mating with XO dpy-2 unc-104/mmc1 males and hermaphrodite recombinants were selected. The genotype of each recombinant was determined by cloning and sequencing regions associated with the tra-2(eg) and tra-2(e2046gfi) mutations.

To separate tra-2(e2531eg) from the closely linked marker, dpy-2, the following strategy was used. Heterozygous dpy-2 tra-2(e2531eg) hermaphrodites were mated to XO tra-2 males and dpy-2 tra-2(e2531eg)/+ tra-2 non-Dpy progeny were individually plated to identify a recombinant that segregated only XX tra-2 pseudomales and tra-2(e2531eg) non-Dpy progeny. 1/43 plates was found to have such a recombinant. Eight hermaphrodites from this plate were individually plated and 2/8 segregated non-pseudomale non-Dpy progeny and thus were likely to be XX tra-2(e2531eg) homoygotes.

Phenotype of animals homoygous for tra-2(eg)

The sexual phenotype of the somatic gonad, hypodermis, tail and germ line of adult XX and XO animals carrying the tra-2(e2531eg) mutation was examined by dissecting microscope and Nomarski DIC optics. Brood sizes were counted by individually plating L4 hermaphrodites on plates and transferring them daily to fresh plates until they stopped producing eggs. Eggs were scored as dead if they failed to hatch within 24 hours. To eliminate potential deleterious XO maternal effects, siblings were chosen from an XX tra-2(e2531eg); him-8 hermaphrodite that produced >200 self-progeny.

Analysis of tra-2(e2531eg)/+ animals

The phenotype of XX and XO animals carrying a single dose of tra-2(e2531eg) was examined by mating tra-2(e2531eg)/unc-4 hermaphrodites to wild-type males and scoring non-Unc cross-progeny. To determine whether masculinised animals were XX or XO, the strain tra-2(e2531eg); unc-7, which carries an X-linked marker, unc-7, was constructed. In constructing this strain, XX tra-2(q270); unc-7/+

Non-Dpy males were used to introduce the unc-7 marker into tra-2(e2531eg). Subsequently, tra-2(e2531eg); unc-7 hermaphrodites were mated with wild-type XO males and the sexual phenotype of Unc and non-Unc progeny scored.

Northern blot analysis

RNA was extracted from synchronised adult XX populations of tra-2(e2531eg) and wild-type hermaphrodites, poly(A)+ selected on sepharose oligo(dT) and electrophoresed on 1.5% formaldehyde-agarose gels for northern analysis. Northern blots were hybridised as described in Sambrook et al. (1989) using a random primed PCR fragment, amplified from lambda JK57 (Kuwabara et al., 1992) with oligonucleotides PK36 and PK81 (see above for sequence). This probe detects all trnA(eg) fragment, amplified from lambda JK57 (Kuwabara et al., 1992) with oligonucleotides PK36 and PK81 (see above for sequence). This probe detects all trnA(eg) fragment, amplified from lambda JK57 (Kuwabara et al., 1992) with oligonucleotides PK36 and PK81 (see above for sequence). This probe detects all trnA(eg) fragment, amplified from lambda JK57 (Kuwabara et al., 1992) with oligonucleotides PK36 and PK81 (see above for sequence). This probe detects all trnA(eg) fragment, amplified from lambda JK57 (Kuwabara et al., 1992) with oligonucleotides PK36 and PK81 (see above for sequence).

RESULTS

Molecular localisation of the tra-2(eg) mutations

The tra-2(gf, eg) double mutations are the first tra-2 mutations that completely feminise both the soma and germ line of XO males (Hodgkin and Albertson, 1995). Previously characterised tra-2(gf) and tra-2(mx) mutations feminise primarily the XX germ line and have little or no effect on XO animals; hermaphrodites produce oocytes, but not sperm (Doniach,
Hydropathy analysis predicts that TRA-2A is a membrane protein with nine potential membrane spanning domains (Kuwabara et al., 1992, named the EG site. Ball and stick symbols refer to potential sites of glycosylation (Kuwabara et al., 1992).

Separation of the tra-2(e2531eg) mutation from the tra-2(e2046gf) mutation by intragenic recombination

XX tra-2(gf, eg) double mutants are females (spermless hermaphrodites), probably because they carry the germ-line feminising tra-2(e2046gf) mutation. To analyse properly the effect of the tra-2(e2531eg) mutation, it was essential to separate it from the tra-2(e2046gf) mutation. Although the distance separating these two mutations is less than 10 kb, it proved possible to isolate the tra-2(eg) mutation by intragenic recombination (see Material and Methods for details). Four female and one hermaphrodite recombinant were obtained in a narrow region flanking tra-2. DNA sequencing revealed that the four female recombinants still carried the germ-line feminising tra-2(e2046gf) mutation, whereas the hermaphrodite recombinant did not. The hermaphrodite recombinant, however, did carry the tra-2(e2531eg) mutation as shown by sequencing. It follows that XX tra-2(e2531eg) animals are self-fertile hermaphrodites, not females.

XX tra-2(e2531eg) hermaphrodites are phenotypically wild type

No phenotypic differences were detected between XX tra-2(e2531eg) mutants and wild-type hermaphrodites. Specifically, no females were detected in a population of XX tra-2(e2531eg) animals (n>100). However, given that all previously identified tra-2 feminising mutations feminise the XX germ line (Doniach, 1986; Schedl and Kimble, 1988), it was important to determine whether tra-2(eg) mutations had any feminising effect on the XX germ line. Brood sizes were counted to investigate the possibility that the tra-2(e2531eg) mutation might partially feminise the XX germ line by reducing sperm number. The number of hermaphrodite self-progeny is limited by sperm number, so animals with a feminised germ line produce fewer self-progeny. XX tra-2(e2531eg) hermaphrodites were found to have a mean brood...
Size comparable to wild-type of 323.3±22.5 (n=6) (Table 1, line 1).

A further test for feminisation of the germ line was made by examining the tra-2(e2531eg); fem-3(gf) double mutant. fem-3(gf) mutations masculinise the XX germ line (sperm, no oocytes) at restrictive temperature, but not the soma (Barton et al., 1987). In contrast, tra-2(gf) mutations feminise the germ line (oocytes, no sperm) (Doniach, 1986; Schedd and Kimble, 1988). Double mutants carrying fem-3(gf) and tra-2(gf) reveal a balancing between the germ-line masculinising and feminising activities of these two genes, because a proportion of these animals are self-fertile (Barton et al., 1987). To test whether a similar interaction might occur between tra-2(e2531eg) and fem-3(gf), the double mutant was constructed. To facilitate identification of the double mutant, this strain also carries the unc-4 and dpy-20 mutations as phenotypic markers closely linked to the tra-2 and fem-3 genes, respectively. XX tra-2(e2531eg) unc-4; fem-3(gf) dpy-20 double mutants grown at restrictive temperature produced only sperm – none were self-fertile (n=120). Thus, the failure to detect any enhanced germ-line feminisation in tra-2(eg) mutants indicates that tra-2(e2531eg) activity remains sensitive to germ-line-specific regulatory controls normally present in XX hermaphrodites.

### Table 1. Brood sizes of tra-2(eg) mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viable</th>
<th>Inivable</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX tra-2(eg)</td>
<td>323.3±22.5*</td>
<td>0.8±0.98</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(Range = 287-350)</td>
<td>(Range = 0-2)</td>
<td></td>
</tr>
<tr>
<td>XX tra-2(eg); him-8†</td>
<td>255.1±32.5</td>
<td>10.3±3.4</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(Range = 192-313)</td>
<td>(Range = 6-16)</td>
<td></td>
</tr>
<tr>
<td>XO tra-2(eg); him-8†</td>
<td>0.3±0.5</td>
<td>14.1±14.6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(Range = 0-1)</td>
<td>(Range = 0-48)</td>
<td></td>
</tr>
<tr>
<td>XO tra-2(eg) unc-4; dpy-26†</td>
<td>49.8±15.0</td>
<td>154.8±19.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(Range = 27-68)</td>
<td>(Range = 117-170)</td>
<td></td>
</tr>
<tr>
<td>XO tra-2(eg); dpy-26</td>
<td>35.6±16.0</td>
<td>107±19.08</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(Range = 17-68)</td>
<td>(Range = 80-138)</td>
<td></td>
</tr>
<tr>
<td>XO tra-2(eg) unc-4; dpy-26 him-8†</td>
<td>31.7±11.7</td>
<td>141.3±20.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(Range = 17-50)</td>
<td>(Range = 117-173)</td>
<td></td>
</tr>
<tr>
<td>XO tra-2(eg); dpy-26 him-8</td>
<td>35.6±14.0</td>
<td>125.6±15.4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(Range = 13-62)</td>
<td>(Range = 107-144)</td>
<td></td>
</tr>
</tbody>
</table>

n = total number of broods counted.
*XX wild-type N2 mean brood size is 330±34, for comparison (Hodgkin et al., 1979).
†37% of him-8 animals are predicted to be XO. Therefore, sibs have been subdivided into XX and XO, based on the observation that 30% of total animals have drastically reduced broods and hence are predicted to be XO. him-8 is a gene closely linked to tra-2 (mRNA messages are incompletely feminised tails. The remaining 10/41 Unc hermaphrodites, which were expected to be XO, had incompletely feminised tails.
§Dead progeny consists of dead embryos, larval lethals and inviable Dpy animals, which are predicted to be XX and nullo-X in genotype.

**tra-2(e2531eg) activity requires wild-type tra-3 activity**

The tra-2 and tra-3 genes are believed to function together to negatively regulate the activity of the fem genes (Fig. 1A). Loss-of-function mutations in either gene transform XX animals into non-mating pseudomales; however XX tra-3 animals are less severely masculinised than XX tra-2 animals (Hodgkin, 1980). Strong hypermorphic tra-2(gf) mutations, which presumably increase the level of wild-type TRA-2 proteins, can largely remove the requirement for tra-3 activity (Doniach, 1986; Hodgkin, 1986). To test whether tra-2(e2531eg) can also function independently of tra-3, the phenotype of XX tra-2(e2531eg) unc-4; tra-3 (m–Z) homzygotes was examined. These animals are identical in phenotype to XX tra-3 mutants (n=82), supporting the notion that tra-2(eg) activity is not hypermorphic because it still requires wild-type tra-3 activity to promote XX hermaphrodite development.

**tra-2(e2531eg) transforms XO males into self-fertile hermaphrodites**

To determine whether the tra-2(e2531eg) mutation, by itself, has XO feminising activity, the phenotype of tra-2(e2531eg); him-8 animals was examined. All tra-2(e2531eg); him-8 mutants examined were hermaphrodites (n>100), although 37% of the progeny of XX him-8 hermaphrodites would normally develop as XO males. This result establishes that XO tra-2(eg) mutants are hermaphrodites with normal regulation of tra-2 germ-line activity and not females as are XO tra-2(gf, eg) double mutants. An example of a young adult XO tra-2(e2531eg); him-8 hermaphrodite is shown in Fig. 3A (compare to XX HERM PHAGE in Fig. 3B).

Brood sizes of twenty-seven tra-2(e2531eg); him-8 siblings were counted to determine if XX and XO tra-2(e2531eg); him-8 hermaphrodites produce similarly sized broods. 18/27 animals had a mean brood size of 255.1±32.5 (Table 1, line 2), which is within the range for XX him-8 hermaphrodites (Hodgkin et al., 1979) and 9/27 animals had a mean brood size of 14 (range = 0-48 dead eggs and 0-1 viable). Given that 37% of him-8 animals are predicted to be XO, the 9/27 hermaphrodites producing only 0-1 live progeny are presumed to be XO animals (Table 1, line 3).

**Dominant feminisation of XO animals by tra-2(e2531eg)**

To ask if a single dose of tra-2(e2531eg) feminises XO animals, tra-2(e2531eg) unc-4 homozygotes were mated with wild-type XO males to produce heterozygous tra-2(e2531eg) unc-4/+ cross progeny. Half of the cross progeny were expected to be XO. All of the cross progeny were hermaphrodite (n=34), indicating that one dose of tra-2(eg) results in dominant feminisation of XO animals. However, the tails of 17 of the 34 animals were incompletely feminised (Fig. 4).

Two further experiments showed that the incomplete tail feminisation of tra-2(e2531eg)/+ heterozygotes is limited to XO animals. First, XX tra-2(e2531eg) unc-4 hermaphrodites were mated with sexually transformed XX tra-2(q276) males to produce XX tra-2(eg) heterozygotes. None of the XX tra-2(e2531eg) unc-4/tra-2(q276) heterozygotes had incompletely feminised tails (n=56), which correlates with the absence of XO animals. Second, tra-2(e2531eg); unc-7 hermaphrodites were mated to wild-type XO males and their progeny examined. unc-7 is an X-linked marker gene: XX unc-7/+ heterozygotes are wild-type in phenotype, whereas XO hemizygotes are uncoordinated (Unc). The tails of all non-Unc XX hermaphrodites were fully feminised (n=47), whereas 31/41 Unc hermaphrodites, which were expected to be XO, had incompletely feminised tails. The remaining 10/41 Unc hermaphrodites had completely feminised tails and thus were likely to be XX self-progeny.
Fig. 3. XO tra-2(eg) mutants are transformed from male to hermaphrodite. (A) Top, Nomarski DIC photomicrograph (400×) of adult XO tra-2(e2531eg) hermaphrodite, lateral view. Bottom, schematic representation of photomicrograph. (B) Top, For comparison, Nomarski DIC photomicrograph (400×) of adult wild-type XX hermaphrodite, lateral view. Bottom, schematic representation of photomicrograph. Scale bar, 20 μm.

Fig. 4. Single-dose of tra-2(eg) produces incomplete tail feminisation of XO tra-2(eg)/+ hermaphrodites. Nomarski DIC photomicrographs (400×). (A) Adult XX hermaphrodite tail spike, for comparison. (B-D) Adult XO tra-2(e2531eg)/+ hermaphrodites showing incomplete tail feminisation, exemplified by truncated tail spikes (B-D) and swelling near anus (C). Scale bar, 10 μm.
Table 2. Relative amounts of tra-2 transcripts*

<table>
<thead>
<tr>
<th>XX wild-type</th>
<th>4.7 kb tra-2</th>
<th>1.8 kb tra-2</th>
<th>myo-1</th>
<th>4.7 kb tra-2</th>
<th>1.8 kb tra-2</th>
<th>myo-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.74</td>
<td>5.38</td>
<td>12.85</td>
<td>1.0†</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>XX tra-2(e2531eg)</td>
<td>7.32</td>
<td>12.5</td>
<td>21.8</td>
<td>1.28</td>
<td>1.97</td>
<td></td>
</tr>
</tbody>
</table>

*Values (in arbitrary units) were determined by scanning laser densitometry of the northern blot in Fig. 6A,B, with an average of two to three scans through different regions of the indicated bands. Values for myo-1 were determined after rehybridising the blot in Fig. 6A with a myo-1 probe (Fig. 7B).

†The ratio of wild-type 4.7 kb tra-2/myo-1 mRNA was set to equal 1.0. All other ratios are expressed as values relative to 1.0.

**tra-2(e2531eg) activity is insensitive to her-1(gf) activity**

XO-specific her-1 activity negatively regulates tra-2 activity in males. The similarity between the XO tra-2(eg) and XO her-1 mutant phenotypes suggests that tra-2(eg) activity is specifically insensitive to negative regulation by her-1. To test this hypothesis, the phenotypes of XX and XO tra-2(e2531eg) unc-4; her-1(gf) him-5 mutants were examined. This strain carries the her-1(gf) mutation, which results in inappropriate expression of her-1 mRNAs and makes it possible to examine the effect of elevated her-1 activity in XX animals (Trent et al., 1991). It was found that all tra-2(e2531eg) unc-4; her-1(gf) him-5 mutants are fully hermaphrodite (n>100); no partially or fully masculinised animals were detected, although these would normally be abundant in a population of animals carrying the her-1(gf) him-5 double mutation. Therefore, the only difference between tra-2(eg) and wild-type tra-2 activities in XX animals, so far detected, is that tra-2(eg) activity can suppress inappropriate her-1(gf) masculinising activity and wild-type tra-2 activity can not. All other aspects of wild-type tra-2 function and regulation appear to be preserved in XX tra-2(eg) mutants.

**The tra-2(e2531eg) mutation does not affect steady-state tra-2 mRNA levels**

Northern blot analysis was used to compare steady-state tra-2 mRNA levels in XX tra-2(eg) mutants to those found in wild-type hermaphrodites. As shown in Fig. 5, adult XX tra-2(e2531eg) and wild-type hermaphrodites each express a 4.7 kb and 1.8 kb tra-2 mRNA, and no novel tra-2 mRNAs are present in XX tra-2(e2531eg) hermaphrodites. Moreover, there is no significant difference in the levels of either the 4.7 kb or 1.8 kb tra-2 mRNA in XX tra-2(e2531eg) or in wild-type hermaphrodites, after normalising for differences in sample loads (see Table 2; Materials and Methods). Therefore, the suppression of her-1(gf) by tra-2(eg) in XX animals is unlikely to result from increased tra-2 mRNA steady-state levels, which might lead to increased TRA-2A protein, or from the expression of novel tra-2 products. By the same logic, it is unlikely that elevated tra-2 mRNA levels are responsible for transforming XO tra-2(eg) mutants into hermaphrodites, although tra-2 mRNA levels have not been measured in XO tra-2(eg) mutants.

**Potential interaction between the sex determination and dosage compensation pathways**

To test the possibility that improper activation of dosage compensation might be responsible for the low fertility of XO tra-2(eg) mothers, a tra-2(e2531eg); dpy-26 strain was constructed. dpy-26 animals are defective in dosage compensation: XX animals are dead or Dpy (dumpy) and XO animals are phenotypically wild type (Hodgkin, 1983; Plenefisch et al., 1989). Surprisingly, the fertility of XO tra-2(e2531eg); dpy-26 (m-ze) hermaphrodites was dramatically improved when compared to XO tra-2(e2531eg) hermaphrodites. The mean brood size of XO tra-2(e2531eg); dpy-26 (m-ze) hermaphrodites was increased from 14 (range = 0-48) to 204±17.4 (n=6) (Table 1, compare line 3 to lines 4, 5). 1/4 of the brood developed into wild-type adults and 3/4 were dead or Dpy (Hodgkin, 1980). To show that the him-8 mutation is not responsible for the low fertility of XO tra-2(eg); him-8 mutants, the mean brood sizes of XO tra-2(eg); dpy-26(m-ze) him-8 and XO tra-2(eg) unc-4; dpy-26(m-ze) him-8 mutants were also determined. As shown in Table 1 (lines 6, 7), the dpy-26 mutation still increases the fertility of XO tra-2(eg) mutants when the him-8 mutation is present. It has been postulated that dpy-26 mutations primarily affect XX animals and have no effect on XO animals (Hodgkin, 1983; Plenefisch et al., 1989). The finding that dpy-26
increases the fertility of XO tra-2(e2531eg); dpy-26 (m-2) mutants suggests that there may be communication between the sex-determination and dosage compensation pathways (Figs 1, 6).

**DISCUSSION**

**tra-2(eg) alleles define a novel class of tra-2 feminising alleles that transforms XO males into hermaphrodites**

The molecular basis of the tra-2(eg) mutation is distinct from that of previously characterised tra-2(gf) and tra-2(mx) feminising mutations (Goodwin et al., 1993; P. Kuwabara, P. Okkema and J. Kimble, unpublished). Each of ten tra-2(eg) mutants has been shown to carry an identical tra-2 (G566A) base change, which is not present in their parent. By contrast, the tra-2(gf) mutations disrupt a 28 nt repeat element in the 3′ UTR (Kuwabara et al., 1992; Goodwin et al., 1993), and the tra-2(mx) mutations encode missense changes in a carboxy terminal domain of TRA-2A (P. Kuwabara, P. Okkema and J. Kimble, unpublished data).

Separation of the tra-2(e2046gf, e2531eg) double mutant by intragenic recombination also shows that, in XX animals, the tra-2(e2531eg) mutation, by itself, promotes entirely wild-type hermaphrodite development and, in combination with either a fem-3(gf) or tra-3 mutation, reveals no enhanced feminising activity. By contrast, tra-2 activity is deregulated and reveals gain-of-function character in XO tra-2(eg) mutants, which are transformed into self-fertile hermaphrodites, identical in phenotype to XO her-1 mutants (Fig. 3A,B). In both XX and XO tra-2(eg) mutants, germ-line-specific controls of tra-2 activity are retained. The sexual transformation of XO animals requires only a single dose of tra-2(eg); however, the tails of XO heterozygotes are incompletely feminised, supporting the notion that the relative amounts of HER-1 and TRA-2A are critical to controlling sexual cell fate (Kuwabara and Kimble, 1995).

**The tra-2(e2531eg) mutation may identify a site that is essential for TRA-2A binding activity**

The tra-2(eg) mutation affects only the 4.7 kb tra-2 mRNA sequence (Fig. 2A), indicating that an alteration in the activity or regulation of TRA-2A is probably responsible for the feminisation of XO males. It has been shown that heat-shock-driven overexpression of TRA-2A transforms XO animals to hermaphrodites, presumably by titrating HER-1 (Kuwabara and Kimble, 1995). There is no evidence, however, that indicates tra-2(eg) activity is hypermorphic and feminises XO animals by titrating HER-1. First, there is no increase in the steady-state levels of tra-2 mRNAs in tra-2(eg) mutants, which might lead to corresponding increases in TRA-2 proteins. Second, tra-2(e2531eg) activity remains dependent on wild-type tra-3 activity, whereas strong hypermorphic tra-2(gf) mutations can function independently of tra-3 (Hodgkin, 1986; Doniach, 1986). Third, unlike strong hypermorphic tra-2(gf) mutations (Goodwin et al., 1993), the tra-2(eg) mutation does not enhance germ-line feminisation.

A more plausible explanation is that the tra-2(e2531eg) mutation encodes a TRA-2A that is insensitive to negative regulation by HER-1. This is supported by the similarity between the XO tra-2(eg) and XO her-1 mutant phenotypes, and also by the finding that inappropriate her-1(gf) activity fails to masculinise XX tra-2(eg) mutants. The simplest model postulates that TRA-2A (R177K) fails to bind HER-1, leaving TRA-2A (R177K) free to negatively regulate the FEM proteins and TRA-1 active (Fig. 1B). It remains possible that HER-1 binds to TRA-2A (R177K), but TRA-2A (R177K) fails to release the FEM proteins from inhibition. This might occur because HER-1 binds poorly and fails to induce an allosteric transition.

It may appear surprising that all ten tra-2(eg) mutations, which are known to be independent isolates, are identical. However, the generation of a tra-2(eg) mutation is probably a rare event, because tra-2(eg) mutants were not recovered in previous genetic screens. It could be argued that only a conservative amino acid change could disrupt a potential TRA-2A/HER-1 interaction and, at the same time, preserve all other aspects of normal TRA-2A function and regulation. This does
Development. However, the dosage compensation genes can be obtained because of the low fertility of sexually transformed X0 (Hodgkin and Albertson, 1995). One might speculate that the mutation in another dosage compensation gene, gene might be a general phenomenon. Indeed, the original hermaphrodites carrying a mutation in a dosage compensation gene, therefore, the increased fertility of sexually transformed X0 hermaphrodites carrying a mutation in a dosage compensation gene might be a general phenomenon. Indeed, the original genetic selection for tra-2(eg) mutations was dependent on a mutation in another dosage compensation gene, dpy-28 (Hodgkin and Albertson, 1995). One might speculate that the X0 tra-2(eg) feminising mutations were not previously obtained because of the low fertility of sexually transformed X0 tra-2(eg) mutants. These observations indicate that dpy-26, and likely the other dosage compensation dpy genes, are active in a feminised X0 tra-2(eg) mutant and have a detrimental effect on germ-line development. However, the dosage compensation genes can not be active throughout the animal. Otherwise, X0 tra-2(eg) mutants would be expected to have a X0 phenotype (for X0 lethal), similar to that observed in X0 xol-1 mutants, which are inviable because the dosage compensation genes are active and inappropriately down-regulate the single X (Miller et al., 1988). The poor fertility of X0 tra-2(eg) mutants might instead be explained by postulating that the XX program of dosage compensation has been activated, but only in the germ line. If so, then germ-line dosage compensation may be controlled by the decision to develop as an oocyte, not the X:A ratio. This could be achieved either by feedback through the xol and sdc genes or by a more direct pathway that bypasses these genes (Fig. 6). Relatively little is known about the role of the dosage compensation dpy genes in the germ line and it remains possible that they affect other processes not directly related to dosage compensation. Future experiments will explore the interactions between the tra-2(eg) mutation and the genes that regulate dosage compensation.

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REFERENCES


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