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Germ-Line Regulation of the *Caenorhabditis elegans* Sex-Determining Gene *tra-2*

Patricia E. Kuwabara,* Peter G. Okkema,† and Judith Kimble‡

**MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom; †Laboratory for Molecular Biology, University of Illinois at Chicago, Chicago, Illinois 60607; and ‡Howard Hughes Medical Institute, Laboratory of Molecular Biology, Department of Biochemistry, and Department of Medical Genetics, University of Wisconsin, Madison, Wisconsin 53706*

The *Caenorhabditis elegans* sex-determining gene *tra-2* promotes female development of the XX hermaphrodite soma and germ line. We previously showed that a 4.7-kb *tra-2* mRNA, which encodes the membrane protein TRA-2A, provides the primary feminizing activity of the *tra-2* locus. This paper focuses on the germ-line activity and regulation of *tra-2*. First, we characterize a 1.8-kb *tra-2* mRNA, which is hermaphrodite-specific and germ-line-dependent. This mRNA encodes TRA-2B, a protein identical to a predicted intracellular domain of TRA-2A. We show that the 1.8-kb mRNA is oocyte-specific, suggesting that it is involved in germ-line or embryonic sex determination. Second, we identify a *tra-2* maternal effect on brood size that may be associated with the 1.8-kb mRNA. Third, we investigate seven dominant *tra-2*(*mx*) (for mixed character) mutations that sexually transform hermaphrodites to females by eliminating hermaphrodite spermatogenesis. Each of the *tra-2*(*mx*) mutants possesses a nonconserved missense change in a 22-amino-acid region common to both TRA-2A and TRA-2B, called the MX region. We propose that the MX region mediates a posttranslational regulation of *tra-2* essential for the onset of hermaphrodite spermatogenesis. Finally, we discuss aspects of *tra-2* function and regulation that are specific to the unusual control of cell fate in the hermaphrodite germ line. © 1998 Academic Press

Key Words: **sex determination; *Caenorhabditis elegans*; hermaphrodite; germ-line; evolution; spermatogenesis.**

INTRODUCTION

Numerous proteins have been implicated in the specification of cell fate during development. Outstanding examples include myoD (Lassar and Munsterberg, 1994) and achaete-scute (Kageyama *et al.*, 1995). Yet the regulation of those proteins, which ensures their function at the right place and time during the development of a complex multicellular organism, is not yet understood in molecular detail for any cell fate decision. We have focused on the specification of sexual fates in the nematode *Caenorhabditis elegans*. The sex-determining genes have been extensively characterized at the genetic level (for reviews, see Kuwabara and Kimble, 1992; Meyer, 1997; Schedl, 1997) and most have been cloned. Genetically, the sex-determining genes function in a cascade of negative regulation (Figs. 1A and 1B). Molecularly, the sex-determining regulatory proteins appear to function by a signal transduction event that controls transcription (Fig. 1C).

The regulatory circuitry for sex determination is fundamentally similar in all tissues; however, striking differences are found between somatic and germ-line tissues. Somatic tissues are essentially female or male: XX hermaphrodites have a female soma, while XO males have a male soma. The sexual specification of somatic tissues depends on the regulatory pathway presented in Fig. 1A, which ultimately controls the activity of TRA-1, a predicted transcriptional regulator. The XX germ line is hermaphroditic: spermatogenesis occurs first to produce a limited number of male gametes, followed by oogenesis, which continues for the life of the animal. Spermatogenesis and oogenesis follow the same sex-determining pathway as somatic differentiation, but with a notable exception: *tra-1* is not the terminal regulator for sexual differentiation in the germ line. In addition, germ-line-specific sex-determining genes and germ-line-specific controls of global sex-determining genes are also involved in regulating the specification of male and female gametes (Fig. 1B).

We have focused on the function and regulation of the *tra-2* gene, which plays a fundamental role in promoting XX

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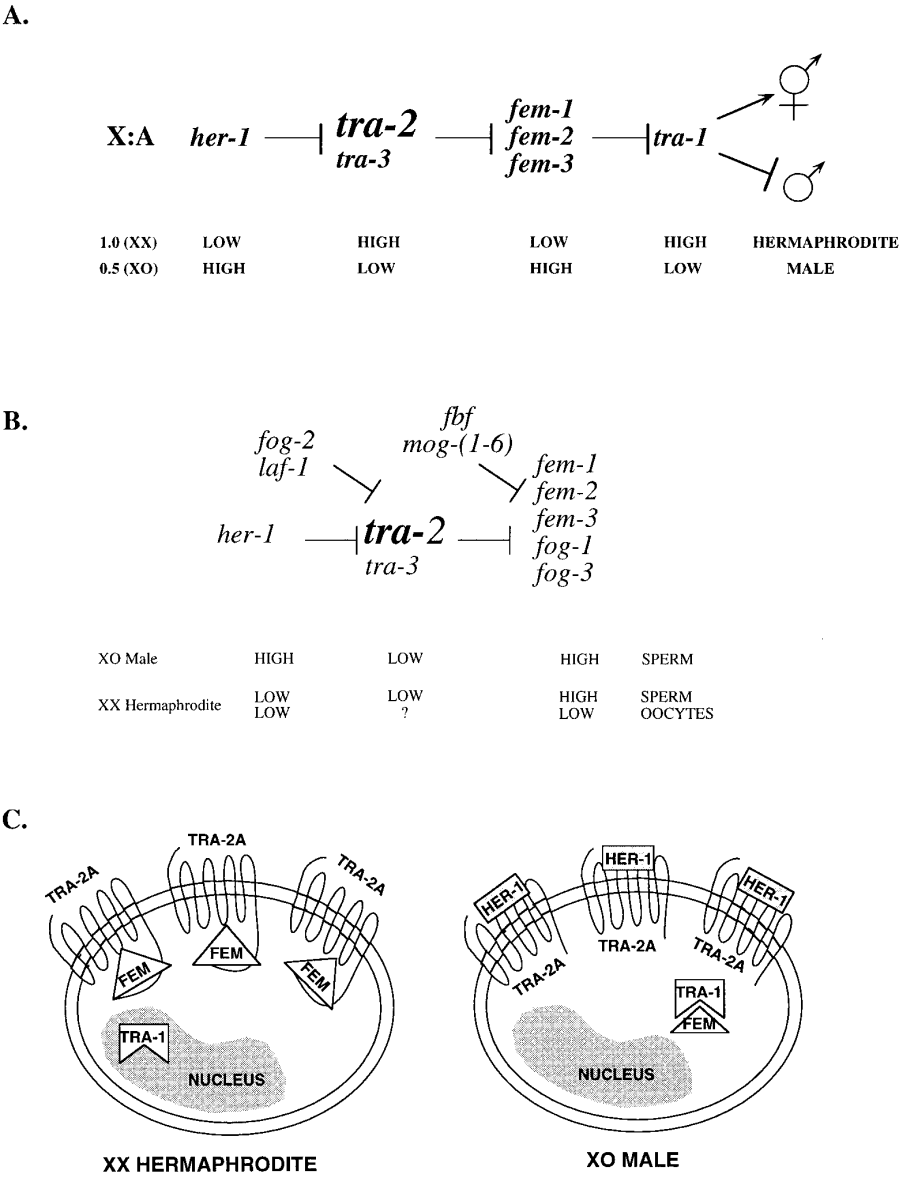

FIG. 1. Regulation of sex determination in the nematode *C. elegans*. (A) Genetic regulation of sex determination in somatic tissues (modified from Hodgkin, 1990; Villeneuve and Meyer, 1990). The X:A ratio is the primary determinant of sex and sets the activity states of the genes controlling both sex determination and dosage compensation; however, only genes whose effect is restricted to sex determination are shown. In XX animals, *tra-2* and *tra-3* (Hodgkin and Brenner, 1977) negatively regulate the *fem* genes. *tra-1* is then free to promote female somatic development. In XO animals, *her-1* (Hodgkin, 1980; Trent *et al.*, 1988) is active and functions to negatively regulate *tra-2* and *tra-3*, permitting the *fem* genes to promote male development by negatively regulating *tra-1*. (B) Genetic regulation of sex determination in the germ line (for reviews, Ellis and Kimble, 1994; Schedl, 1997). Sex determination in the germ line is controlled by global sex-determining genes described in A and by germ-line-specific regulators, *fog-1* (Barton and Kimble, 1990), *fog-2* (Schedl and Kimble, 1988), *fog-3* (Ellis and Kimble, 1995), *mog-(1–6)* (Graham and Kimble, 1993; Graham *et al.*, 1993), and *laf-1* (Goodwin *et al.*, 1996). *tra-1* is not the terminal regulator of germ-line sex determination: although *tra-1* is involved in germ-line sex determination its role remains unclear (Hodgkin, 1987a; Schedl *et al.*, 1989). In XO males, *her-1* negatively regulates *tra-2* and *tra-3*, permitting *fog-1*, *fog-3*, and the *fem* genes to direct spermatogenesis. In XX hermaphrodites, the germ-line produces a limited number of sperm before switching to oogenesis. It has been postulated that *tra-2* is negatively regulated by *fog-2* to allow the onset of hermaphrodite spermatogenesis (Doniach, 1986; Schedl and Kimble, 1988). *laf-1* is also postulated to repress the activity of *tra-2* by functioning as a translational repressor of the *tra-2* 3′ UTR to allow hermaphrodite spermatogenesis (Goodwin *et al.*, 1996). In turn, negative regulation of *fem-3*, by *fbf* (Zhang *et al.*, 1997), allows the switch from spermatogenesis to oogenesis (Barton *et al.*, 1987; Ahringer and Kimble, 1991). Although *her-1* is a negative regulator of *tra-2* activity in the XO male germ line, it is not a regulator of male sex determination in the hermaphrodite germ line. (C) A speculative

female development in both somatic and germ-line tissues by negatively regulating the *fem* genes (Klass *et al.,* 1976; Hodgkin and Brenner, 1977; Kimble *et al.,* 1984; Doniach and Hodgkin, 1984) (Figs. 1A and 1B). The *tra-2* gene generates three transcripts of 4.7, 1.8, and 1.9 kb (Okkema and Kimble, 1991), but hitherto only the 4.7-kb mRNA has been investigated in detail. The 1.8-kb *tra-2* mRNA is hermaphrodite- and germ-line-specific, whereas the 1.9-kb mRNA is male-specific (Okkema and Kimble, 1991). The 4.7-kb *tra-2* mRNA encodes a predicted integral membrane protein, TRA-2A, which provides the major feminizing activity of the *tra-2* locus (Kuwabara *et al.,* 1992; Kuwabara and Kimble, 1995). Figure 1C presents our working model for TRA-2A function in somatic sex determination. In the soma of XX hermaphrodites, TRA-2A is postulated to negatively regulate one or more of the FEM proteins; the predicted intracellular carboxy-terminal region of TRA-2A is likely to play a major role in this regulation (Kuwabara and Kimble, 1995). Consequently, TRA-1 is free to promote female somatic development. In the soma and germ line of XO males, HER-1 is postulated to function cell nonautonomously by inactivating TRA-2A on neighboring cells (Hunter and Wood, 1992; Perry *et al.,* 1993; Kuwabara, 1996a). As a result, the FEM proteins are active, TRA-1 is inactivated, and male development ensues.

The activity of *tra-2* must also be negatively regulated in the germ line of XX animals to achieve hermaphrodite spermatogenesis (Doniach 1986; Schedl and Kimble, 1988). Specifically, dominant *tra-2*(*mx*) mutations inappropriately feminize the hermaphrodite germ line: hermaphrodite spermatogenesis is eliminated and XX animals are transformed into females; by contrast, XO animals are unaffected. In addition to this dominant germ-line effect, *tra-2*(*mx*) mutations reveal a partial loss-of-function somatic masculinization when placed *in trans* to a null mutation (Doniach, 1986); hence, these alleles are called *tra-2*(*mx*) because of their mixed character (both gain-of-function and loss-of-function; Doniach, 1986; Schedl and Kimble, 1988). Distinct from the *tra-2*(*mx*) class are six *tra-2*(*gf*) mutations, which feminize XX and partially feminize XO animals and have no apparent loss-of-function character. The *tra-2*(*gf*) mutations are associated with lesions in the *tra-2* 3′ untranslated region (UTR) and appear to disrupt translational control (Okkema and Kimble, 1991; Kuwabara *et al.,* 1992; Goodwin *et al.,* 1993). A candidate gene involved in this translational repression is *laf-1* (for lethal and feminizing) (Goodwin *et al.,* 1997). The existence of these dominant feminizing mutations has led to the hypothesis that *tra-2* is negatively regulated to achieve the onset of hermaphrodite spermatogenesis; the existence of two classes of such mutations suggests that two different types of control influence the onset of hermaphrodite spermatogenesis.

In this paper, we further characterize the nature of the 1.8-kb *tra-2* transcript and its role in regulating sexual fate decisions. In addition, we examine the molecular basis of the *tra-2*(*mx*) mutations. This work leads to four main conclusions. First, the 1.8-kb *tra-2* mRNA is predicted to encode a protein, TRA-2B, with the same sequence as the intracellular feminizing domain of TRA-2A. Second, the 1.8-kb *tra-2* mRNA is correlated with oocytes rather than sperm. Third, there is a *tra-2* maternal effect on brood size, which is associated with the 1.8-kb *tra-2* mRNA. And fourth, our characterization of the *tra-2*(*mx*) mutants identifies a potential germ-line-specific regulatory site present in both TRA-2A and TRA-2B, which controls the onset of hermaphrodite spermatogenesis. Based on these results, we discuss how multiple *tra-2* gene products and germ-line-specific regulation of *tra-2* activity affect sexual cell fate decisions.

MATERIALS AND METHODS

Worm Strains and Culture

Worms were grown on petri dishes or in liquid as described (Sulston and Hodgkin, 1988). All genetic characterizations were at 20°C unless otherwise indicated, using worms that were not starved or recovering from the dauer state. To synchronize animals, eggs were isolated after hypochlorite treatment of gravid hermaphrodites. For mRNA isolation, XX *fem-1*(*hc17*ts) and *fem-3*(*q20*gf) animals were raised at restrictive temperature (25°C) and harvested as adults. Descriptions of *C. elegans* genes and alleles can be found elsewhere (Hodgkin, 1997).

Linkage groups (LG) were as follows: LGI, *spe-8*(*hc53*); LGII, *tra-2*(*e1095, sc146*); and LGIV, *fem-1*(*e1965, hc17*ts), *mor-2*(*e1125*), *unc-24*(*e138*), *fem-3*(*q20*gf), *dpy-20*(*e1282*).

General Manipulation of Nucleic Acids

General methods for manipulating nucleic acids, gel electrophoresis, and hybridization are described (Sambrook *et al.,* 1989). Nematode DNA was prepared as described (Emmons and Yesner, 1984). RNA was isolated by the guanidinium thiocyanate protocol of Chirgwin *et al.* (1979). Poly(A)$^+$ mRNA was isolated by oligo(dT) chromatography (Pharmacia). The Northern blot in Fig. 5A was hybridized as described by Sambrook *et al.* (1989) using a ^{32}P-labeled random-primed PCR fragment (Feinberg and Vogelstein, 1984), amplified from lambda JK57 (Kuwabara *et al.,* 1992) with

molecular model of somatic sex determination in *C. elegans* (adapted from Kuwabara *et al.,* 1992). TRA-2A is depicted as a membrane protein with an extracellular amino terminus and an intracellular carboxy-terminal tail. In XX hermaphrodites, a carboxy-terminal region of TRA-2A is likely to inhibit the activity of the *fem* gene products, perhaps by sequestration (Kuwabara and Kimble, 1995). In turn, TRA-1 functions in the nucleus to promote hermaphrodite somatic development. In XO male development, the HER-1 protein encodes a predicted secreted protein that functions cell nonautonomously to promote male development (Perry *et al.,* 1993; Hunter and Wood, 1992). *her-1* mRNAs are detected in XO, but not XX animals (Trent *et al.,* 1991). HER-1 is postulated to function as a repressive ligand that inactivates TRA-2A. In turn, the FEM proteins are released from inhibition and promote male development by negatively regulating the activity of TRA-1.

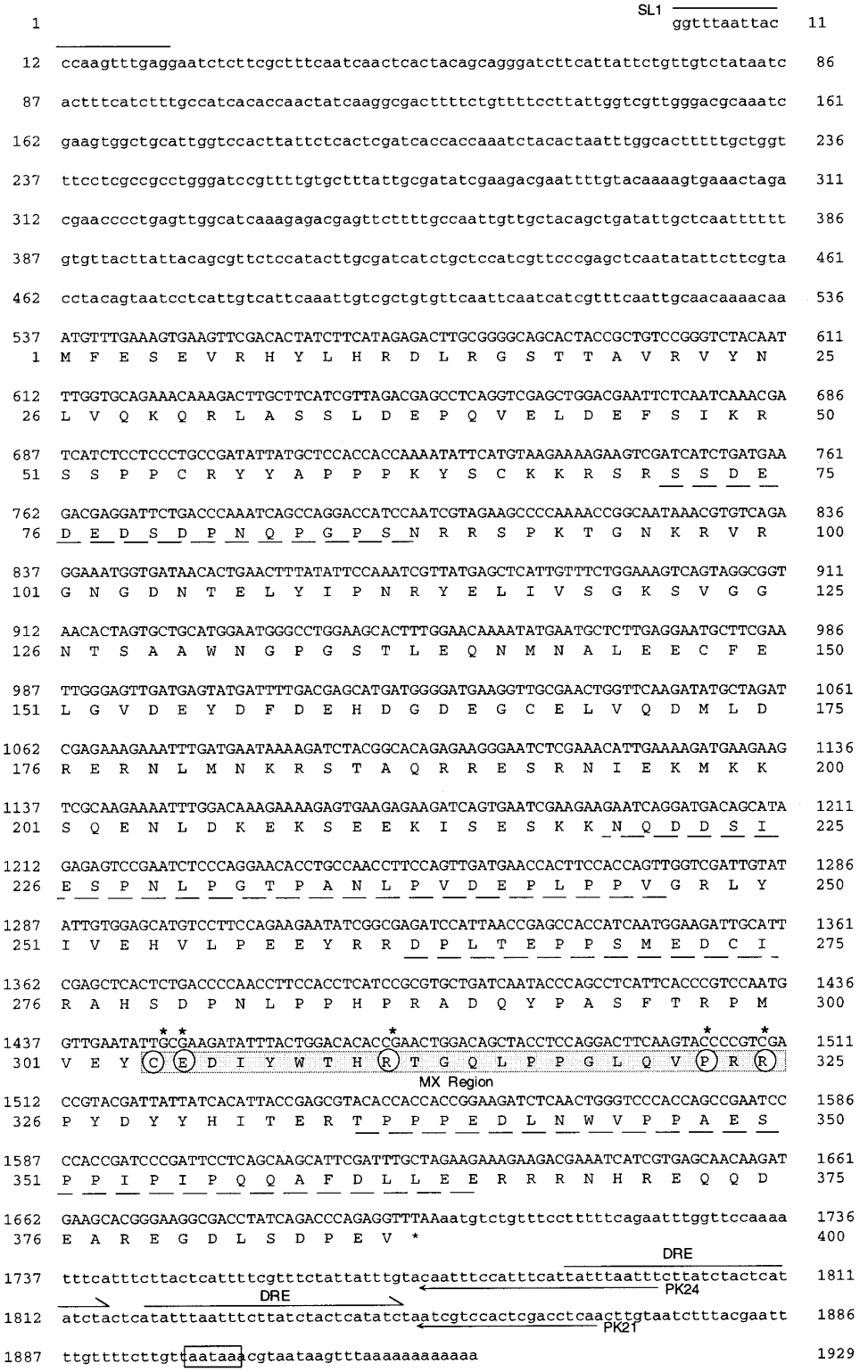

FIG. 2. Sequence analysis of a 1.8-kb *tra-2* cDNA. Sequence of a composite 1.8-kb *tra-2* cDNA. Numbering begins with the first nucleotide of the SL1 *trans*-spliced leader. Nucleotide 23 of the 1.8-kb *tra-2* cDNA corresponds to nt 2787 of the 4.7-kb *tra-2* cDNA (Kuwabara *et al.*, 1992); nt in lowercase correspond to 5′ and 3′ UTR sequences; nt in uppercase correspond to the ORF. The deduced amino acid sequence of TRA-2B is placed below the nucleotide sequence. Amino acids are designated using the single-letter code. Arrows have a 5′ to 3′ polarity

oligonucleotides PK36 and PK81 (see below for sequence). In Fig. 6, Northern blots were hybridized ~16 h at 66°C in 50% formamide, 5× Denhardt's, 5× SSC, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA with ~1 × 10^6 cpm/ml ^{32}P-labeled RNA probe (Melton *et al.*, 1984) synthesized from *tra-2* cDNA clone, pJK111 (Fig. 3). Blots were washed in 1× SSC, 0.1% SDS at 65°C. Rehybridization with *myo-1*, a pharyngeal myosin (Miller *et al.*, 1986), showed that RNA loadings were comparable (Fig. 5B). Densitometry of transcript bands was performed using a Molecular Dynamics Series 300 or Zenith Model SL-504-XL densitometer to calculate the mean peak area (expressed in arbitrary units).

Cloning the 1.8-kb tra-2 cDNA pPK42

To synthesize cDNA, poly(A)$^+$ mRNA was isolated from wild-type adult XX hermaphrodites, which provides a source of abundant 4.7- and 1.8-kb *tra-2* mRNAs, but not the 1.9-kb *tra-2* mRNA. First-strand *tra-2* cDNA was synthesized using the oligonucleotide PK21 (see Fig. 2) and reverse transcriptase (Bethesda Research Labs). This oligonucleotide sequence was selected because the 4.7- and 1.8-kb *tra-2* mRNAs share sequences in the 3′ region of the *tra-2* transcription unit (Okkema and Kimble, 1991). Second-strand replacement was achieved by PCR using either SL1 or SL2 as 5′ sense primers, because many *C. elegans* mRNAs are *trans*-spliced (Krause and Hirsh, 1987; Huang and Hirsh, 1989), and a *tra-2*-specific antisense oligonucleotide (PK24, see Fig. 2), which is nested in the 4.7-kb *tra-2* 3′ UTR. PCR fragments were made blunt using DNA polymerase I Klenow fragment (New England Biolabs) and phosphorylated with T4 polynucleotide kinase (New England Biolabs). PCR fragments were electrophoresed through a 1.2% agarose gel, eluted using GeneClean (Bio 101), and blunt-end ligated into the *Eco*RV site of pBSKSII(+) (Stratagene).

DNA Sequencing

DNA sequencing was performed (Sanger *et al.*, 1977) using Sequenase 2.0 (United States Biochemicals) as directed by the manufacturer for double-stranded DNA templates. DNA sequences were compiled and analyzed using DNA Strider 1.2 (Marck, 1988) and the SEQNET facility at Daresbury Laboratory.

Analysis of a tra-2 Maternal Effect

To examine the brood sizes of *tra-2*/+ XX animals lacking a maternal contribution of wild-type *tra-2 m−z−*, *tra-2; fem-1 mor-2* XX females were mated with N_2 males to generate *tra-2*/+; *fem-1 mor-2*/+ + XX hermaphrodites. Animals of the same genotype were also generated by mating *fem-1 mor-2* females with *tra-2; fem-1 mor-2*/+ + males to ask whether maternal *fem-1* affects brood size. To examine the brood sizes of *tra-2*/+ XX animals maternally inheriting wild-type *tra-2 m + z +*, *unc-24 dpy-20* XX hermaphrodites were mated with *tra-2*; *fem-1 mor-2*/+ + XO males. This mating generates both *tra-2*/+; *unc-24 dpy-20*/+ + and *tra-2*/+; *unc-24 dpy-20*/*fem-1 mor-2* XX hermaphrodites; however, only the latter broods were counted. Each of the crosses described above were performed using the *tra-2* alleles *e1095* and *sc146*. *mor-2* is not involved in sex determination; however, it is included as a recessive marker because it is closely linked to *fem-1*.

Sequence Analysis of tra-2(mx) Mutations

Genomic DNA was isolated from animals homozygous for *tra-2*(*e2021*, *e1939*, *q179*, *e1403*, *e1940*, or *e1941*mx), digested with *Eco*RI, and analyzed by Southern blot analysis. No restriction fragment length polymorphisms were found associated with the *tra-2* locus. A 4.5-kb genomic *Bam*HI–*Hin*dIII DNA fragment containing the *tra-2* 3′ end was subsequently cloned into λ phage vector Charon 17, and later subcloned into pIBI176 (IBI), from all of the *tra-2*(*mx*) mutants, except *tra-2*(*e1403*mx), which was first mapped by hydroxylamine DNA mismatch detection (Cotton *et al.*, 1988).

Oligonucleotides

Oligonucleotide sequences are listed 5′ to 3′. Exon and intron numbering is based on the primary sequence of the 4.7-kb *tra-2* RNA (refer to Fig. 2 and Kuwabara *et al.*, 1992): SL1, GGTTTAATTACCCAAGTTTG; SL2, GGTTTTAACCCAGTTACTCA; PK21, GTTGAGGTCGAGTGGACGAT, exon 23 (3′ UTR); and PK24, AGAAATTAAATAATGAAATGGAAATTG, exon 23 (3′ UTR).

RESULTS

Identification of a cDNA Corresponding to the 1.8-kb tra-2 mRNA

Two approaches were used to obtain cDNA clones corresponding to the hermaphrodite-specific and germ-line-dependent 1.8-kb *tra-2* mRNA. First, 26 *tra-2* cDNAs were examined (isolated from libraries kindly provided by S. Kim and C. Martin); at the level of restriction pattern analysis, all appeared to be shortened versions of the 4.7-kb *tra-2* cDNA. Since the 1.8-kb mRNA is not rare and should have been represented in these libraries, we reasoned that the 4.7- and 1.8-kb *tra-2* mRNAs might be colinear. To support this notion, we showed that oligonucleotides complementary in sequence to exons 18–23 (numbering as in Fig. 3) hybridized to the 1.8-kb mRNA and formed duplexes that were cleaved by RNase H, generating two RNA fragments that could be detected by Northern blot analysis (data not shown). Oligonucleotides complementary to several exon sequences upstream of exon 18 or to intron sequences

(arrowhead is 3′) and are placed below the nucleotide and amino acid sequences to indicate both location and sequence of oligonucleotides discussed in the text. The single-base changes associated with the *tra-2*(*mx*) mutations are indicated by asterisks placed above the nucleotide sequence; amino acids affected by these base changes are circled. The MX region is highlighted in gray. PEST motifs, which are associated with protein instability (Rogers *et al.*, 1986), are underlined with a dashed line. The 28-bp direct repeat element (DRE) in the 3′ UTR is indicated by overhead arrows. The putative polyadenylation sequence AATAAA is boxed. The sequence of pPK42 ends at position 1746 due to the way the cDNA was generated (see text); however, the 3′ UTR of the 1.8-kb *tra-2* mRNA is likely to include the DREs, as shown here (see text).

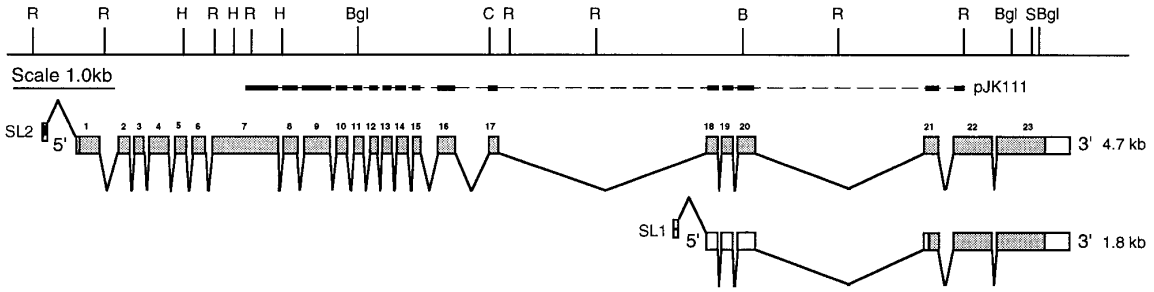

FIG. 3. Comparison of the 1.8- and 4.7-kb *tra-2* mRNAs. Above, a simplified restriction map of the *tra-2* locus (from Kuwabara *et al.*, 1992). Middle, cDNA clone pJK111. pJK111 was used as a probe for Northern blot hybridizations; exon sequences present in pJK111 correspond to filled black boxes (described in Okkema and Kimble, 1991). Below are the 4.7- and 1.8-kb *tra-2* RNAs. Exons are boxes and introns are lines. Exon numbering is based on characterization of the 4.7-kb *tra-2* mRNA (Kuwabara *et al.*, 1992). pPK42 was used to deduce the exon/intron structure of the 1.8-kb *tra-2* RNA. By this convention, the 1.8-kb *tra-2* mRNA contains exons 18–23, but not exons 1–17. Gray filled boxes designate the predicted coding regions; unshaded boxes correspond to the predicted 3′ and 5′ untranslated regions. The 4.7-kb *tra-2* mRNA is *trans*-spliced to SL2, while the 1.8-kb *tra-2* mRNA is *trans*-spliced to SL1. R, *Eco*RI; H, *Hin*dIII; Bgl, *Bgl*II; C, *Cla*I; B, *Bam*HI; S, *Sal*I.

downstream of exon 18 failed to form cleavable duplexes with the 1.8-kb mRNA (data not shown). Therefore, we next generated *tra-2* cDNAs by RT-PCR from *C. elegans* poly(A)$^+$ mRNA enriched for the 1.8-kb and lacking detectable 1.9-kb *tra-2* mRNA (see Materials and Methods). A 1.7-kb RT-PCR product named pPK42 was cloned using SL1 (a spliced leader sequence) and PK24 (Fig. 2) as primers; no products were generated using SL2.

It is likely that pPK42 was generated from the 1.8-kb *tra-2* mRNA for three reasons. First, the presence of the *trans*-spliced leader SL1 at the 5′ end of pPK42 indicates that this cDNA is not simply a truncated cDNA primed from the longer 4.7-kb mRNA. Second, pPK42 was generated from RNA containing abundant 1.8-kb mRNA, but no detectable 1.9-kb mRNA. Third, the size is appropriate.

The Protein Encoded by the 1.8-kb tra-2 mRNA Is Identical to an Intracellular Region of TRA-2A

The DNA sequence of pPK42 is presented in Fig. 2. The 5′ end of pPK42 carries the 22-nt *trans*-spliced leader, SL1. The remaining sequence, nt 23–1747, is identical to that of nt 2787–4511 of the 4.7-kb *tra-2* cDNA. This shared region extends from exon 18 through exon 23 of the 4.7-kb *tra-2* mRNA (Fig. 3). Although the 3′ end should have been defined by the antisense primer used in its synthesis (PK24, Fig. 2), it appears instead to have been partially truncated during blunt-ended cloning. Therefore, because Northern blot analysis indicates that the 1.8- and 4.7-kb *tra-2* mRNAs share the same 3′ UTR (data not shown), then the actual size of the 1.8-kb mRNA with its *trans*-spliced leader is predicted to be 1917 nt.

The longest open reading frame (ORF) associated with pPK42 is 1164 nt long. This ORF begins with the first methionine at nt 537 and is predicted to encode a polypeptide of 387 amino acids (predicted molecular weight 44,534 Da), named TRA-2B (Fig. 2). TRA-2B corresponds in sequence to a predicted intracellular carboxy-terminal region of the membrane protein TRA-2A; hydropathy analysis indicates that TRA-2B is likely to be cytoplasmic because it lacks an N-terminal signal sequence and potential membrane-spanning domains (Kyte and Doolittle, 1982). As was noted already for this stretch of TRA-2A (Kuwabara *et al.*, 1992), TRA-2B contains potential serine and threonine phosphorylation sites and four PEST sequence motifs (Rogers *et al.*, 1986), which have been implicated in controlling protein degradation. BLAST analysis (Altschul *et al.*, 1990) reveals that TRA-2B is significantly similar in sequence to Ce-TRA-2A and Cb-TRA-2A and to two predicted *C. elegans* ORFs, F54F7.4 (LGX) and F17A2.1 (LGX) (Wilson *et al.*, 1994, see text below). Figure 4 shows an alignment of these protein sequences.

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                                          MX Region
                                       **       *          * *
1350/262 RDPLTEPPSM EDCIRAHSDP NLPPHPRADQ YPASFTRPMV EYCEDIYWTH RTGQLPPGLQ VPRRPYDYYH I.TERTPPPE   Ce-TRA-2A/B
     204 RNYFTEPPSI ECCVRE..NQ GLPPHPRANQ YPAHFCKAMV AYCEDPYWTE RTVPLPPGLV VPRRPLDYYN IIPNLSPPPE   F54F7.4
     385 RDYKTEPPII EEVL.ELTRN C...HPKAHL YPPAFSYAMI EYSEDRFWTE RTVDVPPGVL VPNGP                   F17A2.1
    1368 RNPLHDPPSM EEYVQKYDDP NQPPSRRADQ YPPSFTPAMV GYCEDVYWKY NERNLPDNVP MPPRPRDWDQ RRLVELPPPE   Cb-TRA-2A
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FIG. 4. Alignment of sequences detected by BLAST analysis with similarity to TRA-2B. Ce-TRA-2A and TRA-2B are shown as a single protein sequence for the purposes of this comparison. The MX region, identified by the analysis of *tra-2*(*mx*) mutations, is highlighted by a gray box. Individual amino acids affected by the *tra-2*(*mx*) mutations are indicated by asterisks. Amino acids conserved in three of four sequences are in boldface.

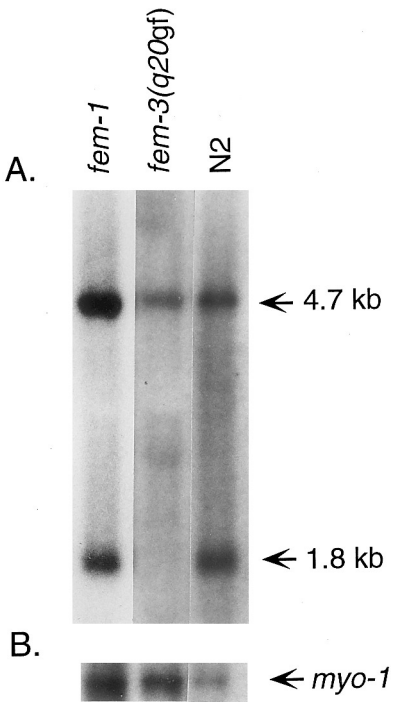

FIG. 5. The 1.8-kb *tra-2* mRNA appears to be oocyte-specific. (A) Northern blot of poly(A)$^+$ mRNA prepared from synchronized adults grown at restrictive temperature (25°C) and hybridized to a ^{32}P-labeled probe that recognizes all *tra-2* mRNAs. Lanes 2 and 3 are overexposed relative to lane 1 to highlight the absence of the 1.8-kb *tra-2* mRNA in lane 2. The 4.7- and 1.8-kb *tra-2* mRNAs are indicated. (B) To normalize the amount of RNA loaded, the blot in (A) was rehybridized with a probe specific for pharyngeal *myo-1*. The *myo-1* mRNA band is indicated.

The 1.8-kb tra-2 mRNA Appears to Be Oocyte-Specific

The 1.8-kb *tra-2* mRNA is normally expressed only in adult hermaphrodites and embryos and is germ-line-dependent (Okkema and Kimble, 1991). To ask whether this RNA is associated with sperm or oocytes preferentially, we examined *tra-2* mRNAs in mutants that make either only sperm or only oocytes. Specifically, XX *fem-3*(*q20*gf) mutants produce only sperm (Barton *et al.*, 1987) and XX *fem-1*(*hc17*) mutants produce only oocytes (Nelson *et al.*, 1978) when raised at restrictive temperature; in contrast to their germ lines, *fem-3*(*q20*gf) and *fem-1*(*hc17*) mutants both possess a typical hermaphrodite soma. We found that the 1.8-kb *tra-2* mRNA is detected only in animals bearing oocytes: XX *fem-1*(*hc17*) animals possess the 1.8-kb *tra-2* mRNA, but XX *fem-3*(*q20*gf) do not (Fig. 5A, compare lanes 1 and 2). We suggest that the 1.8-kb *tra-2* mRNA is not expressed by spermatogenic cells and may be transcribed from an oocyte-specific promoter; consequently, the embryonic 1.8-kb *tra-2* mRNA may be a maternal product inherited with the oocyte.

Maternal Inheritance of the tra-2 1.8-kb mRNA Affects Germ-Line Sex

If *tra-2* products are contributed maternally to the oocyte, then there may be a maternal effect of *tra-2* genotype which has not been noticed previously. To examine this point more closely, we compared the phenotypes of *tra-2*/+; *fem-1*/+ XX animals derived from *tra-2* mothers and from *tra-2*(+) mothers: $m-z+$ compared to $m+z+$ (refer to Table 1 and Materials and Methods for details). Initially we used the *tra-2* allele *e1095*, which carries a nonsense mutation that affects all *tra-2* transcripts (Kuwabara *et al.*, 1992). In both cases, the *tra-2*/+ XX animals are hermaphrodite in phenotype, but there is a significant difference in the level of spermatogenesis, as revealed by different brood sizes: 465 ± 44 for $m-z+$ compared to 353 ± 21 for $m+z+$. The number of hermaphrodite self-progeny is limited by sperm number, so animals with a partially masculinized germ line make more sperm and produce more self-progeny. Therefore, because *tra-2*($m-z+$) mothers produce larger broods than *tra-2*($m+z+$) mothers, this indicates that *tra-2*(+) normally exerts a maternal feminizing effect on germ-line sexual phenotype. This could be due to inheritance of either the 4.7- or the 1.8-kb transcript or both. In order to distinguish between these possibilities, we examined a different allele of *tra-2*, *sc146*, which is a chromosomal rearrangement that affects sequences associated with the 4.7-kb mRNA, but not the 1.8 kb (Okkema and Kimble, 1991). The mean brood size for *sc146*/+ $m-z+$ hermaphrodites is 386 ± 22, compared to 355 ± 23

TABLE 1

Maternal Effect of *tra-2* on Brood Size

Genotype[a]	Brood size[b]	*n*
(m −, z+)[c,d]		
tra-2(*e1095*)/+; *fem-1 mor-2*/++	465 ± 44 Range 399–504	5
(m−, z+)[c,d]		
tra-2(*sc146*)/+; *fem-1 mor-2*/++	386 ± 22 Range 349–411	9
(m+, z+)[c,e]		
tra-2(*e1095*)/+; *fem-1 mor-2*/++	353 ± 21 Range 322–376	5
(m+, z+)[c,e]		
tra-2(*sc146*)/+; *fem-1 mor-2*/++	355 ± 23 Range 313–382	6
(m+, z+)[c,d]		
tra-2(*sc146*)/+; *fem-1 mor-2*/++	359 ± 15 Range 341–378	6

Note. n, total number of broods counted.

[a] All animals were XX hermaphrodites of the specified genotype.

[b] XX wild-type N_2 mean brood size is 327 ± 28, for comparison (Hodgkin and Barnes, 1991).

[c] (m, z) refers to the maternal and zygotic *tra-2* genotypes.

[d] These animals were obtained from *fem-1*/*fem-1* mothers.

[e] These animals were obtained from *fem*(+)/*fem*(+) mothers.

TABLE 2
Molecular Basis of *tra-2*(*mx*) Mutations

Allele	Base change[a]	Amino acid change[b]
e2021	G 4211 → A	C 1392 → Y
e1939	G 4213 → A	E 1393 → K
q179, e2019	G 4235 → A	R 1400 → Q
e1403	C 4268 → T	P 1411 → L
e1940, e1941	G 4274 → A	R 1413 → Q

[a,b] Nucleotide and amino acid coordinates from Kuwabara *et al.* (1992).

for $m + z +$. Both of these means are significantly lower than that obtained with *e1095*/+ $m - z +$. Because the *tra-2*/+ $m - z +$ hermaphrodites were obtained from female mothers that were both *tra-2*(−/−) and *fem-1*(−/−), it was possible that the maternal absence of *fem-1* might also have an effect on brood size. To exclude this possibility, we measured the broods produced by *tra-2*(*sc146*)/+; *fem-1*/+ hermaphrodites, which were obtained by mating *fem-1*(−/−) females with XO *tra-2* males. These animals had a mean brood size of 359 ± 15 (see Materials and Methods for details), which is indistinguishable from the previous number obtained for *tra-2*(*sc146*)/+; *fem-1*/+ animals, when the mother was +/+ for *fem-1* (Table 1, compare lines 4 and 5). Therefore, the absence of maternal *fem-1* does not affect brood size, if *fem-1*(+) is supplied zygotically. These data indicate that maternally derived *tra-2* mRNA, and more specifically the 1.8-kb mRNA, is involved in promoting female development of the hermaphrodite germ line.

Molecular Identification of tra-2(mx) Mutations

To identify molecular defects in the dominant *tra-2*(*mx*) mutants, genomic DNA was analyzed by Southern blot. The *tra-2*(*e1403*) mutant was also included in these studies, because it displays an *mx* phenotype (R. Edgar and T. Schedl, unpublished). Unlike the dominant *tra-2*(*gf*) mutations, which have deletions or insertions in the *tra-2* 3′ UTR (Okkema and Kimble, 1991; Goodwin *et al.*, 1993), no restriction length polymorphism was detected in any *tra-2*(*mx*) allele (data not shown). With the idea that the *tra-2*(*mx*) mutations might nonetheless affect the 3′ UTR, we analyzed a 4.5-kb restriction fragment of *tra-2*(*mx*) genomic DNA encompassing the *tra-2* 3′ end. Although no *tra-2*(*mx*) 3′ UTR sequence changes were found, single-base changes that generated nonconserved amino acid substitutions in the *tra-2* coding region were identified (Table 2). These amino acid changes are clustered in a 22-amino-acid region present in both TRA-2A and TRA-2B, named the "MX region" (Figs. 2 and 4).

tra-2(mx) Alleles Do Not Affect tra-2 mRNA Steady-State Levels

To investigate the idea that the *tra-2*(*mx*) mutations might increase *tra-2* activity by elevating *tra-2* mRNA steady-state levels, *tra-2* mRNAs from *tra-2*(*q179*mx) homozygotes were compared to wild type on Northern blots (Fig. 6A). Because *tra-2*(*q179*mx) mutants must be grown as an obligate male/female strain, the *spe-8* male/female strain was used as a control source of *tra-2*(+) mRNA. No significant difference in the steady-state level of either the 1.8- or the 4.7-kb *tra-2* mRNA was detected when mRNAs from the two strains were compared. In addition, no novel *tra-2* mRNAs were produced by *tra-2*(*q179*mx) mutants (Fig. 6B). Therefore, the germ-line feminization of XX *tra-2*(*mx*) females is unlikely to result from elevated *tra-2* mRNA steady-state levels or from the activation of cryptic splice sites giving rise to novel *tra-2* mRNAs. We suggest that the MX region, which is delineated by the *tra-2*(*mx*) mutations, defines a protein–protein interaction domain that may bind a negative regulator of *tra-2* germ-line activity.

Conservation of the TRA-2 MX Region

BLAST analysis indicates that TRA-2B shares sequence similarity with Ce-TRA-2A and Cb-TRA-2A (Kuwabara,

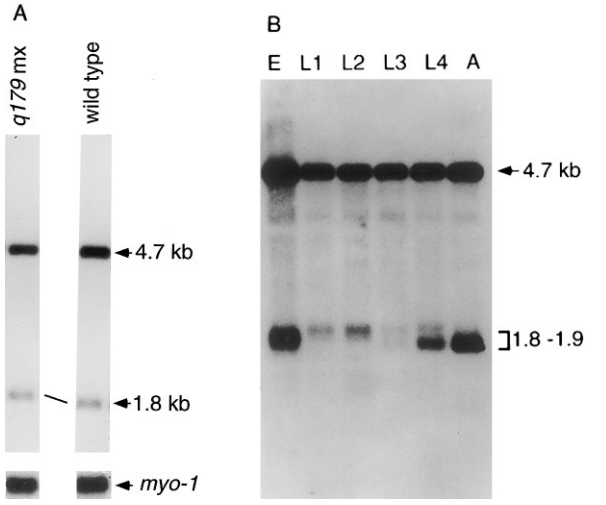

FIG. 6. *tra-2* mRNAs during development of *tra-2*(*q179*mx) mutants. (A) Comparison of steady-state levels of *tra-2* mRNAs isolated from synchronized *tra-2*(*q179*mx) and *spe-8*(*hc53*) adults. Northern blots show *tra-2* mRNAs (top) and *myo-1* mRNAs (bottom) isolated from *tra-2*(*mx*) and *spe-8*(*hc53*) adult animals. Because *tra-2*(*q179*mx) is an obligate male/female strain and because *tra-2* mRNA levels differ between the sexes (Okkema and Kimble, 1991), we used another obligate male/female strain, *spe-8*(*hc53*), as our source of wild-type mRNA. (B) Northern blot of poly(A)$^+$ mRNAs prepared from synchronized *tra-2*(*q179*mx) animals at different developmental stages. E, embryos; L1–L4, first to fourth larval stages; A, adults. Approximate size of each mRNA is marked. This profile is virtually the same as observed with wild type (Okkema and Kimble, 1991). Northern blots were hybridized to a ^{32}P-labeled RNA probe made from *tra-2* cDNA clone pJK111, which detects all *tra-2* mRNAs, as described under Materials and Methods. A *myo-1* ^{32}P-labeled probe (pJK191), which detects pharyngeal myosin, was used to normalize the somatic equivalents of RNA loaded. Approximate size of each mRNA is marked.

1996b), as might be predicted. However, given that the Ce-TRA-2A and Cb-TRA-2A sequences are among the most rapidly evolving of all proteins so far compared between the two species, it was surprising to identify two other predicted *C. elegans* proteins, F54F7.4 and F17A2.1, that also display a striking conservation of the MX region. The sequence alignment shown in Fig. 4 indicates that each of these predicted proteins contains a region with extensive sequence similarity to the TRA-2A/B MX region. In addition, the amino acid residues affected by the *tra-2*(*mx*) mutations are conserved among this set of proteins (Fig. 4). F54F7.4 and F17A2.1 also share an additional 50 amino acids of sequence similarity with TRA-2A outside of the MX region (Fig. 4). We have named the genes associated with F54F7.4 and F17A2.1 *trx-1* and *trx-2* (for *tra-2*(*mx*) related), respectively. The conservation of the MX region reveals that we may have identified a protein interaction motif through our analysis of *tra-2*(*mx*) mutations.

DISCUSSION

TRA-2B, a Predicted Cytoplasmic Protein with Feminizing Activity

The predicted amino acid sequence of TRA-2B protein is colinear with an intracellular portion of TRA-2A, which has been shown to have ectopic feminizing potential when expressed in transgenic animals (Kuwabara *et al.*, 1992; Kuwabara and Kimble, 1995; this paper). By extension, we suggest that TRA-2B also carries the same potential feminizing activity. Recent work indicates that this intracellular domain of TRA-2A/B binds to the FEM-3 protein (A. Spence and P.K., unpublished data); furthermore, this FEM-3 binding region appears to be distinct from the MX region (A. Spence and P.K., unpublished data). Such binding may be the primary biochemical activity of the intracellular domain of TRA-2 that achieves female development.

TRA-2B is likely to be HER-1-independent. Normally in XO animals, secreted HER-1 acts nonautonomously to inactivate TRA-2A (Hunter and Wood, 1992; Perry *et al.*, 1993). Dominant *tra-2*(*eg*) mutations that map to the predicted extracellular domain of TRA-2A render this protein HER-1 insensitive (Kuwabara, 1996a). TRA-2B does not share with TRA-2A the predicted extracellular region that is required for HER-1 regulation, nor does it contain hydrophobic stretches that might act either as a signal sequence or as membrane-spanning domains. Therefore, TRA-2B is likely to escape regulation by HER-1. Moreover, the germline-dependent 1.8-kb *tra-2* mRNA, encoding TRA-2B, is *trans*-spliced to SL1 and is therefore likely to be expressed from a germ-line promoter that is distinct from the 4.7-kb mRNA promoter. We speculate that such a promoter may reside within the unusually large 2.59-kb intron 17 associated with the 4.7-kb mRNA; the *cis*-splice acceptor associated with this intron also functions as the SL1 splice-acceptor for the 1.8-kb mRNA (Fig. 3).

A Potential Negative Regulatory Domain in TRA-2A and TRA-2B

The *tra-2*(*mx*) mutations disrupt a component of *tra-2* regulation that is required specifically to allow the onset of hermaphrodite spermatogenesis (Doniach, 1986; see Introduction). The *tra-2*(*mx*) mutations do not elevate *tra-2* mRNA levels or change the types of mRNAs made. However, Table 2 shows that each *tra-2*(*mx*) mutant carries a nonconserved amino acid substitution in a region common to both TRA-2A and TRA-2B, named the MX region. We suggest that the MX region identifies a potential protein–protein interaction domain involved in negatively regulating *tra-2* activity in the germ line; this negative regulatory site is distinct from that identified by the *tra-2*(*gf*) mutations in the 3′ UTR (Goodwin *et al.*, 1993). We therefore explain the basis of the *tra-2*(*mx*) mixed character phenotype by postulating that the MX amino acid changes have two effects on TRA-2 protein activity: a gain of *tra-2* function, which feminizes the germ line because TRA-2A or TRA-2B is released from negative regulation, and a partial loss of *tra-2* function, which masculinizes the soma because of reduced TRA-2A activity.

Regulation of TRA-2 through the MX region is likely to be posttranslational; a simple interpretation is that the MX region binds a germ-line repressor. The notion that the MX region may identify a protein interaction motif is supported by the observation that two predicted *C. elegans* ORFs, F54F7.4 and F17A2.1 (*trx-1* and *trx-2*), also share sequence similarity with the TRA-2 MX region. A repressor that acts through the MX region is predicted to act specifically in the hermaphrodite germ line. Among various known negative regulators of *tra-2*, only *fog-2* mutants have a phenotype similar to that of *tra-2*(*mx*) mutants: XX mutants are transformed into females and XO mutants are normal males (Schedl and Kimble, 1988). By contrast, two other *tra-2* repressors have distinct phenotypes: *her-1* functions only in XO males (Trent *et al.*, 1991) and *laf-1* apparently acts in both XX and XO animals, probably by repressing *tra-2* at the translational level (Goodwin *et al.*, 1993, 1997).

If FOG-2 is indeed the regulator, we speculate that the *tra-2*(*mx*) mutations interfere with regulation, but do not abolish it, because the *tra-2*(*mx*) mutants remain sensitive to *fog-2* (Schedl and Kimble, 1988). It may be impossible to eliminate the regulatory domain of the *tra-2* protein without completely destroying the feminizing activity of *tra-2*. This view is supported by the *tra-2*(*mx*) mutations themselves, which are already partially defective in promoting female somatic development.

Two Negative Controls of tra-2 Allow the Onset of Hermaphrodite Spermatogenesis

The onset of hermaphrodite spermatogenesis depends on two distinct controls of *tra-2* germ-line activity; disruption of either can eliminate spermatogenesis. Therefore, these are not redundant controls, but instead both must be exercised to achieve hermaphrodite spermatogenesis. One

control occurs through the MX region and is likely to be posttranslational (this paper). The other occurs at the translational level: a 28-nt direct repeat element (DRE) located in the *tra-2* 3′ UTR mediates translational repression (Goodwin *et al.*, 1993). Of these two controls, only that identified by the *tra-2*(*mx*) mutants is specific to the hermaphrodite germ line, because the *tra-2*(*gf*) mutations also feminize XO animals. However, these two controls are likely to act together—translational control exerted through the *tra-2* 3′ UTR may keep *tra-2* protein at a level that can be successfully repressed by a posttranslational inhibitory protein acting through the MX region.

It is intriguing to speculate why the MX region has been conserved in F54F7.4 and F17A2.1 (*trx-1* and *trx-2*). It is possible that these genes have a yet to be determined role in controlling sexual fate or that the activities of these genes are controlled through the MX region. Alternatively, because both genes are found on the X chromosome, these genes may represent the evolutionary remnants of an X-chromosome-based sex-determining mechanism.

Potential Roles of TRA-2A and TRA-2B in Sex Determination

The 1.8-kb *tra-2* mRNA is first detected when the hermaphrodite germ line is switching from spermatogenesis to oogenesis (Okkema and Kimble, 1992); this mRNA also appears to be maternally inherited by the embryo. What role might the 1.8-kb *tra-2* mRNA or its product TRA-2B play in sex determination? We envision two possible roles, which are not mutually exclusive. First, our results indicate that maternally inherited 1.8-kb *tra-2* mRNA may promote hermaphrodite germ-line feminization, with the consequence that brood sizes are reduced. TRA-2B carries two activities that can promote female germ cell fate and hence affect brood sizes: one is the MX region, which might titrate away a repressor of TRA-2A feminizing activity, and the other is the FEM-3 binding site, which may inhibit FEM-3 masculinizing activity (A. Spence and P.K., unpublished data). In addition, the 1.8-kb *tra-2* mRNA might also titrate the repressor that functions through the *tra-2* 3′ UTR away from the 4.7-kb mRNA in order to relieve translational repression of TRA-2A. Finally, expression of the 1.8-kb mRNA and TRA-2B may be part of a feedback loop that reinforces the switch from spermatogenesis to oogenesis.

Second, the 1.8-kb *tra-2* mRNA might also have an important maternal function in the embryo. Because all three *fem* genes show maternal effects, it may be necessary to repress maternal *fem* activity to prevent inappropriate masculinization of XX embryos. Consistent with this hypothesis, polyadenylation of the 1.8-kb *tra-2* mRNA in XX embryos (Okkema and Kimble, 1991) may reflect its translational activation (Rosenthal and Ruderman, 1987). Thus, TRA-2B may function in both XX and XO embryos to keep the FEM proteins at bay until the X/A ratio has been critically assessed.

Control of tra-2 and the Evolution of Hermaphroditism

The regulation that permits sperm and oocytes to be produced in the same XX animal is likely to have been a key regulatory step in the evolution of hermaphrodites from females. In two closely related *Caenorhabditis* species, *C. briggsae* and *C. remanei*, the first is hermaphroditic, but the second reproduces as a typical male/female strain (Fitch *et al.*, 1995). Little is known about *C. remanei* sex-determining genes. Of particular note for this paper, however, *tra-2* has been cloned from *C. briggsae* (Kuwabara, 1996b).

Comparison of the *C. briggsae* and *C. elegans tra-2* sequences is relevant for three aspects of *tra-2* germ-line regulation. First, *Cb-tra-2* produces only a single transcript, which encodes a TRA-2A and not a TRA-2B protein. Therefore, the 1.8-kb transcript and TRA-2B may not be essential for either oogenesis or early embryonic sex determination, or the 1.8-kb mRNA may have evolved to play a specific role in *C. elegans* sex determination (see discussion below). Second, the Cb-TRA-2A sequence carries a well-conserved MX region and therefore is likely to be subject to the same posttranslational control as Ce-TRA-2A/B. Third, the *Cb-tra-2* sequence does not possess an obvious pair of DREs, although it may contain a single functionally similar regulatory element (Jan *et al.*, 1997). We suggest that the translational control mediated through the *tra-2* DREs may have evolved more recently to help maintain *tra-2* protein at levels low enough to permit posttranslational regulation.

It has been suggested that there may be an optimal hermaphrodite brood size, which may influence species survival in the wild (Hodgkin and Barnes, 1991). Thus, mutations in sex-determining genes that lead to an increase in brood size may do so at a cost, because oogenesis is delayed and the egg-to-egg generation time is increased. In *C. elegans*, we and others have shown that a number of genes, including *tra-2*, play primary roles in regulating germ-line sexual fate and hence brood size (Fig. 1B). As discussed in this paper, *tra-2* germ-line activity can be regulated at many levels. Therefore, these multiple controls may have evolved, in part, to provide opportunities to fine-tune brood sizes. These controls may be evolving independently in different hermaphroditic species. In *C. briggsae*, the mean brood size is 234 ± 25 (Fodor *et al.*, 1983), which is considerably lower than the mean for *C. elegans*, which is 327 ± 28 (Hodgkin and Barnes, 1991). Because the 1.8-kb *tra-2* mRNA is only detected in *C. elegans*, it is possible that expression of this transcript is a recent evolutionary event that has occurred to prevent further increases in the mean brood size of *C. elegans*.

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