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

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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) 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). Molecurally, 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
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 fbfl (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). 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(mg) mutations inappropriately feminize the hermaphrodite germ line: hermaphrodite spermatogenesis is eliminated and XX animals are transformed into males; by contrast, XO animals are unaffected. In addition to this dominant germ-line effect, tra-2(mg) 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(mg) because of their mixed character (both gain-of-function and loss-of-function; Doniach, 1986; Schedl and Kimble, 1988). Distinct from the tra-2(mg) 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 Iaf-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(mg) 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(mg) 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(hc17ts) and fem-3(q20gf) 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, hc17ts), mor-2(e1125), unc-24(e138), fem-3(q20gf), 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 32p-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.

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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 59 and 39 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 59 to 39 polarity.
oligonucleotides PK36 and PK81 (see below for sequence). In Fig. 6, Northern blots were hybridized to a 16 h at 66°C in 50% formamide, 5× Denhardt’s, 5× SSC, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA with 10^6 cpm/ml 32P-labeled RNA probe (Melton et al., 1984) synthesized from tra-2 cDNA clone, pK311 (Fig. 3). Blots were washed in 1× SSC, 0.1% SDS at 65°C. Redhybridization 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 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 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 EcoRV 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 Darbyshire 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 N2 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 female 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(e1021, e1939, e1403, e1940, or e1941mx), digested with EcoRI, and analyzed by Southern blot analysis. No restriction fragment length polymorphisms were found associated with the tra-2 locus. A 4.5-kb genomic BamHI–HindIII DNA fragment containing the tra-2 3′ end was subsequently cloned into λ phage vector Charon 17, and then subcloned into pBluescript (pBl), from all of the tra-2(mx) mutants, except tra-2(e1403mx), which was first mapped by hydroxylamine DNA mismatch detection (Cotton et al., 1988).

Oligonucleotides

Oligonucleotides 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, GGTATTAATCACAAGTGG; SL2, GGTATTAACCGATCTCA; PK21, GTTAGCTGAGTGACGAT, exon 23 (3′ UTR); and PK24, AGAATATGAAATGGAAATTG, 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).
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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**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, EcoRI; H, HindIII; Bgl, BglII; C, Clal; B, BamHI; S, SalI.

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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.
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(q20gf) 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(q20gf) 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(q20gf) 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/ and 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

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Brood sizeb</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>(m–, z+)c,d</td>
<td>tra-2(e1095)/+; fem-1 mor-2/++</td>
<td>465 ± 44</td>
</tr>
<tr>
<td>(m–, z+)c,d</td>
<td>tra-2(sc146)/+; fem-1 mor-2/++</td>
<td>386 ± 22</td>
</tr>
<tr>
<td>(m+, z+)f,e</td>
<td>tra-2(e1095)/+; fem-1 mor-2/++</td>
<td>353 ± 21</td>
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<td>tra-2(sc146)/+; fem-1 mor-2/++</td>
<td>355 ± 23</td>
</tr>
<tr>
<td>(m+, z+)f,e</td>
<td>tra-2(sc146)/+; fem-1 mor-2/++</td>
<td>359 ± 15</td>
</tr>
</tbody>
</table>

Note. n, total number of broods counted.

a All animals were XX hermaphrodites of the specified genotype. 
b XX wild-type N2 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

<table>
<thead>
<tr>
<th>Allele</th>
<th>Base changea</th>
<th>Amino acid changeb</th>
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<tbody>
<tr>
<td>e2021</td>
<td>G 4211 → A</td>
<td>C 1392 → Y</td>
</tr>
<tr>
<td>e1939</td>
<td>G 4213 → A</td>
<td>E 1393 → K</td>
</tr>
<tr>
<td>q179, e2019</td>
<td>G 4235 → A</td>
<td>R 1400 → Q</td>
</tr>
<tr>
<td>e1403</td>
<td>C 4268 → T</td>
<td>P 1411 → L</td>
</tr>
<tr>
<td>e1940, e1941</td>
<td>G 4274 → A</td>
<td>R 1413 → Q</td>
</tr>
</tbody>
</table>

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 also might 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(mx)/ +/-; 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(q179mx) homozygotes were compared to wild type on Northern blots (Fig. 6A). Because tra-2(q179mx) 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(q179mx) 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,
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 with TRA-2A outside of the MX region (Fig. 4). We have named the genes associated with F54F7.4 and F17A2.1, that also display a striking conservation of the MX region. The predicted 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 of the MX region (Fig. 4). We have named the genes associated with F54F7.4 and F17A2.1 as MX region (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 germ-line-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, also share sequence similarity with TRA-2A and TRA-2B outside of the MX region (Fig. 4). We therefore speculate that 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, 1996).

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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