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A predicted membrane protein, TRA-2A, directs hermaphrodite development in *Caenorhabditis elegans*

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SUMMARY

The nematode *C. elegans* naturally develops as either an XO male or XX hermaphrodite. The sex-determining gene, *tra-2*, promotes hermaphrodite development in XX animals. This gene encodes a predicted membrane protein, named TRA-2A, which has been proposed to provide the primary feminising activity of the *tra-2* locus. Here, we show that transgenic TRA-2A driven from a heat shock promoter can fully feminise the somatic tissues of XX *tra-2* loss-of-function mutants, which would otherwise develop as male. TRA-2A is thus likely to provide a component of the *tra-2* locus that is both necessary and sufficient to promote female somatic development. Transgenic TRA-2A driven by the heat shock promoter can also transform XO animals from male to self-fertile hermaphrodite. This result establishes the role of *tra-2* as a developmental switch that controls somatic sexual cell fate. We show that a carboxy-terminal region of TRA-2A, predicted to be intracellular, can partially feminise XX *tra-2* loss-of-function mutants and XO *tra-2*(+) males. We suggest that this intracellular domain of TRA-2A promotes hermaphrodite development by negatively regulating the FEM proteins.

Key words: sex determination, *Caenorhabditis elegans*, *tra-2*, cell-cell signalling, feedback regulation

INTRODUCTION

Sex determination in the nematode *C. elegans* has been extensively characterised at the genetic level. Our current understanding of the genetic pathway that controls sexual fate is summarised in Fig. 1A. The primary determinant of sex is the ratio of X chromosomes to sets of autosomes (X:A ratio) (Madl and Herman, 1979). Diploid XX animals develop as hermaphrodites, whereas XO animals become males. A *C. elegans* hermaphrodite is essentially a self-fertile female: the hermaphrodite soma is indistinguishable from that of closely related female nematodes (Baird et al., 1994), but its germ line produces sperm first and then oocytes. A number of genes control sexual fate in response to the X:A ratio (refer to Fig. 1A, for details). These genes have been ordered into a regulatory hierarchy, whereby each gene negatively regulates the activity of genes positioned immediately downstream in the pathway. Genes at the beginning of the pathway control both sex determination and dosage compensation. The pathway then bifurcates; one branch controls sexual phenotype, while the other branch controls dosage compensation. This paper focuses on the pathway controlling sexual phenotype, and more specifically on the role of the *tra-2* gene.

The *tra-2* gene promotes female development in an XX hermaphrodite (Fig. 1A) (Klass et al., 1976, Hodgkin and Brenner, 1977). In the absence of wild-type *tra-2* activity, XX animals are sexually transformed from hermaphrodite to male; XO *tra-2* mutants develop normally as males. *tra-2* activity is required throughout XX larval development (Klass et al., 1976). In somatic tissues, *tra-2* directs XX hermaphrodite development, which is the same as female development. However, the situation is more complex in the germ line, because the feminising activity of *tra-2* must be negatively regulated to allow the onset of XX hermaphrodite spermatogenesis (Doniach, 1986, Schedl and Kimble, 1988). Dominant mutations of *tra-2*, which escape this germline negative regulation, transform the XX germline from hermaphrodite to female. Therefore, *tra-2* can promote female development in both somatic and germline tissues, and must be regulated to achieve hermaphrodite germline development.

A molecular analysis of the *tra-2* locus reveals a complex developmental pattern of transcripts (Okkema and Kimble, 1991). A 4.7 kb *tra-2* mRNA is detected in both XO and XX animals throughout development; however, it is 15-fold more abundant in XX than in XO animals (Okkema and Kimble, 1991). This sex-specific difference in the amount of 4.7 kb *tra-2* mRNA is dependent on the activity of the downstream *tra-1* gene, and provides evidence for a positive feedback loop in the pathway (Fig. 1A) (Okkema and Kimble, 1991). Two other *tra-2* transcripts have also been detected. One is a 1.8 kb *tra-2* mRNA found in XX animals, which appears to be germline-specific in L4 and adult hermaphrodites and is also present in early embryos. The other is a 1.9 kb *tra-2* mRNA found in adult XO males and during larval stages of XX hermaphrodite

development when the germline is undergoing spermatogenesis. The structures and possible functions of the 1.8 kb and 1.9 kb *tra-2* mRNAs will be discussed elsewhere (P. Kuwabara, P. Okkema, and J. Kimble, in preparation). We have proposed that the 4.7 kb *tra-2* mRNA provides the primary feminising component of the *tra-2* locus, based on the developmental pattern of *tra-2* transcripts and on the analysis of *tra-2(lf)* mutations (Kuwabara et al., 1992). The 4.7 kb *tra-2* mRNA encodes a predicted membrane protein called TRA-2A (Kuwabara et al., 1992).

A molecular model for the control of somatic sexual phenotype has been proposed (Fig. 1B; for review, see Kuwabara and Kimble, 1992). The main features of this model are based on the deduced amino acid sequences of cloned genes (*tra-2*, Kuwabara et al., 1992; *her-1*, Perry et al., 1993; *fem-1*, Spence et al., 1990; *fem-3*, Ahringer et al., 1992; *tra-1*, Zarkower and Hodgkin, 1992) and on evidence that specification of sexual fate depends on cell-to-cell communication (Villeneuve and Meyer, 1990; Schedin et al., 1991; Hunter and Wood, 1992). In XX animals, the predicted membrane protein TRA-2A is proposed to promote female development constitutively, because HER-1 is absent (refer to Fig. 1A,B) (Kuwabara et al., 1992; Trent et al., 1991). An intracellular carboxy-terminal region of TRA-2A is postulated to bind and to suppress one or more of the FEM proteins, perhaps through sequestration (Fig. 1B) (Kuwabara et al., 1992). As a consequence, TRA-1, a zinc finger protein and putative transcriptional regulator (Zarkower and Hodgkin, 1992), is free to direct hermaphrodite development.

In XO males, genetic arguments predict that *tra-2* is negatively regulated by *her-1* (Fig.1A) (Hodgkin, 1980). The *her-1* gene functions cell non-autonomously to promote XO male development (Hunter and Wood, 1992). In addition, the HER-1 protein appears to be secreted (Perry et al., 1993). Therefore, HER-1 is an excellent candidate for an antagonist that directly binds to and negatively regulates TRA-2A. This interaction

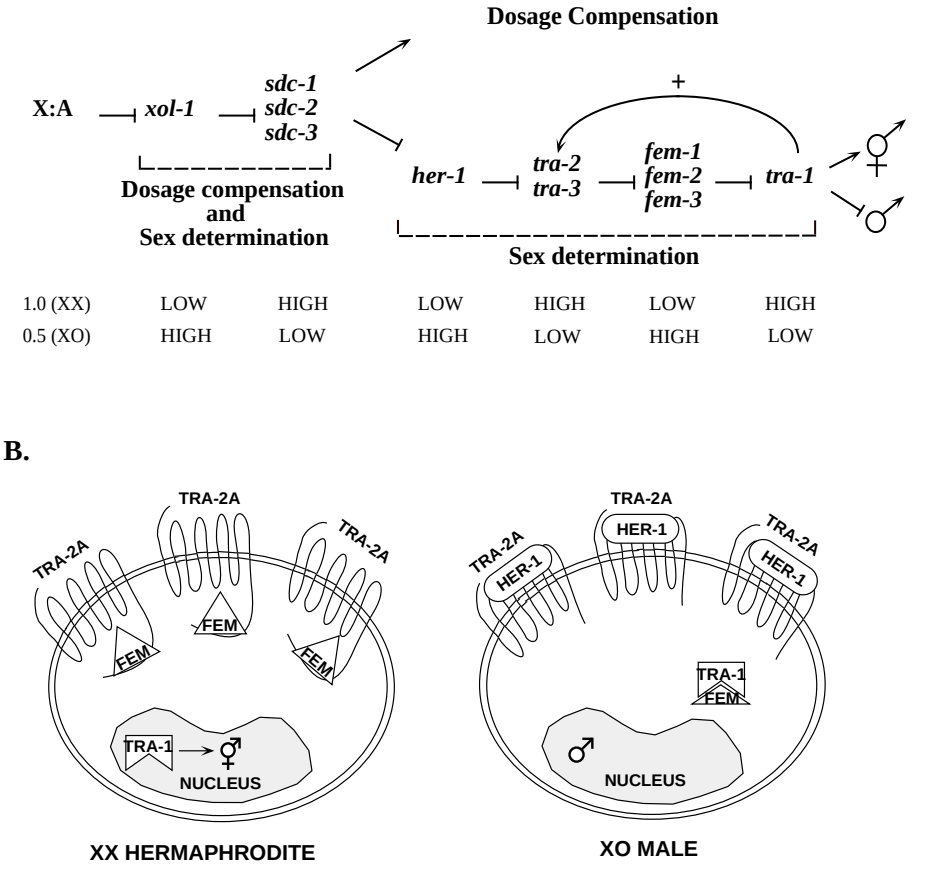

Fig. 1. Regulation of somatic sexual fate in the nematode *C. elegans.* (A) Genetic pathway of dosage compensation and somatic sex determination (from Hodgkin, 1990; Villeneuve and Meyer, 1990). The X:A ratio is the primary determinant of sex – XX animals develop as hermaphrodites and XO animals develop as males (Madl and Herman, 1979). In response to the X:A ratio, genes that regulate dosage compensation and somatic sex determination function as a series of HIGH/LOW switches. At the beginning of the pathway, *xol-1* and *sdc-1, sdc-2 and sdc-3* control both sex determination and dosage compensation. Details of the dosage compensation pathway are beyond the scope of this paper (readers are referred to Villeneuve and Meyer, 1990, for details). In XX hermaphrodites, high *sdc* levels negatively regulate *her-1*. In turn, *tra-2 and tra-3* negatively regulate the *fem* genes. Low *fem* activity permits *tra-1* to promote hermaphrodite development. In somatic tissues, *tra-1* is the terminal regulator of somatic sex determination: high levels of *tra-1* promote hermaphrodite development, whereas low levels result in male development. Included in this model is a proposed positive feedback loop in which *tra-1* activates *tra-2* and reinforces a commitment to the hermaphrodite pathway of differentiation. In XO males, *xol-1* negatively regulates the *sdc* genes, resulting in high *her-1* activity. In turn, *her-1* negatively regulates *tra-2 and tra-3*. Consequently, the *fem* genes are free to negatively regulate *tra-1* and male development ensues. The germline pathway of sex determination involves the same genes described above and additional genes that have germline-specific activities (see Schedl, 1991; Ellis and Kimble, 1994, for details of the germline pathway). (B) Speculative model of protein-protein interactions controlling somatic sex determination, focusing on the role of TRA-2A. Central to this model is the prediction that the 4.7 kb *tra-2* RNA encodes a transmembrane protein, TRA-2A, which provides the primary feminising activity of *tra-2* (Kuwabara et al., 1992). In XX animals, TRA-2A negatively regulates the activity of one or more FEM proteins, thereby allowing TRA-1, a predicted transcription factor (Zarkower and Hodgkin, 1992) to promote hermaphrodite development. In XO males, the activity of TRA-2A is predicted to be negatively regulated by binding HER-1, which functions cell non-autonomously and encodes a secreted protein (Perry et al., 1993, Hunter and Wood, 1992). TRA-2A and HER-1 are thus postulated to mediate cell-to-cell signalling (Kuwabara et al., 1992, Hunter and Wood, 1992). In turn, the FEM proteins repress TRA-1 and male development ensues. In this working model, we suggest that negative regulation of the FEM proteins by TRA-2A and TRA-1 by the FEM proteins may involve sequestration, however, other models exist. A role for TRA-3 in our model for sex determination has been omitted, because *tra-3* has been postulated to be an almost dispensable positive co-factor of *tra-2* (Hodgkin, 1980).

ensures that all cells in a region follow one of the two sexual fates. Inactivation of TRA-2A removes the inhibition of the FEM proteins, and hence allows the FEM proteins to negatively regulate TRA-1 and to promote male development (Fig. 1B).

This paper tests three hypotheses on which the model in Fig. 1B is based. First, we have proposed that the 4.7 kb *tra-2* mRNA provides the primary feminising component of the *tra-2* locus (Kuwabara et al., 1992). We demonstrate here that TRA-2A is both necessary and sufficient to provide *tra-2* somatic feminising activity. Transgenic expression of a full-length cDNA corresponding to the 4.7 kb *tra-2* transcript feminises the somatic tissues of XX animals that lack wild-type *tra-2* activity. Second, we postulate that an increased level of TRA-2A activity is sufficient to transform XO animals into hermaphrodites. We show that expression of TRA-2A driven from a strong promoter can fully transform XO animals from male to hermaphrodite. This suggests that the relative amounts of HER-1 and TRA-2A are crucial for sex determination. Third, we test the hypothesis that an intracellular domain of TRA-2A might, by itself, have feminising activity. We find that an intracellular domain of TRA-2A can indeed partially feminise XX *tra-2* and XO *tra-2*(+) males. Therefore, the TRA-2A intracellular domain appears to be an essential part for regulating the FEM proteins.

MATERIALS AND METHODS

Nematode culture, strains and general handling methods

General methods for genetic manipulation, culturing and microscopy of nematodes have been described (Sulston and Hodgkin, 1988). Standard nomenclature is used in this paper (Horvitz et al., 1979). The suffix *gf* designates gain-of-function and unless otherwise stated it is implicit that all other alleles are loss-of-function (*lf*). Extrachromosomal arrays were transferred to different genetic backgrounds using standard genetic techniques. For information regarding *C. elegans* genes and alleles refer to (Hodgkin et al., 1988). A brief description of genes and alleles used in this paper follows.

tra-2(e1095) II: a putative null allele of the *tra-2* gene (Hodgkin and Brenner, 1977), encodes a TRA-2A protein with a nonsense mutation at amino acid 1290 (Kuwabara et al., 1992). This nonsense change is also present in all other TRA-2 proteins (P. Kuwabara, P. Okkema, and J. Kimble, in preparation).

unc-4(e120) II: a closely linked gene to *tra-2*. Most *tra-2(lf)* strains were maintained as *tra-2unc-4*/*mnC1*. Hence, *tra-2unc-4* homozygotes have an uncoordinated (Unc) phenotype, which permits simple recognition of *tra-2* homozygotes regardless of the sexual phenotype.

mnC1: a rearrangement of chromosome *II*, which suppresses recombination. All strains heterozygous for *tra-2(lf)* were maintained as *tra-2/mnC1*.

him-8(e1489) IV: increases the frequency of XO male progeny from 0.2 to 37%.

dpy-21(e428) V: distinguishes XX from XO animals. XX are dumpy (Dpy), while XO have a normal wild-type length.

Construction of expression plasmids

Methods used for manipulating nucleic acids are described by Sambrook et al. (1989). To construct pJK349, a fragment containing a full-length wild-type 4.7 kb *tra-2* cDNA with 5 bp of 5′ UTR and the entire 3′ untranslated region (3′ UTR) (Kuwabara et al., 1992), flanked by *Xba*I and *Sma*I restriction sites, was ligated into the *Nhe*I-*Eco*RV site of pPD49.83 (D. Dixon, S. White-Harrison, and A. Fire, unpublished data). Translation is predicted to begin at the TRA-2A initiation codon (Kuwabara et al., 1992). A similar *tra-2* expression clone was also constructed in the heat shock vector, pPD49.79 (D. Dixon, S. White-Harrison, and A. Fire, unpublished data). However, this clone and pJK349 function similarly in transgenic animals, hence only results with pJK349 are described. pPK81 is a derivative of pJK349 that replaces the wild-type *tra-2* 3′ UTR with the *tra-2*(*e2020*gf) 3′ UTR (Goodwin et al., 1993). pPK83 is a derivative of pPK81 that replaces the *Sph*I-*Xma*I fragment of the heat shock promoter with a 2.9 kb *Sph*I-*Xma*I *her-1* P2 promoter fragment from pWLG1(Perry et al., 1993). The *Sph*I-*Xma*I fragment provides *her-1* P2 promoter activity, although it removes ~560 bp from the 5′ end of the originally defined *her-1* P2 promoter (Perry et al., 1993). pPK58 was constructed by ligating a *Kpn*I-*Sma*I restriction fragment, containing bases 2787-4464 of the 4.7 kb *tra-2* cDNA coding sequence (Kuwabara et al., 1992), into the *Kpn*I-*Eco*RV site of pPD49.83. Expression of pPK58 is predicted to produce a 387 amino acid protein named TRA-2B, which starts at M^{1089}, based on the numbering of amino acids in TRA-2A (Kuwabara et al., 1992). All plasmids contain both a *tra-2* and *unc-54* 3′ UTR and an artificial intron between the promoter and coding region (D. Dixon, S. White-Harrison, and A. Fire, unpublished data).

Generation of transgenic nematodes

Standard methods were used to generate transgenic worms (Fire, 1986; Mello et al., 1991). All DNA injection solutions contained 100 μg/ml pRF4 and 10-20 μg/ml of appropriate expression plasmid. pRF4 carries a dominant marker, *rol-6(su1006)*, which is used to identify transgenic animals, because they have a Rol phenotype (Mello et al., 1991). Only transgenic animals that heritably transmit extrachromosomal arrays to F_2 progeny were maintained for further analysis. Single-worm PCR, using a unique plasmid primer and *tra-2* primer, was used to verify that transgenic animals carried not only pRF4, but also the expression plasmid (Williams et al., 1992). The composition of extrachromosomal arrays is described below (see Fig. 2 and above for description of plasmids).

Extrachromosomal array	Transgene (plasmid)
qEx32	HS-TRA-2A (pJK349) + pRF 4
crEx2	HS-TRA-2A (3′ UTRgf) (pPK81) + pRF4
crEx1	P2-TRA-2A (pPK83) + pRF4
qEx35	HS-TRA-2B (pPK58) + pRF4

Heat shock and phenotypic analysis of transgenic nematodes

Unless otherwise stated, the progeny of gravid Rol hermaphrodites, carrying heat shock driven transgenes, were subjected to a total of three heat shocks beginning at the late embryo/early L1 stage of development. Each heat shock consisted of a 2 hour incubation at 33°C, followed by a recovery period of 24 hours at 23°C. The effects of single heat shocks were observed by subjecting a mixed-stage population to a single 2 hour incubation at 33°C and examining animals of the appropriate genotype when they reached the adult stage. The sexual phenotypes of the somatic gonad, tail, hypodermis, intestine, and germ line were scored in adult transgenic animals using Nomarski DIC optics (400× or 630×). Somatic tissues were considered feminised if they displayed one or more of the following hermaphroditic or intersexual characteristics.

somatic gonad: complete or partial bi-lobed arms.

tail: hermaphrodite spike; truncated or missing fan, rays, or spicules.

hypodermis: complete or partial vulval induction.

intestine: yolk protein accumulation in the pseudocoelom.

germ line: oocytes.

For some experiments, adult male animals were heat shocked once at 33°C for 2 hours and scored for germline and intestinal phenotypes 24 hours after heat shock.

Controls: feminisation by *tra-2* transgenes is heat shock dependent (n>>100), except pPK83, which is driven by the *her-1* P2 promoter.

Heat shock does not feminise either XX *tra-2* or *tra-2*(+) XO transgenic animals that carry a heat shock driven *lacZ* transgene, which has no *tra-2* activity (kindly provided by A. Fire) (n>>100).

SDS polyacrylamide gel electrophoresis

A 7% polyacrylamide gel with a 4.75% stacking gel was prepared as described (Sambrook et al., 1989). Samples were prepared by washing hand-picked worms in M9 salts three times before resuspending each worm pellet in 2× SDS gel sample buffer. Prior to loading, samples were heated to 95°C for 10 minutes. Gels were stained after electrophoresis with Coomassie Blue.

RESULTS

HS-TRA-2A feminises the soma of XX *tra-2* mutants

To test whether TRA-2A promotes hermaphrodite development when introduced as a transgene, we examined the effect of HS-TRA-2A (Fig. 2) on the sexual phenotype among the self-progeny of XX *tra-2unc-4*/ + +;*qEx32* hermaphrodites. Without heat shock, transgenic roller animals are either non-Unc hermaphrodites of genotype *tra-2unc-4*/++;*qEx32* or ++/++;*qEx32* or Unc pseudomales of genotype *tra-2unc-4;qEx32* (n>>100). However, after a series of heat shocks, many of the XX *tra-2unc-4;qEx32* homozygotes (identified by their uncoordinated phenotype) are clearly feminised (see Materials and Methods for heat shock regime and scoring criteria for sexual phenotypes). HS-TRA-2A extensively feminises the gonad, tail, hypodermis, and intestine of XX *tra-2unc-4;qEx32* animals, but does not feminise the germline of XX *tra-2unc-4;qEx32* animals: the somatic gonad contains sperm but not oocytes (Table 1, line 1). An example of an XX *tra-2unc-4;qEx32* transformant is shown in Fig. 3A. This transformant has a virtually wild-type hermaphrodite soma, yet its germ line produces only sperm. XX *tra-2unc-4;qEx32* mutants that receive only a single heat shock (see Materials and Methods) also show somatic feminisation. However, these animals are not as extensively feminised as animals that receive multiple heat shocks (data not shown).

Two additional approaches were taken in an attempt to see an effect of HS-TRA-2A on the XX germ line. First, we searched among XX *tra-2unc-4*/++;*qEx32* and +/+;*qEx32* animals after heat shock for the presence of phenotypic females. We reasoned that the combination of endogenous *tra-2*(+) activity plus transgenic HS-TRA-2A might produce a dominant gain-of-function phenotype similar to that observed in *tra-2(gf)* mutants (Doniach, 1986; Schedl and Kimble, 1988). However, no females were detected (n>>100). Second, we generated the transgene HS-TRA-2A(3′ UTRgf) by methods similar to those used to generate HS-TRA-2A (see Materials and Methods). HS-TRA-2A(3′ UTRgf) carries a deletion within the *tra-2* 3′ UTR, which permits *tra-2* to escape negative translational control (Goodwin et al., 1993). We

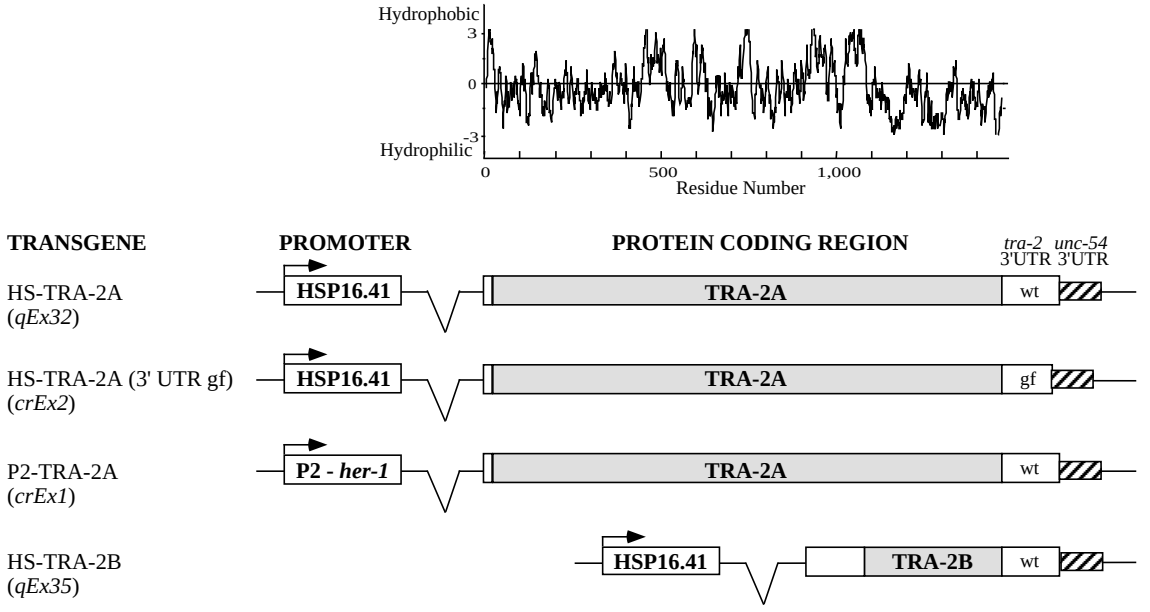

Fig. 2. Transgenes carrying *tra-2* cDNA sequences. Above, Kyte-Doolittle hydropathy plot (Kyte and Doolittle, 1982) indicates that TRA-2A is likely to be a transmembrane protein, because it contains a potential secretion signal sequence and transmembrane helices (Kuwabara et al., 1992). Below, *tra-2* transgenes (for details on construction, see Materials and Methods). The HS-TRA-2A transgene expresses a full-length TRA-2A protein driven by the *C. elegans* heat shock promoter *hsp16* (Stringham et al., 1992; D. Dixon, S. White-Harrison, and A. Fire, unpublished data). The HS-TRA-2A (3′ UTR gf) transgene is identical to HS-TRA-2A, except that it carries the *tra-2(e2020*gf) 3′ UTR (Goodwin et al., 1993), in place of a wild-type *tra-2* 3′ UTR. P2-TRA-2A expresses a full-length TRA-2A protein driven by the *C. elegans her-1* P2 promoter, which is XO-specific (Perry et al., 1993). The HS-TRA-2B transgene expresses the carboxy-terminal 387 amino acids of TRA-2A driven from the *C. elegans* heat shock promoter *hsp-16.* Hydropathy analysis indicates that HS-TRA-2B is likely to be cytoplasmic, because it lacks any hydrophobic domains. This construct has been named HS-TRA-2B, because it is predicted to have the same sequence as TRA-2B, the product of the 1.8 kb *tra-2* mRNA (Kuwabara, Okkema, and Kimble, unpublished data). The extrachromosomal array that was used to study the expression of a specific transgene is listed in parentheses. All constructs carry both a *tra-2* 3′ UTR and *unc-54* 3′ UTR, and an artificial intron placed between the promoter and *tra-2* coding sequence. P, promoter with arrow indicating direction of transcription. Stippled boxes, TRA-2A coding sequence: unshaded boxes, untranslated regions; hatched box, *unc-54* 3′ UTR.

Table 1. HS-TRA-2A feminises the soma of XX and XO *tra-2* mutants and sexually transforms XO *tra-2*(+) nematodes from male to hermaphrodite

Genotype*	Number of animals feminised after heat shock/total animals examined†				
	Somatic gonad	Tail	Hypodermis	Intestine	Oocytes
(Multiple heat shocks)					
XX *tra-2;qEx32*	21/22	19/22	21/22	21/22	0/22
XO *tra-2*(+);*qEx32*	18/20	11/20	13/20	17/20	14/20
(Single heat shock)					
XO *tra-2*(+);*qEx32*	NA	NA	NA	20/20	14/20
XX *tra-2;qEx32*‡	NA	NA	NA	6/30	0/30
XO *tra-2;qEx32*	NA	NA	NA	19/20	0/20

NA, not applicable.

*Descriptions of genes, alleles, and transgenes are provided in Materials and Methods. Line 1, XX *tra-2unc-4;qEx32*. Line 2, XO *tra-2(+);dpy-21;him-8;qEx32*. Line 3, XO *tra-2(+);qEx32*. Line 4, XX *tra-2unc-4;qEx32*. Line 5, XO *tra-2;qEx32*.

†Refer to Materials and Methods for heat shock regime and scoring criteria to assay tissue feminisation. No feminisation was detected in transgenic animals not subjected to heat shock, n>>100 for each genotype listed.

‡XX *tra-2* pseudomale, non-mating.

found that the soma of XX *tra-2unc-4;crEx2* homozygous animals expressing HS-TRA-2A(3′ UTRgf) is feminised to the same extent as XX *tra-2unc-4;qEx32* transgenic animals (data not shown), and the germ line is not feminised. HS-TRA-2A(3′ UTRgf) also fails to feminise the germ line of wild-type animals (eg. ++/++; *crEx2*).

We conclude that HS-TRA2A can provide the major somatic feminising activity of *tra-2*. The failure to detect oocytes in XX *tra-2unc-4;qEx32* animals suggests that either the heat shock promoter does not function in the germ line (Stringham et al., 1992) or that an additional *tra-2* gene product is required for oogenesis.

Transgenic TRA-2A transforms XO males into hermaphrodites

In XO males, secreted HER-1 is postulated to bind and to inactivate TRA-2A (Fig. 1B) (Kuwabara et al., 1992, Hunter and Wood, 1992). From this model, we predict that an elevated level of TRA-2A might escape HER-1 regulation and hence feminise XO animals (Kuwabara et al., 1992). To test this hypothesis, we asked if HS-TRA-2A could feminise XO animals. The strain constructed for this experiment was *dpy-21;him-8;qEx32*. This strain carries *dpy-21* to permit us to distinguish XX (Dpy) from XO (non-Dpy) animals (Hodgkin, 1983), *him-8*, which generates 37% XO animals, and *qEx32*, the extrachromosomal array bearing HS-TRA-2A. After a series of heat shocks, we examined non-Dpy adult XO animals by Nomarski DIC optics. We found that HS-TRA-2A feminised not only somatic tissues, but also the germ line of XO animals (Table 1, line 2). All XO animals with feminised germ lines produced sperm first, then oocytes - indicating that HS-TRA-2A expression results in hermaphrodite rather than female germline development. These animals are often self-fertile, albeit with low brood sizes <10. Many of the brood die as embryos, but occasional animals develop into adult males (data not shown). An example of an XO *tra-2*(+);*qEx32* transgenic animal is shown in Fig. 3B. This XO animal is fully transformed from a male to a self-fertile hermaphrodite by HS-TRA-2A, although it has a slightly snubbed tail. We conclude that HS-TRA-2A is capable of feminising all XO tissues, including the germ line.

In a separate set of experiments, the 4.7 kb *tra-2* cDNA was expressed in XO animals under control of the *her-1* P2 promoter (P2-TRA-2A) (Fig. 2). The purpose of this experiment was to verify that expression of P2-TRA-2A from the extrachromosomal array, *crEx1*, could feminise XO males under non-heat shock conditions. P2-TRA-2A was not expected to transform XO males completely into hermaphrodites. *tra-2* activity is required throughout larval development (Klass et al., 1976), whereas the *her-1* P2 promoter is active primarily during early stages of XO, but not XX development (Perry et al., 1993). An example of an XO *tra-2*(+);*crEx1* male with intersexual somatic development is provided in Fig. 3. Thus, we have shown in two independent experiments that transgenic TRA-2A feminises somatic tissues of XO animals, presumably by overcoming negative regulation by HER-1.

Feminising activity associated with a putative intracellular carboxy-terminal domain of TRA-2A

We have hypothesised that a carboxy-terminal domain of TRA-2A negatively regulates one or more of the FEM proteins through a protein-protein interaction in the cytoplasm (Fig. 1B) (Kuwabara et al., 1992). If true, then expression of the carboxy-terminal region of TRA-2A, by itself, might have feminising activity. To test this possibility, we asked if HS-TRA-2B could feminise XX *tra-2unc-4;qEx35* mutants. HS-TRA-2B consists of the carboxy-terminal 387 amino acids of TRA-2A and is predicted to be cytoplasmic, because it lacks any hydrophobic region that might function as a signal sequence or as a membrane spanning domain (Fig. 2). HS-TRA-2B is also predicted to encode the same TRA-2B protein as the 1.8 kb *tra-2* mRNA (P. Kuwabara, P. Okkema, and J. Kimble, in preparation). We found that a number of XX *tra-2unc-4;qEx35* Unc homozygotes had partially feminised tails after a series of heat shocks. However, these feminised animals did not also display the Rol phenotype, which is diagnostic of animals carrying an extrachromosomal array. Nomarski DIC optics revealed that these feminised animals probably failed to roll because they suffered from severe defecation defects, the result of intersexual tail development. Therefore, we initially examined all Unc animals (*tra-2unc-4;qEx35* and *tra-2unc-4*) to determine the range of XX *tra-2unc-4;qEx35* phenotypes, although not all Unc animals are expected to express the transgene. We found that 9/41 animals had partially feminised

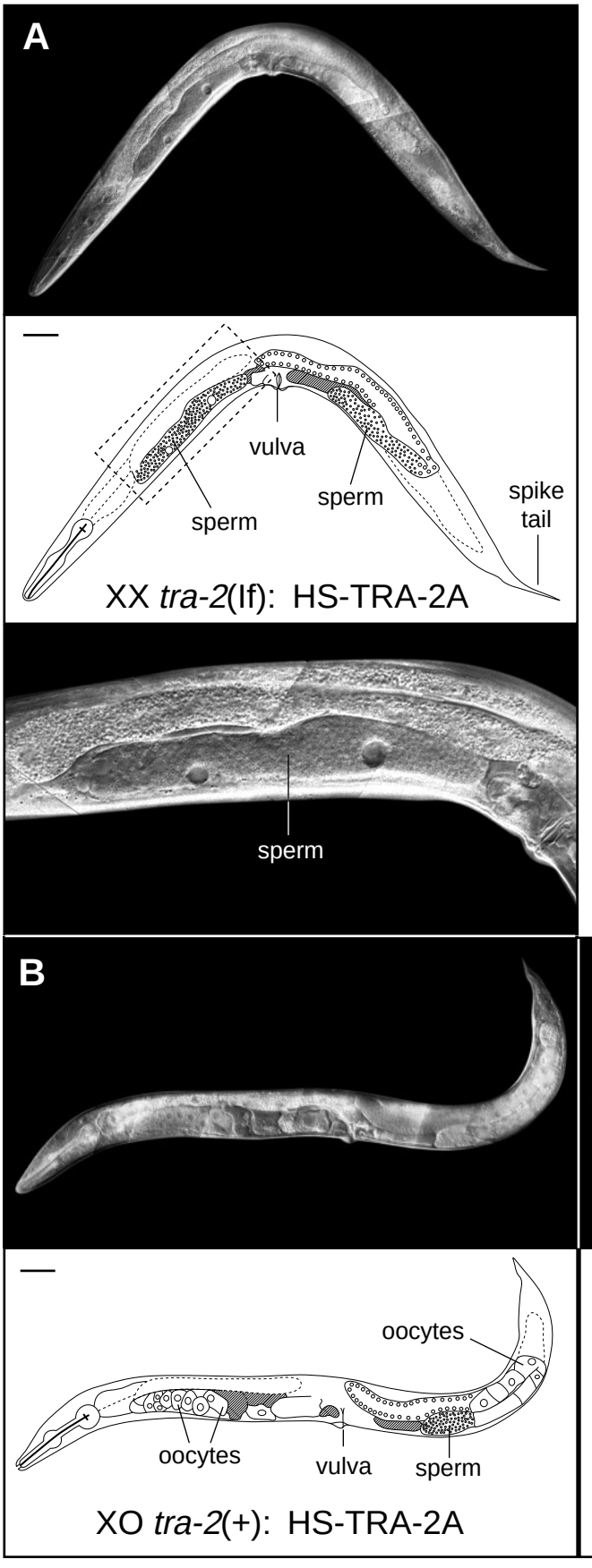

Fig. 3. Transgenic TRA-2A promotes feminisation of XX *tra-2* and XO *tra-2*(+) animals. Top, Nomarski DIC photomicrograph (630×) of adult transgenic animal, lateral view. Bottom, schematic representation of photomicrograph, except panel A. (A) Adult XX *tra-2unc-4;qEx32* mutant transformed from pseudomale to hermaphrodite by HS-TRA-2A after a series of heat shocks. Feminised somatic tissues include: bi-lobed somatic gonad, vulva, spiked tail, and intestine (yolk). Sperm, but not oocytes are present in each lobe of the somatic gonad. Middle, schematic representation of photomicrograph. Bottom, enlargement of photomicrograph, which corresponds to boxed region in middle panel, details the presence of sperm, but not oocytes in the germ line. (B) Adult XO *tra-2*(+);*dpy-21;him-8;qEx32* animal transformed by HS-TRA-2A from male to fertile hermaphrodite after a series of heat shocks. Feminised somatic tissues include: bi-lobed somatic gonad, vulva, spiked tail, and intestine (yolk). The snub tail is incompletely feminised. Sperm and oocytes are present in each lobe of the somatic gonad. (C) Adult XO *tra-2*(+);*him-8;crEx1* intersexual animal. The somatic gonad, vulva, tail, and intestine (yolk) are partially feminised by P2-HER-1, however, masculinised tail structures such as a ray and truncated fan are also visible. Sperm, but not oocytes are visible. Scale bar, 10 μm.

Table 2. HS-TRA-2B feminises the intestine of XX *tra-2* and XO *tra-2*(+) males

Genotype*	Number animals with intestinal feminisation† / Total number examined	
	+	–
XX *tra-2;qEx35*‡	26/30	0/50
XO *tra-2*(+);*qEx35*	35/35	0/50

*Descriptions of genes, alleles, and transgenes are provided in Fig. 2 and Materials and Methods. Line 1, XX *tra-2unc-4; qEx35*. Line 2, XO *tra-2*(+);*him-8; qEx35*.

†Intestinal feminisation was assayed in non-constipated animals by the accumulation of yolk in the pseudocoelom. Refer to Materials and Methods for heat shock regime. (+/–), with or without heat shock, respectively.

‡XX *tra-2* pseudomale, non-mating.

tails or defecation defects arising from intersexual tail development, 14/41 animals accumulated yolk in the pseudocoelom, which is indicative of intestinal feminisation, and 2/41 had partially feminised tails and accumulated yolk. In addition, 16/41 Unc animals were not feminised, as might be expected if they did not carry or express the transgene. None of the animals examined showed feminisation of the germ line.

The number of XX *tra-2unc-4;qEx35* animals accumulating yolk in response to heat shock was probably underestimated, because it is difficult to score visually for yolk in nematodes with severe defecation defects. Therefore, in a second experiment, we used Nomarski DIC optics and SDS polyacrylamide gel electrophoresis to examine transgenic animals that were clearly XX *tra-2;qEx35* Unc rollers and not defecation defective. We found that 26/30 XX *tra-2unc-4;qEx35* Unc rollers accumulated yolk after heat shock (Table 2, line 1). Analysis by SDS polyacrylamide electrophoresis verified that XX *tra-2unc-4;qEx35* mutants produce yolk only in response to heat shock (Fig. 4, compare lanes 3,4). We also found that HS-TRA-2B induced yolk accumulation in 35/35 XO *tra-2*(+);*him-8;qEx35* males (Table 2, line 2). Again, yolk accumulation in these animals is heat shock dependent (Fig. 4, compare lanes 1, 2). In addition, none of the XO *tra-2*(+);*him-8;qEx35* males showed feminisation of the germ line. We conclude that ectopic expression of the carboxy terminus of TRA-2A has feminising activity in both XX *tra-2unc-4;qEx35* and XO *tra-2*(+);*him-8;qEx35* males. Therefore, these results support the hypothesis that the carboxy-terminal region of TRA-2A contains a domain involved in negatively regulating the FEM proteins.

HS-TRA-2A requires an endogenous wild-type *tra-2* gene to promote germline feminisation

It might be predicted that HS-TRA2A should feminise XX *tra-2* animals more efficiently than XO *tra-2*(+) animals, because TRA-2A is not inactivated by HER-1 in XX animals. Therefore, it was a surprise to find that HS-TRA-2A feminised both the soma and germ line of XO *tra-2*(+);*qEx32* transgenic animals, yet failed to feminise the germ line of XX *tra-2;qEx32* mutants. These results suggested that the feminising effects of HS-TRA-2A were more extensive in XO *tra-2*(+);*qEx32* animals than in XX *tra-2; qEx32* mutants, because the former carried a wild-type *tra-2* gene. To test this idea, we asked if HS-TRA-2A could feminise the germline of XO mutants that lack a wild-type *tra-2* gene. For this study, we constructed the strain *tra-2;tra-1(e1575*gf)/+;*qEx32*, using methods similar to those described by Hodgkin (1980). This strain produces two kinds of males: XO *tra-2;qEx32* and XX *tra-2;qEx32*. An XO *tra-2;qEx32* male can be distinguished from an XX *tra-2;qEx32* male by adult tail morphology and mating behaviour. Therefore, for this experiment we selected adult males of the appropriate genotype and subjected them to a single heat shock. First, we established that applying a single heat shock to an adult XO *tra-2*(+);*qEx32* or XX *tra-2;qEx32* male has the same effect on germline and intestinal phenotype as applying a series of heat shocks throughout development. We found that after a single heat shock, both the intestine and germ line of adult XO *tra-2*(+);*him-8; qEx32* males were feminised by HS-TRA-2A (Table 1, line 3). An example of an adult XO *tra-2*(+);*him-8;qEx32* male that produced both yolk and oocytes in response to HS-TRA-2A is shown in Fig. 5. Under the same conditions, HS-TRA-2A feminised the intestine (yolk) of adult XX *tra-2;qEx32* males, but again failed to feminise the germ line (Table 1, line 4). Thus, applying a single heat shock to an adult, which carries the *qEx32* transgene, appears to have the same effect on the germline and intestinal phenotype as applying a series of developmental heat shocks. Next, we examined the effect of HS-TRA-2A on the phenotype of adult XO *tra-2;qEx32* males, which lack a wild-type *tra-2* gene. We found that HS-TRA-2A feminised the intestine (yolk) of XO *tra-2;qEx32* males, but failed to feminise the germ line (Table 1, line 5). Therefore, we conclude that HS-

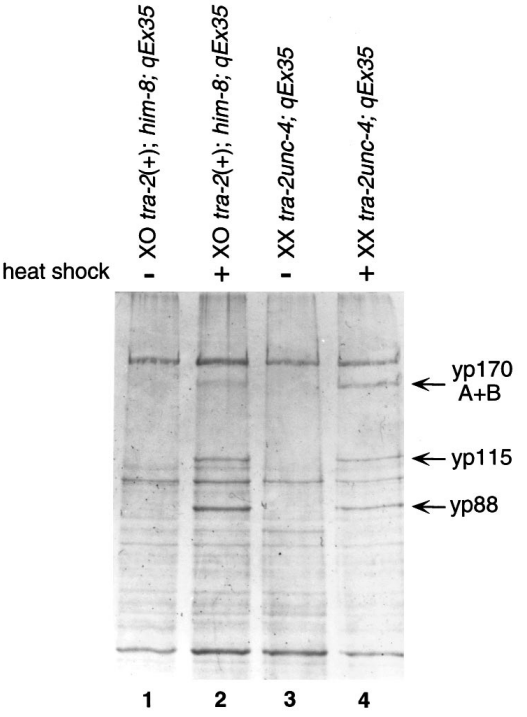

Fig. 4. HS-TRA-2B promotes partial feminisation of XO *tra-2*(+);*qEx35* and XX *tra-2unc-4;qEx35* transgenic nematodes. SDS polyacrylamide gel showing the accumulation of four yolk proteins: yp170A, yp170B, yp115, and yp88 (Sharrock, 1983) in response to heat shock driven expression of HS-TRA-2B. Each lane contains 30 animals of the specified genotype. The position and molecular mass ($\times 10^{-3}$) of each yolk protein is marked by an arrow. (+/–) indicates with or without heat shock.

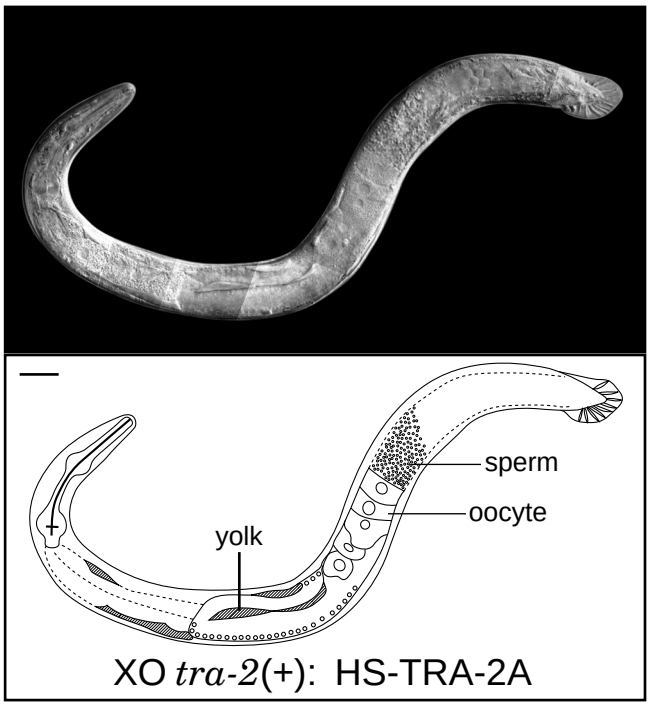

Fig. 5. HS-TRA-2A feminises the intestine and germ line of adult XO males. Top, Nomarski DIC photomicrograph (630×) of adult XO *him-8;qEx32* male, oblique view. Bottom, schematic representation of photomicrograph. Feminised intestine (yolk) and germline (oocytes) are indicated. No other changes in somatic structures are observed. Scale bar, 10 μm.

TRA-2A cannot feminise the germ line if an endogenous wild-type *tra-2* gene is absent. This suggests that additional wild-type *tra-2* products may be required to elicit germ line feminisation. These results also indicate that HS-TRA-2A can reverse sexual cell fate decisions in animals that are already committed to following the male fate. A similar plasticity in sexual cell fate maintenance was also noted by Schedin et al. (1994). They found that the intestine and germline of adult XO *her-1(ts)* males could be feminised by shifting animals from permissive to restrictive temperature.

DISCUSSION

TRA-2A is necessary and sufficient to promote feminisation of XX somatic tissues

The *tra-2* locus expresses multiple transcripts (Okkema and Kimble, 1991). Our model for sex determination (Fig. 1B) proposes that the predicted membrane protein, TRA-2A, encoded by the 4.7 kb *tra-2* mRNA, provides the primary feminising activity of the *tra-2* locus (Kuwabara et al., 1992). Here, we show that transgenic TRA-2A does provide a *tra-2*(+) activity that directs somatic cells of XX *tra-2* mutants to follow the hermaphrodite fate. We argue that TRA-2A is both a necessary and sufficient component of somatic *tra-2* feminising activity for three reasons. First, mutations that disrupt only the TRA-2A coding sequence and not other predicted TRA-2 proteins abolish *tra-2* activity (Okkema and Kimble, 1991; Kuwabara et al., 1992). Second, HS-TRA-2A alone is sufficient to feminise the soma of XX *tra-2* null mutants (this study). Finally, like *tra-2*(+), which is required throughout hermaphrodite larval development (Klass et al., 1976), ectopic TRA-2A can affect sexual cell fates at multiple points during development.

Cell-to-cell signalling mediated by HER-1 and TRA-2A

Cell-to-cell signalling is an important mechanism for regulating cell fates during the development of many organisms. In *C. elegans*, we have proposed that TRA-2A and HER-1 mediate cell-to-cell communication to regulate sexual cell fate decisions and to ensure that all cells follow the same sexual fate (Kuwabara et al., 1992). *tra-2* mRNAs are found in both XX hermaphrodites and in adult XO males, however, *tra-2* mRNA levels are 15-fold lower in XO males than in XX hermaphrodites (Okkema and Kimble, 1991). We have suggested that HER-1 functions as a TRA-2A antagonist to ensure that even low levels of TRA-2A remain inactive in XO males (Kuwabara et al., 1992). Otherwise, inappropriate TRA-2A activity in XO animals might activate a positive feed-back loop that leads to increased *tra-2* mRNA steady-state levels and probably TRA-2A protein (Fig. 1A) (Okkema and Kimble, 1991). As a result, an XO cell might be driven to follow the hermaphrodite fate, if insufficient HER-1 is present to negatively regulate TRA-2A (Kuwabara et al., 1992). We have shown that HS-TRA-2A driven from a strong promoter does indeed transform XO animals into fertile hermaphrodites, although HER-1 is presumably present in these animals. We suggest that the level of HS-TRA-2A is sufficiently elevated to titrate HER-1 and to allow some HS-TRA-2A activity to escape negative regulation, because the transformation of XO males into hermaphrodites mimics the XO *her-1* loss-of-function phenotype. Therefore, the relative ratio of HER-1 to TRA-2A may be crucial in determining sexual cell fate. It might also be predicted that mutant TRA-2A proteins, which are essentially wild-type in activity except that they are insensitive to negative regulation by HER-1, would also transform XO animals to the hermaphrodite fate. *tra-2* alleles with such properties have been identified and their characterisation will be reported elsewhere (J. Hodgkin, submitted; P. Kuwabara, submitted).

The carboxy-terminal domain of TRA-2A contains feminising activity that may mediate signal transduction

It has been hypothesised that TRA-2A promotes XX hermaphrodite development by negatively regulating one or more of the predicted cytoplasmic FEM proteins (Spence et al., 1990; Ahringer et al., 1992). We have proposed that an intracellular carboxy-terminal region of TRA-2A plays a crucial role in this regulation (Kuwabara et al., 1992). In this study, we have demonstrated that HS-TRA-2B, which contains only a carboxy-terminal region of TRA-2A, has feminising activity on its own. HS-TRA-2B is so named because it is identical in sequence to TRA-2B, the predicted protein encoded by the 1.8 kb *tra-2* mRNA. The normal role of the 1.8 kb *tra-2* mRNA in *C. elegans* sex determination will be discussed elsewhere (P. Kuwabara, P. Okkema, and J. Kimble, in preparation). HS-TRA-2B is likely to be cytoplasmic because it lacks any hydrophobic domains or other sub-cellular localisation signals

(Fig. 2). We found that HS-TRA-2B expression in XX *tra-2;qEx35* mutants led to intersexual tail development and yolk protein accumulation. In addition, HS-TRA-2B induced yolk accumulation in XO *tra-2*(+);*qEx35* males. These results indicate that the TRA-2A carboxy terminus probably contains a regulatory domain that represses the activity of one or more of the cytoplasmic FEM proteins. This interaction is proposed to occur when TRA-2A is not repressed by HER-1 and implies that TRA-2A is constitutively active in a signal transduction process during XX hermaphrodite somatic development.

HS-TRA-2B does not feminise the soma of animals to the same extent as HS-TRA-2A. This difference can be attributed to a number of factors such as protein topology, intracellular localisation, or protein stability. For example, TRA-2A might be better at sequestering the FEM proteins, because its carboxy terminus is anchored to the membrane; in contrast, HS-TRA-2B is likely to be freely cytoplasmic. In addition, HS-TRA-2B does not feminise the germline, possibly because the heat shock promoter fails to function in the germ line (Stringham et al., 1992; see below).

HS-TRA-2A may reinforce a commitment to the hermaphrodite fate

We have shown that HS-TRA-2A does not feminise the germ line of XX *tra-2* mutants. This could be because the heat shock promoter does not function in the germ line (Stringham et al. 1992) or because TRA-2A is not the only *tra-2* gene product needed to support hermaphrodite germline development. However, if the heat shock promoter does not function in the germline, it becomes necessary to explain how the germline of XO *tra-2*(+) animals can be feminised by HS-TRA-2A. One possibility is that somatic HS-TRA-2A titrates HER-1 protein, which would otherwise bind to and repress endogenous germline TRA-2A. As a consequence, the endogenous germline TRA-2 proteins are freed from repression and can promote hermaphrodite germline development. This model would be consistent with the finding that in XO animals mosaic for *her-1*, certain *her-1*(+) cells can be induced to follow the female fate (Hunter and Wood, 1992), presumably because of influences exerted by neighbouring cells (Kuwabara and Kimble, 1992).

Alternatively, it remains possible that HS-TRA-2A is expressed in the germline, but that in addition, the endogenous *tra-2* gene products are required to promote hermaphrodite germline development. Evidence that the heat shock promoter does not function in the germline is based primarily on the failure to observe germline *lacZ* reporter activity (Stringham et al., 1992); this does not rule out the possibility that the heat shock promoter may function in the germ line, but at a level lower than that found in somatic tissues. It is tempting to speculate that HS-TRA-2A may indeed be expressed in the germ line, albeit poorly, and that HS-TRA-2A can thereby recruit endogenous *tra-2* gene products by activating the same positive feedback loop that is likely to be responsible for the sex-specific differences in *tra-2* mRNA levels (Fig. 1A) (Okkema and Kimble, 1991). In this scenario, HS-TRA-2A expression is predicted to elevate the steady-state levels of both the 4.7 kb and 1.8 kb *tra-2* mRNAs. Either or both of these *tra-2* mRNAs might play an important role in promoting hermaphrodite germline development.

Our results indicate that TRA-2A plays a central role in regulating sexual fate decisions in both XX and XO animals. Now that all of the known major regulatory genes that control sex determination in *C. elegans* have been cloned, we have the tools to investigate how sexual cell fate decisions are controlled at the biochemical level. Future experiments will focus on demonstrating whether a direct binding interaction can be detected between TRA-2A and HER-1. In addition, it should be possible to determine how the intracellular carboxy-terminal domain of TRA-2A interacts with one or more of the FEM proteins to mediate signal transduction.

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