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The TRA-1 transcription factor binds TRA-2 to regulate sexual fates in *Caenorhabditis elegans*

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The *tra-1* and *tra-2* sex-determining genes promote female fates in *Caenorhabditis elegans*. Classical genetic studies placed *tra-1* as the terminal regulator of the pathway with *tra-2* acting upstream as a regulator of regulators of *tra-1*. Here we report the surprising result that the TRA-1 transcription factor binds the intracellular domain of the TRA-2 membrane protein. This binding is dependent on the MX regulatory domain, a region of the TRA-2 intracellular domain shown previously to be critical for the onset of hermaphrodite spermatogenesis. The functional importance of the TRA-1–TRA-2 physical interaction is supported by genetic interactions between *tra-1(0)* and *tra-2(mx)* mutations: a reduction of *tra-1* gene dose from two copies to one copy enhances the *tra-2(mx)* feminization phenotype, but has no apparent somatic effect. In *Caenorhabditis briggsae*, we also find an MX-dependent interaction between Cb-TRA-1 and Cb-TRA-2, but intriguingly, no cross-species interactions are seen. The conservation of the TRA-1–TRA-2 interaction underscores its importance in sex determination.

Keywords: *Caenorhabditis briggsae*/*Caenorhabditis elegans*/sex determination/TRA-1/TRA-2

Introduction

Caenorhabditis elegans XX animals are self-fertilizing hermaphrodites, making sperm first and then oocytes; XO animals are male. Specification as hermaphrodite or male relies initially on the X:A ratio to control a pathway of sex-determining genes to direct sexual cell fates (reviewed in Meyer, 1997). The transient generation of sperm in an otherwise female XX animal is regulated by modulating the activities of sex-determining genes that direct male or female development (Puoti *et al*., 1997). Of particular importance to this paper are two sex-determining genes, *tra-1* and *tra-2*, which specify female development (Hodgkin and Brenner, 1977). We have found an unexpected physical interaction between the TRA-1 and TRA-2 proteins, which suggests a functional relationship between these two regulators that was not predicted by previous analyses.

Figure 1 shows an abbreviated sex determination pathway that highlights functional relationships among genes at the end of the pathway. In somatic tissues, the pathway ultimately controls activity of TRA-1 (Hodgkin and Brenner, 1977; Hodgkin, 1986) (Figure 1A). The *tra-1* gene encodes two proteins: TRA-1A with five zinc fingers and TRA-1B with only two zinc fingers (Zarkower and Hodgkin, 1992). TRA-1A is homologous to *Drosophila cubitus interuptus* (*ci*) and vertebrate GLI proteins, and is essential for *tra-1* activity; no role is known for TRA-1B (Zarkower and Hodgkin, 1992). TRA-1A functions as a transcription factor (Conradt and Horvitz, 1999; Chen and Ellis, 2000), and also promotes transport of *tra-2* mRNA to the cytoplasm (Graves *et al*., 1999). In *tra-1* XX null mutants, somatic tissues are masculinized, and the germ line makes a reduced number of sperm (Hodgkin and Brenner, 1977; Hodgkin, 1987; Schedl *et al*., 1989). Therefore, wild-type TRA-1 promotes female development in somatic tissues and abundant spermatogenesis in the germ line.

The *tra-2* gene encodes multiple *tra-2* transcripts to generate TRA-2A, a membrane protein with similarity to *Drosophila* and vertebrate *patched*, and the oocyte-specific TRA-2B, which corresponds to the intracellular domain (ic) of TRA-2A (Kuwabara *et al*., 1992, 1998). In addition, TRA-2A can be cleaved by the TRA-3 protease to free the TRA-2ic from its membrane attachment (Sokol and Kuwabara, 2000). Genetic studies have shown that *tra-2* negatively regulates the *fem* genes and promotes female development in both somatic and germ-line tissues (Hodgkin and Brenner, 1977; Hodgkin, 1986) (Figure 1).

TRA-2ic, whether generated as a cleavage product of TRA-2A (Sokol and Kuwabara, 2000) or as the separate translation product TRA-2B (Kuwabara *et al*., 1998), carries the feminizing activity of TRA-2 (Kuwabara and Kimble, 1995). One aspect of that feminizing activity resides in the N-terminal portion, which binds and inhibits FEM-3 (Mehra *et al*., 1999). Intriguingly, the C-terminal region contains a 22-amino-acid MX region that is also critical for sex determination (Kuwabara *et al*., 1998). This MX region was defined by a series of *tra-2(mx)* missense mutations, which have both gain-of-function and loss-of-function character and were designated 'mx' for mixed character. The germ lines of both *tra-2(mx)*/+ and *tra-2(mx)* XX animals are feminized, albeit with greater penetrance in the homozygotes; in contrast, *tra-2(mx)* XO males make sperm continuously (Doniach, 1986; Schedl and Kimble, 1988). In addition, the somatic tissues of *tra-2(mx)* mutants can be weakly masculinized (Doniach, 1986; Schedl and Kimble, 1988). Therefore, the TRA-2(MX) region has a major role in the onset of hermaphrodite spermatogenesis and a minor role in somatic tissues.

In this paper, we report the unexpected finding that TRA-1A binds TRA-2ic. This binding occurs in the C-terminal portion of TRA-2ic and depends on the TRA-2(MX) regulatory region. TRA-1–TRA-2ic binding was discovered and analyzed using yeast two-hybrid

assays, and confirmed as a direct physical interaction *in vitro*. To test its biological function, we examined the effect of reducing *tra-1* gene dose on the *tra-2(mx)* phenotype. We find that *tra-1(0)* is a dominant enhancer of *tra-2(mx)*/+ feminization in the germ line, but have not detected a similar enhancement of *tra-2(mx)* masculinization of somatic tissues. Finally, we demonstrate that the TRA-1–TRA-2ic interaction is conserved: *Caenorhabditis briggsae* TRA-1 binds the intracellular domain of *C.briggsae* TRA-2 in an MX-dependent manner. This conservation over millions of years supports the idea that TRA-1–TRA-2ic binding plays a key role in sex determination.

Results

Identification of TRA-1 in a yeast two-hybrid screen for TRA-2 interactors

To search for proteins that regulate or are regulated by TRA-2, we performed a yeast two-hybrid screen using a TRA-2ic fragment as bait (Figure 2A). From 1.2 million transformants, we identified one positive clone that encodes the C-terminal portion [amino acids (aa) 647–1110] of TRA-1A; we call this fragment TRA-1c (Figure 2B). As GAL4 DNA-binding domain (DB) fusions, TRA-2ic has weak activating activity and TRA-1c has strong activity (data not shown). By contrast, full-length DB–TRA-1A protein possesses little activation activity (Figure 2C, sector 3). We therefore used DB–TRA-1A for subsequent analyses of the TRA-1–TRA-2 interaction (Figure 2C and D).

Our yeast two-hybrid assays relied on growth on selective medium (see Materials and methods); growth was scored as positive for the interaction and lack of growth scored as negative. By this assay, yeast transformed with DB–p53, and activation domain (AD)–SV40 T-antigen fusions grew on this medium (Figure 2C, sector 1). DB–TRA-1A did not interact with SV40 T antigen fused to an AD (Figure 2C, sector 2) and did not self-activate (Figure 2C, sector 3), but DB–TRA-1A did interact with AD–TRA-2ic (Figure 2C, sector 4). Conversely, AD–TRA-2ic interacted with TRA-1A (Figure 2D, sector 1), but did not self-activate (Figure 2D, sector 2) or interact with p53 or LAM5 (Figure 2D, sectors 3 and 4). The relative strength of the TRA-1–TRA-1ic interaction compared with that of p53/SV40 T antigen was determined by measuring yeast growth rates (Figure 2E). These results suggest that TRA-1A interacts specifically with the cytoplasmic portion of TRA-2.

Distinct TRA-2 regions bind FEM-3 and TRA-1

The FEM-3-binding region of TRA-2 extends from aa 1133 to 1273 (Mehra *et al*., 1999) (Figures 2A and 7A). To ask where TRA-1A binds, we employed a series of N-terminal deletions of AD–TRA-2ic (Figure 3A). Specifically, TRA-2icΔ1 removed 100 amino acids, TRA-2icΔ2 removed 200 amino acids, TRA-2icΔ3 removed 233 amino acids and TRA-2icΔ4 removed 300 amino acids. We found that removal of 100, 200 or 233 amino acids from the TRA-2ic N-terminus had little or no effect on interactions with TRA-1A (Figure 3B). However, deletion of 300 amino acids disrupted the interaction (Figure 3B, sector Δ4). Therefore, the TRA-1–TRA-2ic interaction does not require the FEM-3-binding domain (aa 1133–1273), but instead requires a region of at most 154 amino acids (aa 1322–1475), which contains the MX regulatory region (aa 1392–1413).

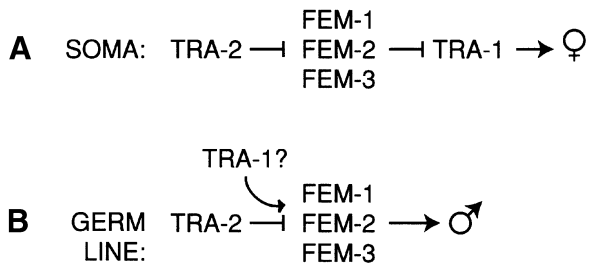

Fig. 1. Genetic regulation of sex determination in *C.elegans*. Only genes essential to this paper are presented; for a full description of the pathway see Meyer (1997). For references see the text. (**A**) Somatic tissues. TRA-1 is the terminal regulator and directs female development. TRA-2 promotes female development by negatively regulating the FEM proteins. (**B**) Germ line. TRA-1 is not the terminal regulator and promotes spermatogenesis, perhaps by positively regulating activity of the FEM proteins.

The TRA-2 MX region is required for the TRA-1–TRA-2 interaction

To ask whether the TRA-2 MX region is required for TRA-1 binding, we engineered *mx* mutations into TRA-2ic (Figure 4A). The MXΔ deletion removes 21 of the 22 MX amino acids, and three point mutations, E1393K, R1400Q and P1411L, were made corresponding to *tra-2(mx)* alleles *e1939*, *q179* and *e1403*, respectively (Figure 4A). The size of each MX mutant protein fused with the GAL4 AD was confirmed by examination of *in vitro* translation products (Figure 4B and data not shown); in addition, expression of the TRA-2(MX) mutant proteins in yeast was verified by western blotting (data not shown). The interaction of TRA-1A with wild-type TRA-2ic and the TRA-2(MX) mutant proteins was assayed in yeast. In contrast to wild-type TRA-2ic, which interacted well with TRA-1 (Figure 4C, sector 2), the three TRA-2(MX) mutants all failed to interact (Figure 4C, sectors 3 and 4 and data not shown). We conclude that the MX region of TRA-2 is critical for the TRA-1–TRA-2 interaction.

We tested further the importance of the MX region for TRA-1–TRA-2 binding using deletions TRA-2icΔ1 and TRA-2icΔ2 (Figure 3A). To this end, the MXΔ deletion was engineered into each of two constructs to generate AD–TRA-2icΔ1ΔMX and AD–TRA-2icΔ2ΔMX. We found that yeast co-transformed with plasmids encoding DB–TRA-1A, AD–TRA-2icΔ1 and AD–TRA-2icΔ2 grew well (Figure 4D, sectors 1 and 3), whereas those co-transformed with plasmids encoding DB–TRA-1A, AD–TRA-2icΔ1ΔMX and AD–TRA-2icΔ2ΔMX did not grow (Figure 4D, sectors 2 and 4). Expression of TRA-2icΔ2 and TRA-2bΔ2ΔMX proteins in yeast was verified by western blotting (Figure 4E, arrow). This result substantiates the finding that the TRA-2(MX) region is required for the TRA-1–TRA-2 interaction.

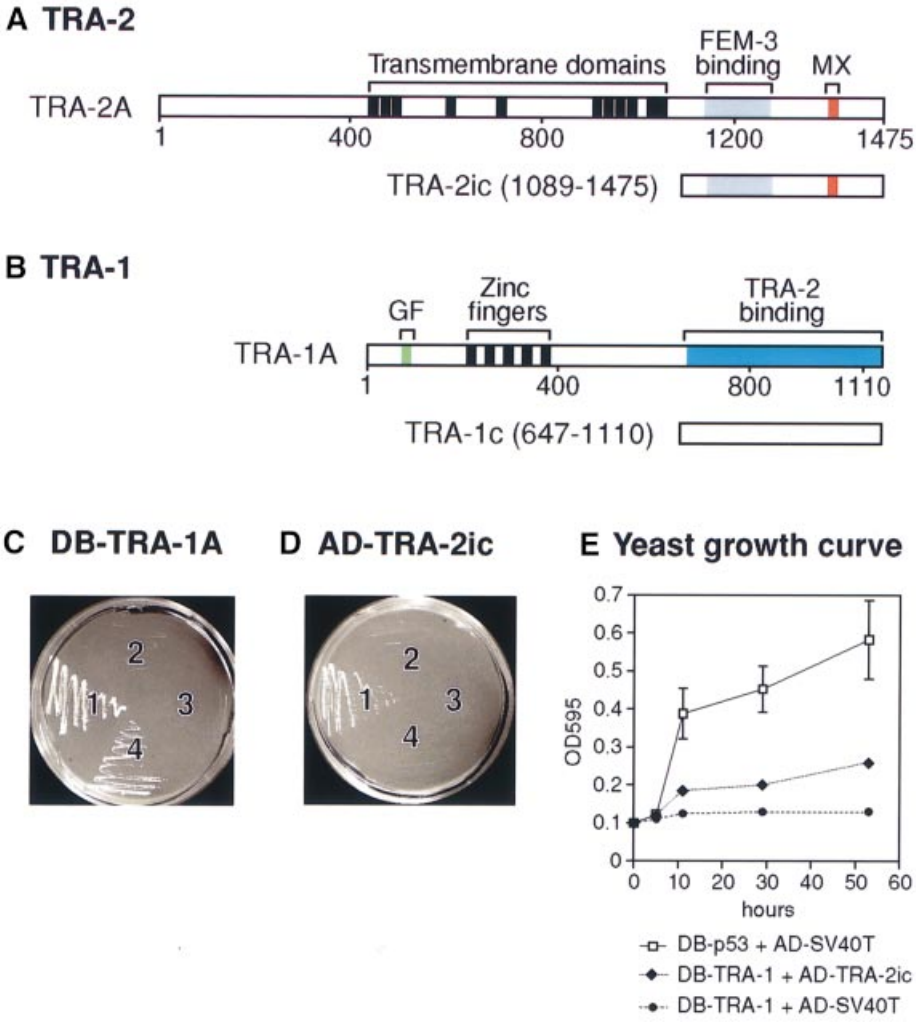

Fig. 2. *Caenorhabditis elegans* TRA-1 interacts with *C.elegans* TRA-2ic in yeast. (**A**) The *tra-2* gene encodes two products: TRA-2A is a transmembrane protein and TRA-2ic corresponds to the TRA-2A cytoplasmic region (aa 1089–1475, with numbering from the TRA-2A sequence) (Kuwabara *et al*., 1992). The region that binds FEM-3 extends from aa 1133 to 1273 (Mehra *et al*., 1999); the MX domain is defined as aa 1392–1413 (Kuwabara *et al*., 1998). TRA-2ic was derived from a TRA-2A cDNA, and is equivalent to TRA-2B. (**B**) The *tra-1* gene encodes two products: the longer TRA-1A shown here contains five zinc fingers; the shorter TRA-1B possesses only the two N-terminal fingers and lacks the region corresponding to TRA-1c (Zarkower and Hodgkin, 1992). The GF region of TRA-1 was defined by missense gain-of-function mutations clustered in a region encoding aa 73–88 (de Bono *et al*., 1995); it should be noted that the TRA-1(GF) region resides in the N-terminal part of TRA-1A, whereas the TRA-2-binding region resides in the C-terminal part of TRA-1A. TRA-1c extends from aa 647 to 1110. (**C** and **D**) Yeast were cultured on –His/–Trp/–Leu + 2.5 mM 3-AT medium at 30°C for 4–5 days. A two-hybrid interaction was scored as positive by growth on this selective medium, and as negative by a lack of growth. (C) Yeast were transformed with plasmid pairs and streaked in four sectors: sector 1, pDB-p53 and AD–SV40T; sector 2, pDB-TRA-1A and pAD-SV40T; sector 3, pDB-TRA-1A and pAD-vector; sector 4, pDB-TRA-1A and pAD-TRA-2ic. (D) Yeast were transformed with plasmid pairs and streaked in four sectors: sector 1, pDB-TRA-1A and pAD-TRA-2ic; sector 2, pDB-vector and pAD-TRA-2ic; sector 3, pDB-p53 and pAD-TRA-2ic; and sector 4, pDB-LAM5′ and pAD-TRA-2ic. (**E**) Yeast growth curves in liquid media SD/–His/–Trp/–Leu + 2 mM 3-AT. Each point is an average of three cultures; standard deviations are shown for the DB-p53 + AD-SV40T curve; standard deviations for other curves are within the size of the triangles/dots used for the graphic and are therefore not visible.

TRA-1 and TRA-2 interact in vitro

To confirm the TRA-1–TRA-2 interaction *in vitro*, a fragment encoding TRA-2icΔ2 (Figure 3A) was fused in-frame to a sequence encoding a T7 tag; this fragment either harbored the wild-type MX region or the MXΔ deletion (Figure 4A). The resultant proteins, T7-TRA-2icΔ2 and T7-TRA-2icΔ2ΔMX, were expressed in *Escherichia coli* and purified with T7 antibody-conjugated agarose beads (Figure 5A). To tag TRA-1, the TRA-1c fragment (Figure 2B) was fused in-frame to an *S*-tag. The *in vitro* translated, *S*-tagged TRA-1c was added to either T7-TRA-2icΔ2 (Figure 5B, lane 2) or T7-TRA-2icΔ2ΔMX (Figure 5B, lane 3) and incubated to permit binding. Subsequently, beads were washed and pelleted, and associated proteins were analyzed by western blotting using alkaline phosphatase conjugated to *S*-protein (see Materials and methods). We found that T7-TRA-2icΔ2 protein pulled down *S*-tagged TRA-1c (Figure 5B, lane 4), but that T7-TRA-2icΔ2ΔMX did not (Figure 5B, lane 5). We conclude that TRA-1 and TRA-2 bind each other *in vitro* and that this binding requires the MX region.

A functional test for tra-1/tra-2(mx) interactions

Normally, *tra-2(mx)*/+ XX animals are either hermaphrodite or female with the percentage of females character-

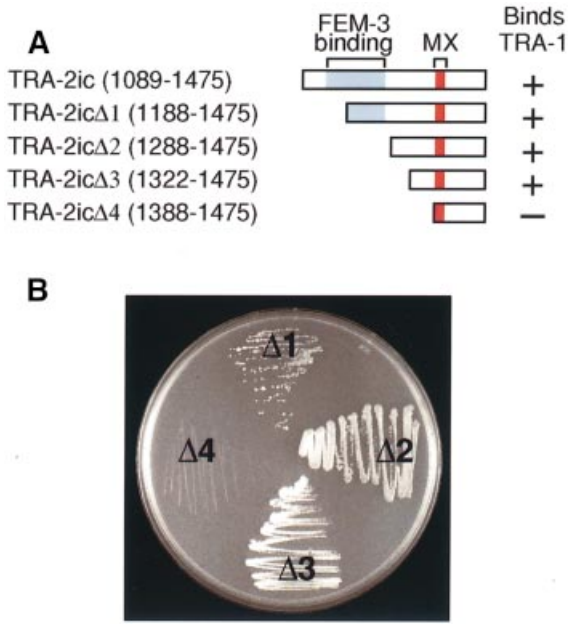

Fig. 3. The part of TRA-2ic that binds TRA-1 includes the MX region and does not overlap with the FEM-3-binding region. (**A**) TRA-2 N-terminal deletions used to define the TRA-1 binding domain; amino acid numbering and motif colors are the same as in Figure 2. (**B**) Yeast were transformed with one plasmid encoding DB–TRA-1A and one plasmid bearing an N-terminal deletion as depicted in (A). All yeast were cultured on –His/–Trp/–Leu + 2.5 mM 3-AT at 30°C for 4–5 days and then scored for growth. Yeast growth was robust for two of the deletions (TRA-2icΔ2 and TRA-2icΔ3) and no growth was observed for TRA-2icΔ4. For TRA-2icΔ1, growth was less robust than for TRA-2icΔ2 and TRA-2icΔ3, but was still easily detectable. In repeats of this experiment, growth was always easily detectable using TRA-2icΔ1, TRA-2icΔ2 and TRA-2icΔ3, and never detectable using TRA-2icΔ4.

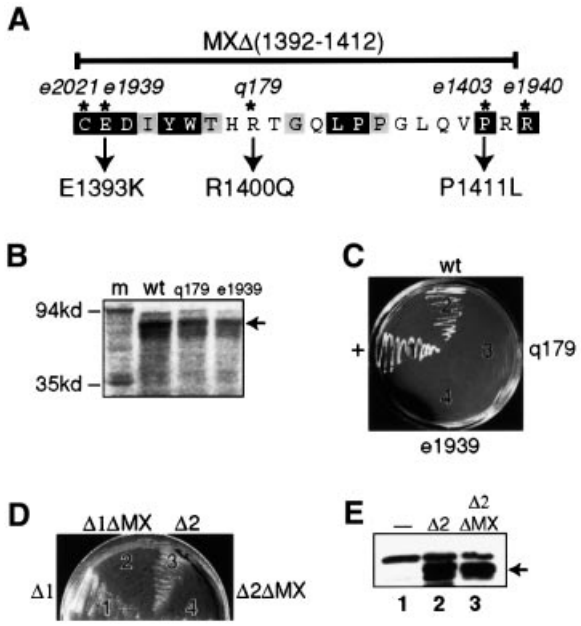

Fig. 4. The TRA-2 MX region is required for the TRA-1–TRA-2 interaction. (**A**) Amino acid sequence of the TRA-2 MX region. The MX region extends from the cysteine at aa 1392 to the arginine at aa 1413. Amino acids that are identical in *C.elegans*, *C.briggsae* and *C.remanei* are shown in black; amino acids identical in two of these species, including *C.elegans*, are shown in gray. The amino acids changed in *tra-2(mx)* mutants are marked by asterisks and include: *tra-2(e2021)*, a C to Y transition at aa 1392; *tra-2(e1939)*, an E to K transition at aa 1393; *tra-2(q179)* or *tra-2(e2019)*, an R to Q transition at aa 1400; *tra-2(e1403)*, a P to L transition at aa 1411; and *tra-2(e1940)*, an R to Q transition at aa 1413 (Kuwabara *et al*., 1998). The MX mutants employed in this study are: MXΔ, which removes aa 1392–1412; MX-E1393K, the *tra-2(e1939)* change; MX-R1400Q, the *tra-2(q179)* change; and MX-P1411L, the *tra-2(e1403)* change. Data are presented only for MX-E1393K and MX-R1400Q. (**B**) MX mutant proteins. Clones encoding GAL4 AD fusion proteins with either wild-type (+) TRA-2ic or one of three mutant TRA-2ic proteins were tested by expression *in vitro* for generation of protein of the correct size (see Materials and methods). Lanes are as follows: m, molecular weight markers; wt, wild-type AD–TRA-2ic fusion; *q179*, AD–TRA-2icR1400Q fusion; *e1939*, AD–TRA-2icE1393K fusion. Molecular weights are indicated on the left. (**C**) Two-hybrid results with TRA-2ic-MX mutants. Yeast were co-transformed with one plasmid encoding TRA-1A as a GAL4 DNA-binding hybrid and one of three plasmids encoding TRA-2ic or a TRA-2ic-MX mutant as AD hybrids; they were then cultured on SD/–His/–Trp/–Leu with 2.5 mM 3-AT medium at 30°C for 4–5 days. Sector 1, pDB-p53 and pAD-SV40T; sector 2, AD–TRA-2ic; sector 3, AD–TRA-2ic(*q179*); sector 4, AD–TRA-2ic(*e1939*). (**D**) Two-hybrid results with TRA-2icΔ and TRA-2icΔ-MXΔ proteins. Yeast were co-transformed with one plasmid encoding TRA-1A as a GAL4 DNA-binding hybrid and one of four plasmids encoding AD–TRA-2icΔ1 (sector 1), AD–TRA-2icΔ1ΔMX (sector 2), AD–TRA-2icΔ2 (sector 3) or AD–TRA-2icΔ2ΔMX (sector 4); the yeast were then cultured on –His/–Trp/–Leu + 2.5 mM 3-AT medium for 4–5 days. (**E**) Expression of TRA-2 mutant proteins in yeast. Immunoblot of yeast proteins probed with mouse anti-HA antibody. Lane 1, yeast CG1945; lane 2, yeast CG1945 transformed with pAD-TRA-2icΔ2; lane 3, yeast CG1945 transformed with pAD-TRA-2icΔ2ΔMX. The GAL4AD–TRA-2ic fusion proteins (either wild type or deletion) are indicated by the arrow.

istic of an individual allele (Doniach, 1986; Schedl *et al*., 1989); *tra-1(0)*/+ XX animals are all hermaphrodite (Hodgkin and Brenner, 1977). To test the *in vivo* importance of the TRA-1–TRA-2 interaction, we compared the percentage females among *tra-2(mx)*/+ XX animals with that among *tra-2(mx)*/+; *tra-1(0)*/+ XX animals. For each of three different alleles, *tra-2(e1939mx)*, *tra-2(q179mx)* and *tra-2(e1940mx)*, XX animals bearing only one copy of wild-type *tra-1* were significantly more feminized than those with two copies of wild-type *tra-1* (Figure 6). In contrast to the effect of *tra-1(0)* on the *tra-2(mx)* mutations, *tra-1(0)*/+ did not feminize *fem-3(0)*/+ XX animals (data not shown). Therefore, *tra-1(0)* is a dominant enhancer of *tra-2(mx)*/+, consistent with the idea that TRA-1–TRA-2ic binding promotes spermatogenesis in wild-type animals (see Discussion).

To examine the interaction between *tra-1* and *tra-2(mx)* in somatic tissues, we examined *tra-2(q179mx)* homozygotes that were either *tra-1(0)*/+ or *tra-1(0)* homozygotes. Animals of these genotypes were distinguished by the presence or absence of green fluorescent protein (GFP) expressed in the pharynx, which was carried by the *hT2[qIs48]* chromosome used to balance *tra-1(0)*. We found that *tra-1(0)*/+; *tra-2(mx)* XX animals retained their hermaphrodite morphology: tails had the whip-like morphology typical of hermaphrodites and vulvas appeared normal (n = 342). In *tra-1(0)*; *tra-2(mx)* double homozygotes, XX animals were male, as expected for *tra-1(0)* homozygotes: no vulva or hermaphrodite-like tail was observed (n = 120). To see some more subtle effects of *tra-2(mx)* on *tra-1(0)* males, we examined them at the L4 or early adult stage by Nomarski microscopy. The somatic gonad was not feminized: no hermaphrodite-like somatic gonads were found in *tra-2(q179)*; *tra-1(0)* double

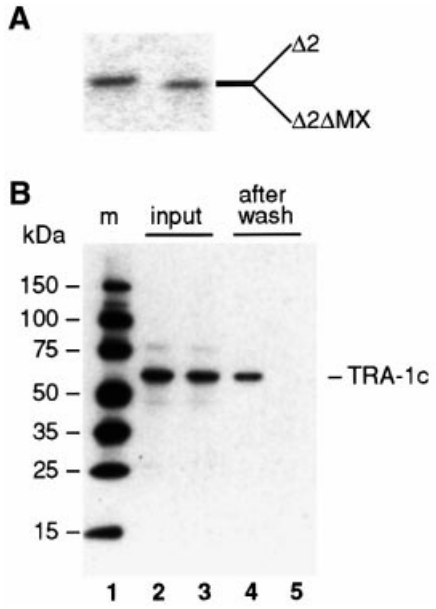

Fig. 5. TRA-1 interacts with TRA-2ic *in vitro*. (**A**) The TRA-2 proteins used in the *in vitro* assay were purified T7-tagged TRA-2icΔ2 and T7-tagged TRA-2icΔ2ΔMX. TRA-2icΔ2 lacks the FEM-3-binding domain, but possesses the MX domain, as shown in Figure 3A; TRA-2icΔ2ΔMX is an N-terminal deletion corresponding to TRA-2icΔ2, but it also has the deletion of MX, MXΔ, as shown in Figure 4A. (**B**) *S*-tagged TRA-1c was incubated with T7-tagged TRA-2ic proteins. Lane 1, molecular weight markers; lane 2, input *S*-tagged TRA-1c incubated with T7-tagged TRA-2icΔ2; lane 3, input *S*-tagged TRA-1c incubated with T7-tagged TRA-2icΔ2ΔMX; lane 4, the same as lane 2 but after washing; lane 5, the same as lane 3 but after washing.

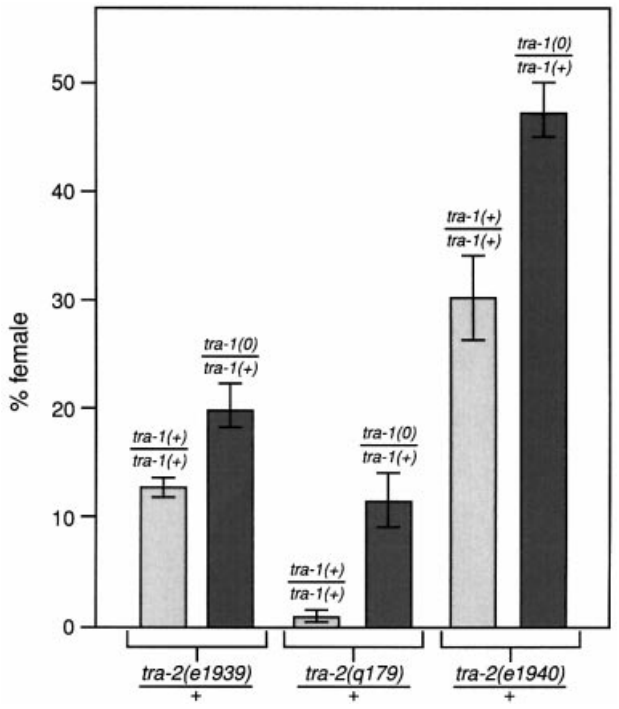

Fig. 6. Enhancement of *tra-2(mx)* by removal of one copy of *tra-1*. The percentage of females among XX animals of genotype *tra-2(mx)*/+ is compared with the percentage of females among XX animals of genotype *tra-2(mx)*/+; *tra-1(0)*/+. Each column represents results from at least three matings. The experiments compared were performed in the same incubator and in the same box of plates. For *tra-2(e1939mx)*/+, 13 ± 1% females were observed (n = 100) and for *tra-2(e1939mx)*/+; *tra-1(0)*/+, 20 ± 2% females were found (n = 175). For *tra-2(q179)*/+, 1.5 ± 0.4% females were observed (n = 486), and for *tra-2(q179mx)*/+; *tra-1(0)*/+, 11 ± 3.5% females were found (n = 303). For *tra-2(e1940mx)*/+, 31 ± 4% females were observed (n = 100) and for *tra-2(e1940mx)*/+; *tra-1(0)*/+, 47 ± 3% females were found (n = 104).

mutants (n = 31). We therefore found no effect of *tra-2(mx)* on somatic tissues, either when the *tra-1* dose was reduced by half or when *tra-1* was removed entirely.

TRA-1–TRA-2ic binding is conserved

The *C.briggsae tra-1* and *tra-2* genes are functionally similar to their *C.elegans* relatives (Kuwabara and Shah, 1994; de Bono and Hodgkin, 1996) and the MX region is conserved (Kuwabara and Shah, 1994) (Figure 7A). If the TRA-1–TRA-2 interaction is critical for sex determination, that interaction should also be conserved. We therefore tested a nearly full-length TRA-1A from *C.briggsae* for interactions with Cb-TRA-2ic, a fragment equivalent to Ce-TRA-2ic (Figure 2A). Whereas yeast transformed with plasmids encoding DB–Cb-TRA-1 and AD–Cb-TRA-2ic were able to grow on selective medium, those transformed with pDB-Cb-TRA-1 and a pAD empty vector or pAD-SV40 T antigen failed to grow on the same medium (data not shown). Furthermore, yeast transformed with plasmids encoding DB–p53 or the DB empty vector together with pAD-CbTRA-2ic could not grow on this medium (data not shown). Therefore, *C.briggsae* TRA-1 interacts with *C.briggsae* TRA-2c specifically in yeast.

To ask whether the Cb-TRA-1–Cb-TRA-2 interaction relied on the MX region, we tested N-terminal deletions of TRA-2ic similar to those described above for *C.elegans* TRA-2ic and obtained similar results: deletions of either 100 or 200 amino acids from the N-terminus of *C.briggsae* TRA-2ic had no effect, but an N-terminal deletion of 300 amino acids disrupted the interaction (data not shown). Finally, we deleted the MX region from Cb-TRA-2icΔ2 (a deletion analogous to Ce-TRA-2icΔ2; Figure 3A). The Cb-MX deletion was equivalent to Ce-MXΔ (Figure 4A). Whereas yeast transformed with pAD-Cb-TRA-2icΔ2 and pDB-Cb-TRA-1 grew well on selective medium (Figure 7B, sectors 1 and 3), those transformed with pAD-Cb-TRA-2icΔ2ΔMX and pDB-Cb-TRA-1 did not grow on the same medium (Figure 7B, sectors 2 and 4). Expression in yeast of the AD–Cb-TRA-2icΔ2 and AD–Cb-TRA-2icΔ2ΔMX fusion proteins was verified by western blotting (data not shown). We conclude that the *C.briggsae* TRA-1–TRA-2 interaction requires the MX region.

Finally, we asked whether *C.elegans* TRA-1 could interact with *C.briggsae* TRA-2ic and vice versa. Experiments were carried out similarly to those described above and are summarized in Figure 7C. We found that the *C.elegans* DB–TRA-1 fusion protein did not interact with the *C.briggsae* AD–TRA-2ic fusion protein, and conversely that the *C.briggsae* DB–TRA-1 fusion protein did not interact with the *C.elegans* AD–TRA-2ic fusion protein (data not shown). We conclude that the *C.elegans* and *C.briggsae* proteins have diverged too far to interact.

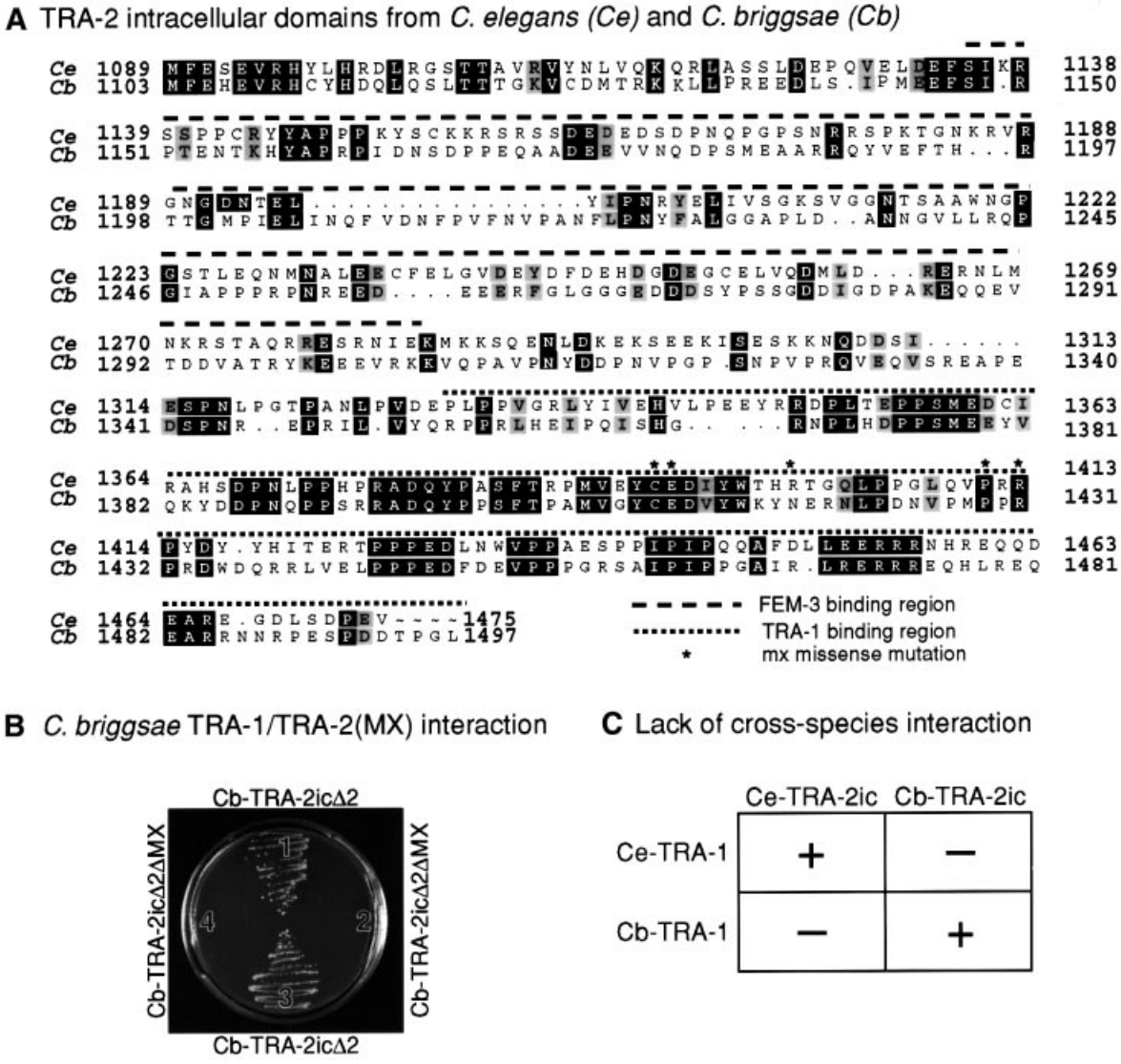

Fig. 7. Conservation of TRA-1–TRA-2 interaction. (**A**) Alignment of amino acid sequences of TRA-2ic for *C.elegans* (*Ce*) and *C.briggsae* (*Cb*). Numbering for *C.elegans* TRA-2 is from Kuwabara *et al*. (1992); numbering for *C.briggsae* TRA-2 is from Kuwabara (1996). Identical amino acids are black; similar amino acids are gray; the FEM-3-binding region is indicated by a dashed line over the sequence (Mehra *et al*., 1999); the TRA-1-binding region by a dotted line over the sequence (this work); amino acids altered by *tra-2(mx)* missense mutations are marked by an asterisk (Kuwabara *et al*., 1998). (**B**) Yeast two-hybrid results. Yeast were co-transformed with pDB-Cb-TRA-1, a plasmid encoding *C.briggsae* TRA-1A as a GAL4 DNA-binding hybrid, and either of two plasmids as AD hybrids: pAD-Cb-TRA-2icΔ2 encodes a fragment of *C.briggsae* TRA-2ic deleted for its N-terminal 200 amino acids; pCb-TRA-2icΔ2ΔMX encodes a form of Cb-TRA-2icΔ2 that also deletes the MX region. Yeast were streaked on SD/–His/–Trp/–Leu + 5 mM 3-AT plates and scored for growth after 4 days. Sectors 1 and 3, Cb-TRA-2icΔ2 interacts with pDB-Cb-TRA-1; sectors 2 and 4, Cb-TRA-2icΔ2ΔMX does not interact with pDB-Cb-TRA-1. (**C**) Summary of yeast two-hybrid results to test cross-species interactions between TRA-1 and TRA-2. See text for explanation.

Discussion

In this paper, we report three conclusions about nematode sex determination. First, the TRA-1 transcription factor binds the C-terminal region of TRA-2 in an MX-dependent manner. Secondly, a *tra-1* null mutation dominantly enhances the semi-dominant *tra-2(mx)*/+ feminization of germ-line tissues. In contrast, no enhancement of the recessive *tra-2(mx)* weak masculinization of somatic tissues was detected. And thirdly, the TRA-1–TRA-2 interaction is conserved in *C.briggsae*. In the following sections, we discuss the functional significance of the TRA-1–TRA-2 interaction in controlling sexual fates and possible mechanisms by which it may do so.

The TRA-1–TRA-2 interaction is critical for promoting spermatogenesis

We have found that TRA-1 binds TRA-2ic in a region that includes the MX regulatory region. Furthermore, TRA-1 does not bind TRA-2(MX) mutant proteins. The precise site of TRA-1 binding within TRA-2ic is not known, but must involve the MX region either directly or indirectly. The TRA-1–TRA-2ic interaction occurs between two proteins that were previously predicted to reside in distinct subcellular compartments (Kuwabara *et al*., 1992; Zarkower and Hodgkin, 1992). However, the TRA-1 transcription factor has been detected in both nucleus and cytoplasm (Graves *et al*., 1999), and an overexpressed TRA-2ic::GFP fusion protein is nuclear (Lum *et al*., 2000).

Because TRA-2ic can be generated by translation of the smaller *tra-2* transcript (Kuwabara *et al*., 1998) or by cleavage of the TRA-2A membrane protein (Sokol and Kuwabara, 2000), TRA-2ic may indeed be nuclear in wild-type animals. Therefore, TRA-1 and TRA-2 are present in the same subcellular compartments within *C.elegans* and are likely to interact there as well.

The TRA-2 MX region is critical for control of sexual fates: the germ line of *tra-2(mx)* XX mutants is feminized, making no sperm and only oocytes, and somatic tissues are weakly masculinized (Doniach, 1986; Schedl and Kimble, 1988). To explore the importance of TRA-1–TRA-2 binding in *C.elegans*, we asked whether genetic interactions between *tra-1* null and *tra-2(mx)* mutations could be observed. This type of experiment assumes a correlation between gene dosage and amount of protein made. Normally, one *tra-1* copy is sufficient for proper sex determination (Hodgkin and Brenner, 1977; Hodgkin, 1986). Therefore, the wild-type TRA-2 protein is not sensitive to a reduction of *tra-1* gene dose. However, we reasoned that a *tra-2(mx)* mutation might provide a more sensitized background, which might be dependent on *tra-1* gene dosage. Indeed, the *tra-2(mx)*/+; *tra-1(0)*/+ germ line was more often feminized than that of *tra-2(mx)*/+ animals. An effect on somatic tissues, however, was not observed in animals of the same genotype. We conclude that the *tra-2(mx)* mutant protein is sensitive to *tra-1* gene dose, consistent with an *in vivo* role for the TRA-1–TRA-2 interaction in germ-line sex determination.

tra-1 and germ-line sex determination

TRA-1 is not the terminal regulator of sexual fates in the germ line (Hodgkin, 1986) (see Figure 1). In *tra-1* null mutants, somatic tissues are strongly masculinized, but the germ line makes fewer sperm than are made in wild-type males (Hodgkin and Brenner, 1977; Hodgkin, 1987; Schedl *et al*., 1989). Therefore, TRA-1 promotes female development in somatic tissues and abundant spermatogenesis in males. In *tra-1* gain-of-function mutants, both somatic and germ-line tissues are feminized, suggesting that TRA-1 may also direct oogenesis (Hodgkin, 1987; de Bono *et al*., 1995). We propose a third role for TRA-1 in germ-line sex determination. *tra-2(mx)* mutants are defective in the onset of hermaphrodite spermatogenesis, and *tra-1* is a dominant enhancer of that defect. Therefore, in addition to its previously suggested roles, TRA-1 participates in controlling the onset of hermaphrodite spermatogenesis. This third role may be mechanistically similar to the role of *tra-1* in promoting continued spermatogenesis in males.

How does the TRA-1–TRA-2 interaction influence sex determination?

The binding of TRA-1 to TRA-2ic in an MX-dependent manner provides evidence for a new mechanism by which TRA-1 and TRA-2 act together to influence sex determination. Molecular analyses of *tra-2(mx)* mutations led to the idea that a repressor might bind the TRA-2 MX region and thereby inhibit the feminizing activity of TRA-2 to promote male development (Kuwabara *et al*., 1998). One possibility is that TRA-1 is that repressor; alternatively, TRA-2 might repress TRA-1. How might repression of TRA-1 be consistent with a requirement for TRA-1 in promoting spermatogenesis? One mechanism by which TRA-1 controls germ-line sexual fates appears to be transcriptional. The *fog-3* gene is required for specification of sperm (Ellis and Kimble, 1995), and harbors a series of TRA-1 binding sites in its promoter (Chen and Ellis, 2000). Intriguingly, these TRA-1 sites can mediate either repression or activation of the *fog-3* promoter (Chen and Ellis, 2000). One simple idea is that TRA-1 activates *fog-3* at one level of expression or in one state which exists in males and larval hermaphrodites as sperm are specified, and represses *fog-3* at a second level or state which exists during oogenesis. The binding of TRA-2 to TRA-1 might promote the TRA-1 level or state critical for spermatogenesis. For example, sequestration of TRA-1 by TRA-2 could decrease available TRA-1 and keep TRA-1 at a level appropriate for *fog-3* activation.

An alternative scenario brings in FEM-3, a protein required for male development in both somatic and germline tissues (Hodgkin, 1986; Barton *et al*., 1987). The N-terminal region of TRA-2ic binds FEM-3 and inhibits its ability to direct male development (Mehra *et al*., 1999). TRA-1 binding to the C-terminal region of TRA-2ic may activate FEM-3. Possible mechanisms for such activation include enhancing a modification required for activity, promoting assembly into an active complex, or competing with FEM-3 binding and thereby freeing FEM-3 to promote male development. Such a complex relationship among regulatory proteins would be difficult to unravel by conventional genetic tests.

Does the TRA-1–TRA-2ic interaction affect somatic sex determination?

The TRA-1 and TRA-2 proteins are present in somatic tissues and direct female development there (Hodgkin and Brenner, 1977). Does the TRA-1–TRA-2(MX) interaction play a role in somatic sex determination? The somatic effects of *tra-2(mx)* mutants are extremely weak: for three alleles (*e1939*, *q179* and *e1940*), somatic masculinization is observed only in *tra-2(mx)/tra-2(0)* heterozygotes; for the other two alleles (*e2021* and *e1403*), masculinization is so weak in homozygotes that most XX animals are self-fertile (Doniach, 1986; R.Edgar and T.Schedl, personal communication; S.Wang and J.Kimble, unpublished). Such weak masculinization might result from a specific effect on the TRA-1–TRA-2 interaction or from a non-specific defect, such as reduced stability of the TRA-2 mutant protein.

In an attempt to observe a role of the TRA-1–TRA-2 interaction in somatic sex determination, we examined somatic tissues in *tra-2(mx)*; *tra-1(0)*/+ mutants. We used *tra-2(q179)*, an allele that is strongly feminized in the germ line (Schedl *et al*., 1989), which is reduced in its ability to bind TRA-1 (this work) and which exhibits no somatic masculinization in XX homozygotes. Because this allele was clearly sensitive to the dose of *tra-1* in the germ line, we reasoned that it might also be sensitive in somatic tissues. However, *tra-2(q179)*; *tra-1(0)*/+ XX animals showed no somatic masculinization. In particular, the tail had the typical whip-like morphology of the hermaphrodite. Since the tail is a particularly sensitive monitor of sexual fate, a cautious interpretation is that the TRA-1–TRA-2 interaction has no major role in somatic sexual fates.

Conservation of the TRA-1–TRA-2 interaction

The *tra-1* and *tra-2* genes are among the most divergent genes in *C.elegans* and *C.briggsae* (de Bono and Hodgkin, 1996; Kuwabara, 1996). Each *C.elegans* protein is only ~40% identical to its *C.briggsae* homolog. Nonetheless, the TRA-1–TRA-2 interaction has been conserved (this work), which argues strongly that it plays a critical role in sex determination. Within TRA-2, the TRA-2(MX) region is highly conserved between *C.elegans* and *C.briggsae* (Kuwabara, 1996), and we have found that this region is required for the interaction between TRA-1 and TRA-2 in both species (this work). Nonetheless, the *C.elegans* proteins do not interact with their *C.briggsae* partners. This lack of interaction between proteins of different species underscores the divergence between the sex-determining proteins of these two species, and argues that the TRA-1–TRA-2 interaction is sufficiently critical that it has been retained by co-evolution of the *tra-1* and *tra-2* genes.

In *C.elegans*, the TRA-1–TRA-2 interaction has been implicated in the onset of hermaphrodite spermatogenesis, and a similar function in *C.briggsae* seems plausible. An intriguing question is whether the TRA-1–TRA-2 interaction has been conserved in *Caenorhabditis remanei*, a female/male species. Analysis of that interaction awaits cloning of the *Cr-tra-1* gene. However, we do know that the TRA-2 MX region has been conserved in *C.remanei* (Haag and Kimble, 2000). If the TRA-1–TRA-2 interaction has been similarly conserved, it is likely to play a more general role in nematode sex determination that is not limited to hermaphrodite/male species. Possibilities include continued spermatogenesis in XO males or control of sexual fates in somatic tissues.

Materials and methods

DNA manipulation

Most DNA manipulations were performed according to Sambrook *et al.* (1989). Primers and their position in the respective genes used in this study are listed in Table I.

To construct pAD-TRA-2ic, a C-terminal fragment corresponding to nucleotides (nt) 3301–4464 in the *tra-2* gene was PCR amplified from pJK349 (Kuwabara and Kimble, 1995) with primers P1 and P6. The resulting PCR fragment was digested with *Bam*HI and *Xho*I and ligated to pACTII (Clontech) digested with the same restriction enzymes. A PCR-based strategy was used to make deletions at the N-terminus of TRA-2ic. pAD-TRA-2icΔ1 was constructed by PCR amplifying a *Ce-tra-2* cDNA fragment from nt 3601–4464 with primers P2 and P6, and then cloned into *Bam*HI- and *Xho*I-cut pACTII. pAD-TRA-2icΔ2, pAD-TRA-2icΔ3 and pAD-TRA-2icΔ4 were constructed in the same way, except that primer sets P3 and P6, P4 and P6 or P5 and P6 were used, respectively.

To make Ce-TRA-2ic *mx* mutants, mutations were introduced in primers, and *Ce-tra-2* cDNA amplified and cloned into an intermediate vector, pGEM-TRA-2ic. pGEM-TRA-2ic was made by ligating a *Bam*HI–*Xho*I fragment from pAD-TRA-2ic into pGEM7zf(+) (Promega) digested with the same enzymes. To construct pAD-TRA-2icMX-E1393K, PCR product amplified from *Ce-tra-2ic* with P1 and P8 was digested with *Bam*HI and *Sal*I and then cloned into pGEM-TRA-2ic digested with the same enzymes. Then the *Bam*HI–*Xho*I fragment of TRA-2ic was cloned into pACTII. pAD-TRA-2ic R1400Q and pAD-TRA-2ic P1411L were constructed with the same strategy, but with primer pairs P1 and P9, or P1 and P7 to amplify *tra-2ic* cDNA. To construct pAD-TRA-2icΔ1ΔMX, a PCR product amplified from pAD-TRA-2icMXΔ(1392–1412) by primer pair P2 and P6 was cloned into *Bam*HI- and *Xho*I-digested pACTII. pAD-TRA-2icΔ2ΔMX was built with the same strategy as pAD-TRA-2icΔ1ΔMX except that primer pair P3 and P6 was used to amplify from pAD-TRA-2cMXΔ(1392–1412).

To make pET-TRA-2icΔ2 and pET-TRA-2icΔ2ΔMX, primers P15 and P16 were used to amplify the *tra-2ic* (nt 3901–4464) fragment with pAD-TRA-2icΔ2 and pAD-TRA-2icΔ2ΔMX as template, respectively. PCR products were digested with *Eco*RI and *Not*I and ligated to pET28a(+) (Novagen), to make pET-TRA-2icΔ2 and pET-TRA-2icΔ2ΔMX, respectively. Expression in *E.coli* produced T7-tagged TRA-2icΔ2Δ and TRA-2icΔ2ΔMX proteins.

Table I. Primers and their positions

Primer sequence	Annealing start position
P1: 5′-CGC*GGATCC*GTATGTTTGAAAGTGAAGTTCGACAC-3′	*Ce-tra-2*, nt 3301–3324
P2: 5′-CGC*GGATCC*GTGGAAATGGTGATAACACTGAAC-3′	*Ce-tra-2*, nt 3601–3621
P3: 5′-CGC*GGATCC*GTTCGCAAGAAAATTTGGACAAAG-3′	*Ce-tra-2*, nt 3901–3922
P4: 5′-CGC*GGATCC*GTCCTGCCAACCTTCCAGTTGA-3′	*Ce-tra-2*, nt 4000–4019
P5: 5′-CGC*GGATCC*GTGTTGAATATTGCGAAGATA-3′	*Ce-tra-2*, nt 4201–4219
P6: 5′-CCG*CTCGAG*TTAAACCTCTGGGTCTGATAGGTC-3′	*Ce-tra-2*, nt 4464–4441
P7: 5′-ACGC*GTCGAC*GG<u>A</u>GTACTTGAAGTCCTGGAGGTAGC-3′	*Ce-tra-2*, nt 4268–4245
P8: 5′-ACGC*GTCGAC*GGGGTACTTGAAGTCCTGGAGGTAGCTGTCCAGTT<u>T</u>GGTGTGTCCAGTAAATATC-3′	*Ce-tra-2*, nt 4268–4245
P9: 5′-ACGC*GTCGAC*GGGGTACTTGAAGTCCTGGAGGTAGCTGTCCAGTTCGGTGTGTCCAGTAAATATCTT <u>T</u>GCAATATTCAACCATTGGACGG-3′	*Ce-tra-2*, nt 4268–4245
P10: 5′-ACGC*GTCGAC*GATATTCAACCATTGGACGGG 3′	*Ce-tra-2*, nt 4208–4190
P11: 5′-CG*GGATCC*GTATGTTCGAACACGAAGTTCG-3′	*Cb-tra-2*, nt 3339–3358
P12: 5′-CCG*CTCGAG*CTAAAGACCAGGAGTGTC-3′	*Cb-tra-2*, nt 4526–4509
P13: 5′-CGC*GGATCC*GGATTCGGTGGATCGGGATCG-3′	*Ce-tra-1*, nt 2098–2108
P14: 5′-ATAAGAAT*GCGGCCGC*TTAAAATTGATGACGTGGCTTTTTGGG-3′	*Ce-tra-1*, nt 3492–3466
P15: 5′-G*GAATTC*TCGCAAGAAAATTTGGACAAAG-3′	*Ce-tra-2*, nt 3901–3922
P16: 5′-ATAAGAAT*GCGGCCGC*TTAAACCTCTGGGTCTGATAGGTC-3′	*Ce-tra-2*, nt 4464–4441
P17: 5′-CATG*CCATGG*AGATGTACCCATACGACGTCCCAGACTACGCTACCAGTCATGGAGAAGAGACT-3′	*Cb-tra-1*, nt 841–861
P18: 5′-G*GAATTC*TTAAAAACTGCGTGGCTTC-3′	*Cb-tra-1*, nt 3705–3687
P19: 5′-CG*GGATCC*GAGAAGTTCGGAAGAAAGTACA-3′	*Cb-tra-2*, nt 3939–3958
P20: 5′-A*AGGCCT*GTAACCAACCATTGCCGGTG-3′	*Cb-tra-2*, nt 4259–4240
P21: 5′-CTATCTATTCGATGATGAAG-3′	–
P22: 5′-ACAGTTGAAGTGAACTTGCG-3′	–

The numbering in *C.elegans tra-2* was according to Kuwabara *et al.* (1992). The numbering in *C.briggsae* was according to Kuwabara (1996). The numbering of *C.elegans tra-1* was according to Zarkower and Hodgkin (1992). The numbering of *C.briggsae tra-1* was according to de Bono and Hodgkin (1996). Restriction enzymes used in cloning are italicized; nucleotide changes associated with *tra-2(mx)* mutations are underlined.

To construct pCITE-TRA-1c, a 1.4 kb PCR product (nt 2098–3492) amplified from pDZ120 (a gift from D.Zarkower) with primers P13 and P14 was digested with *Bam*HI and *Not*I and ligated to pCITE4a(+) (Novagen) digested with the same enzymes. *In vitro* translation in TNT Quick System (Promega) produced an *S*-tagged TRA-1c protein.

pAD-Cb-TRA-2ic was constructed in two steps. First, a 1.2 kb PCR fragment (nt 3339–4526) amplified from a *C.briggsae* cDNA library by primers P11 and P12 was digested with *Bam*HI and *Xho*I (a *Bam*HI site exists at nt 3573), and the released 1 kb fragment was ligated to pACTII digested with *Bam*HI and *Xho*I, to make pAD-Cb-TRA-2icΔN. Then, the same PCR fragment was digested with *Bam*HI and the released 0.2 kb fragment was ligated to *Bam*HI-digested pAD-Cb-TRA-2cNΔ to make pAD-Cb-TRA-2ic.

To make pDB-Cb-TRA-1, restriction sites were added at the 5′ end of primers P17 and P18, which were used to amplify *C.briggsae tra-1* cDNA. The 3 kb PCR fragment (*C.briggsae tra-1* cDNA sequence nt 841–3705) was digested with *Nco*I and *Eco*RI and ligated to pAS2-1 cut with the same enzymes.

To delete the N-terminus from Cb-TRA-2ic, primers P11 and P20 were used to amplify a C-terminus of Cb-TRA-2ic; the resulting PCR fragment was digested with *Bam*HI and *Xho*I and ligated into pACTII digested with the same enzymes, to make pAD-Cb-TRA-2icΔ2. To delete the MX region from Cb-TRA-2Δ2, primers P12 and P20 were used to amplify the Cb-TRA-2ic fragment; the PCR product was digested with *Stu*I and *Bam*HI, and then ligated into pAD-Cb-TRA-2Δ2 digested with the same enzymes, to make pAD-Cb-TRA-2icΔ2ΔMX.

Yeast two-hybrid assays

pDB-TRA-2ic was used as bait to screen the ACT-RB-2 *C.elegans* cDNA library (Kraemer *et al*., 1999). The large-scale yeast transformation procedure followed Clontech protocol. Because pDB-TRA-2c had weak self-activation, the screen was performed on medium SD/–His/–Trp/–Leu supplemented with 20 mM 3-aminotriazole (3-AT). Approximately 200 yeast colonies grew on this medium from 1.2 million transformants, and 19 were analyzed by sequencing the inserts. Yeast plasmid DNA was isolated and transformed into *E.coli* strain KC-10 according to the manufacturer's instructions (Clontech). Inserts were PCR amplified with primers P21 and P22, sequenced, and used to search the *C.elegans* genomic sequence database with the BLAST program.

Yeast two-hybrid assays were carried out by introducing relevant plasmids into yeast strain CG1945 (Clontech) and plated on SD/–His/–Trp/–Leu medium supplemented with 2.5–5 mM 3-AT.

Yeast growth rate in liquid media was determined by inoculating a colony in the SD/–His/–Trp/–Leu + 2 mM 3-AT and monitoring the OD at 595 nm at indicated time points.

Purification of recombinant protein from E.coli

pET-TRA-2icΔ2 and pET-TRA-2icΔ2ΔMX were transformed in *E.coli* strain λDE3(LysS) (Novagen) and grown in 5 ml of Luria–Bertani (LB) medium + 50 μg/ml ampicillin overnight at 37°C. Bacteria were diluted to 100 ml of + 50 μg/ml medium and cultured for a further 3 h; after adding 1 mM isopropyl-β-D-thiogalactopyranoside, they were shaken at 37°C for 2 h. Bacteria were collected by centrifugation, resuspended in 5 ml of phosphate-buffered saline (PBS), sonicated, and centrifuged to remove cell debris. T7 antibody-conjugated agarose beads (100 μl) were mixed with supernatant, washed and resuspended according to the manufacturer's protocol (Novagen). Purified proteins were examined on 4–20% gradient polyacrylamide gels (Bio-Rad) with Coomassie Blue staining.

Immunoblot hybridization

Yeast was grown overnight in 5 ml of culture at 30°C, and collected by spinning in a microfuge for 30 s. Preparation of yeast extract was modified from Clontech's protocol. Briefly, yeast pellet was resuspended in 100 μl of disruption buffer (Clontech), mixed with glass beads and vortexed for 1 min. The crude extract was spun in a microfuge for 30 s, and 50 μl of supernatant transferred to a fresh tube. Clear extract (20 μl) was mixed with loading buffer and boiled for 2 min. Boiled samples were examined on a 4–20% gradient polyacrylamide precast gel (Bio-Rad). Protein was transferred to a polyvinylidene difluoride (PVDF) membrane according to the manufacturer's instructions (Amersham). The membrane was washed briefly with PBS buffer and incubated in 5% non-fat milk in PBS solution for 2 h with rotation. Then, the membrane was transferred to 5% non-fat milk in PBS + 0.25% Tween-20 solution containing anti-HA antibody (Boehringer) (1:1000 dilution), and incubated for 1 h with rotation. After the membrane was washed three times with PBS + 0.25% Tween-20 solution, each for 10 min with rotation, it was incubated in 5% non-fat milk in PBS + 0.25% Tween-20 solution containing horseradish peroxidase-conjugated, anti-mouse secondary antibody for 1 h with rotation. Then the membrane was washed a further three times before exposure to X-ray film for 0.5–5 min.

In vitro transcription and in vitro translation

pGEM-AD-TRA-2ic, pGEM-AD-TRA-2icMX-P1411L, pGEM-AD-TRA-2icMX-E1393K and pGEM-AD-TRA-2icMXR1400Q were digested with *Sma*I and transcribed using an SP6 Megascript kit (Ambion). RNA (0.1 μg) was used for *in vitro* translation in wheat germ extract (Promega) in the presence of [^{35}S]methionine (Amersham) according to the manufacturer's instructions. Translation product (5 μl) was subjected to SDS–PAGE analysis on 10% separation polyacrylamide gel. The gel was treated with Amplify® (Amersham) before drying and exposure to X-ray film (Kodak).

In vitro pull-down assay

S-tagged TRA-1c was synthesized in TNT Quick® System (Promega) in a total volume of 50 μl according to the manufacturer's instructions. Translation product (10 μl) was mixed with ~0.1 μg (10 μl) of purified T7-tagged TRA-2icΔ2, either wild-type or MX deletion, in a total volume of 50 μl, and incubated at room temperature for 30 min. The mixture (10 μl) was removed as an inoculation amount control. The rest of the mixture was washed five times with 1× binding/washing buffer from the T7 purification kit (Novagen). After the final spin, the sample was resuspended in 10 μl of 1× binding/washing buffer mixed with 10 μl of loading buffer. The sample was boiled for 5 min before loading onto 4–20% gradient precast polyacrylamide gel (Bio-Rad) for SDS–PAGE analysis. Protein was transferred to PVDF membrane according to the manufacturer's instruction. Alkaline phosphatase-conjugated *S*-protein (Novagen) was used to detect *S*-tagged TRA-1c according to the manufacturer's instruction.

Genetics

Strains. The nonsense mutant *tra-1(e1099)* was used for *tra-1(0).* The nonsense mutant *fem-3(e1996)* was used for *fem-3(0).* The *tra-2(mx)* strains *tra-2(e1939mx)*, *tra-2(q179mx)* and *tra-2(e1940mx)* were maintained as homozygotes. The double mutant strain, *tra-2(q179mx); tra-1(e1099)/hT2[qIs48](I;III)* was made by standard methods; *tra-1(e1099)/hT2[qIs48](I;III)* was used for controls. The *hT2* translocation chromosome balances *tra-1*; the *qIs48* insertion onto *hT2* allows *tra-1(e1099)/+* heterozygotes to be distinguished from *tra-1(e1099)* homozygotes by the presence or absence of the GFP marker inserted onto *hT2*.

Assays. tra-1(e1099) males or N2 males were mated to *tra-2(e1939)*, *tra-2(e1940)* or *tra-2(q179)* females at 20°C. To assess an animal as hermaphrodite or female, L4s were picked individually onto separate plates, and scored as self-fertile (hermaphrodite) or sterile with stacked oocytes and an otherwise female morphology (female). To assess the effect of *tra-2(mx)* on somatic sexual characters, green and non-green adult progeny from a *tra-2(q179mx); tra-1(e1099)/hT2[qIs48](I;III)* parent were scored by dissecting scope for vulva and tail morphology. In addition, *tra-2(q179mx); tra-1(e1099)* and *tra-1(e1099)* homozygotes were compared by Nomarski microscopy for effects on somatic gonadal morphology; these males were either L4 larvae or young adults within 1 day after their L4 to adult molt.

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