***tra-2* Encodes a Membrane Protein and May Mediate Cell Communication in the *Caenorhabditis elegans* Sex Determination Pathway**

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The *Caenorhabditis elegans* sex-determining gene, *tra-2*, promotes female development in XX animals. In this paper we report the cDNA sequence corresponding to a 4.7 kb *tra-2* mRNA and show that it is composed of 23 exons, is trans-spliced to SL2, and contains a perfect direct repeat in the 3' untranslated region. This mRNA is predicted to encode a 1475 amino acid protein, named pTra2A, that has a secretory signal and several potential membrane-spanning domains. The molecular analysis of *tra-2* loss-of-function mutations supports our open reading frame identification and suggests that the carboxy-terminal domain is important for *tra-2* activity. We propose that in XX animals the carboxy-terminal domain of pTra2A negatively regulates the downstream male promoting *fem* genes. In XO animals, *tra-2* is negatively regulated by *her-1*, which acts cell nonautonomously. Because hydropathy predictions suggest that pTra2A is an integral membrane protein, pTra2A might act as a receptor for the *her-1* protein. We propose that in XO animals, the *her-1* protein promotes male development by binding and inactivating pTra2A. The role of cell communication in *C. elegans* sex determination might be to ensure unified sexual development throughout the animal. If so, then regulation of sexual fate by *her-1* and *tra-2* might provide a general model for the coordination of groups of cells to follow a single cell fate.

INTRODUCTION

Sex determination involves the specification of cells as one of two sexual fates. In the nematode *Caenorhabditis elegans*, sexual fate is controlled by a cascade of regulatory genes (Figure 1) (for reviews, see Hodgkin, 1990; Villeneuve and Meyer, 1990b). The primary determinant of sex is the ratio of X chromosomes to sets of autosomes: the X:A ratio (Madl and Herman, 1979). Diploid animals with two X chromosomes (XX) are hermaphrodite, whereas those with a single X chromosome (XO) are male. Three genes in the sex-determining cascade regulate both sexual fate and dosage compensation: *xol-1* (Miller *et al.*, 1988), *sdc-1* (Villeneuve and Meyer, 1987), and *sdc-2* (Nusbaum and Meyer, 1989). Seven genes specify sexual fate only: *her-1* (Hodgkin, 1980; Trent *et al.*, 1988), *tra-1* (Hodgkin and Brenner, 1977;

Hodgkin, 1987; Schedl *et al.*, 1989), *tra-2* (Klass *et al.*, 1976; Hodgkin and Brenner, 1977), *tra-3* (Hodgkin and Brenner, 1977), *fem-1* (Nelson *et al.*, 1978; Doniach and Hodgkin, 1984), *fem-2* (Kimble *et al.*, 1984; Hodgkin, 1986), and *fem-3* (Hodgkin, 1986; Barton *et al.*, 1987). These regulatory genes appear to function as a series of alternating on/off switches, with the activity of each gene controlled by the state of one or more upstream genes (Figure 1).

Many genes in the *C. elegans* sex-determination pathway have now been identified at the molecular level. An early-acting gene, *sdc-1*, encodes a putative zinc-finger protein (Nonet and Meyer, 1991). Because the next gene in the pathway, *her-1*, appears to be transcriptionally regulated (Trent *et al.*, 1991) and because zinc-finger motifs are found in numerous proteins that regulate transcription (Berg, 1990), *sdc-1* is an excellent candidate for regulating *her-1* at the transcriptional level. *tra-1*, the final gene in the somatic pathway, encodes two putative zinc-finger proteins (Zarkower and Hodg-

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kin, personal communication). Based on similar logic described for *sd*-1, *tra*-1 is an excellent candidate for regulating downstream genes at the transcriptional level.

The *tra*-2 gene lies in the middle of the sex-determining hierarchy and functions in XX animals to promote female development (Hodgkin and Brenner, 1977; Hodgkin, 1980, 1986) (Figure 1). Loss-of-function *tra*-2 mutations transform XX animals into nonmating pseudomales (Hodgkin and Brenner, 1977) but do not alter XO male development. In contrast, dominant gain-of-function *tra*-2 mutations transform hermaphrodites into females (spermless hermaphrodites) (Doniach, 1986; Schedl and Kimble, 1988).

The cloning of *tra*-2 and characterization of its transcripts by Northern analysis were previously reported (Okkema and Kimble, 1991). Both hermaphrodites and males contain *tra*-2 mRNAs, but there are two sex-specific differences. First, although both sexes express a 4.7-kb *tra*-2 mRNA, this mRNA is ≥ 15 -fold more abundant in hermaphrodites than in males. Second, adult hermaphrodites have a 1.8-kb *tra*-2 mRNA, whereas adult males have a 1.9-kb *tra*-2 mRNA. These sex-specific differences depend on phenotypic sex rather than the X:A ratio. For example, the *tra*-2 mRNAs in XX *tra*-1 males are typical of XO males, even though *tra*-1 is downstream of *tra*-2. Based on this finding, the existence of positive feedback regulation in the sex-determination pathway was proposed (Okkema and Kimble, 1991).

In this paper we focus on a molecular characterization of the 4.7-kb *tra*-2 mRNA, because it appears to mediate the primary function of *tra*-2 in sex determination. We find that the 4.7-kb *tra*-2 mRNA encodes a large protein with several putative membrane-spanning domains; we call this protein pTra2A. We propose that the carboxy-terminal domain of pTra2A, which we predict to be intracellular, is essential in XX animals to direct female development. Work by others has shown that genes upstream of *tra*-2 act nonautonomously (Villeneuve and Meyer, 1990a; Schedl *et al.*, 1991; Hunter and Wood, 1992; see DISCUSSION). We postulate that in XO animals, pTra2A acts as a receptor for the protein product

of one such gene, *her*-1. Because *her*-1 is a negative regulator of *tra*-2, we propose that pTra2A is inactivated by binding the *her*-1 protein, thus allowing XO animals to adopt the male fate.

MATERIALS AND METHODS

General Manipulation of Nucleic Acids

General methods for manipulating nucleic acids are described by Maniatis *et al.* (1989). DNA fragments generated by exonuclease III digestion, restriction enzyme digestion, or by polymerase chain reaction (PCR) were subcloned into either pBI76 (International Biotechnologies, New Haven, CT), pGEM-7Z(+) (Promega, Madison, WI), or pBSKSII(+) (Stratagene, La Jolla, CA). Plasmid DNA was isolated by alkaline lysis and purified by cesium chloride density gradient centrifugation or by Qiagen (Chatsworth, CA) column chromatography. DNA fragments were separated by electrophoresis on 1.0% agarose gels, and fragments for cloning were purified using GeneClean (Bio 101, La Jolla, CA).

Nematode DNA was prepared as described by Emmons and Yesner (1984). RNA was isolated from worms by the guanidinium thiocyanate protocol of Chirgwin *et al.* (1979). Poly(A⁺) RNA was selected by a single round of selection on oligo(dT) cellulose (Pharmacia, Piscataway, NJ).

cDNA Isolation and Sequencing of cDNA and Genomic Clones

A λ gt10 cDNA library, prepared from mixed-stage N2 hermaphrodite RNA and kindly provided by S. Kim (Stanford University, Palo Alto, CA) and H.R. Horvitz (MIT, Cambridge, MA), was screened using RNA probes synthesized from plasmids pJK88 and pJK67 (Okkema and Kimble, 1991). Mixed-stage N2 library 2a prepared by J. Ahringer (University of Wisconsin, Madison, WI), and two N2 cDNA libraries containing size-fractionated inserts (1–2 kb and 2–3 kb), prepared by C. Martin (Columbia University, N.Y., N.Y.), were screened with RNA probes synthesized from pJK67. pJK88 hybridizes only to the 4.7-kb *tra*-2 mRNA, whereas pJK67 hybridizes to all identified *tra*-2 mRNAs (Okkema and Kimble, 1991). A *tra*-2 cDNA clone, λ JK57, hybridized to both probes and was selected from the S. Kim library for further characterization. The remaining *tra*-2 cDNA clones were shown to share internal restriction fragments with λ JK57 after digestion with *Rsa* I or *Bst*NI and were thus believed to be incomplete cDNA products. The insert from λ JK57 was subcloned into plasmid pBI76 and sequenced at least once on each strand using Sequenase (United States Biochemicals, Cleveland, OH), as directed for double-stranded templates by the manufacturer. Nested deletions were generated using Exonuclease III (Henikoff, 1987), and sequence specific primers were synthesized to complete the sequencing of first and second strands.

Figure 1. The regulatory pathway of sex determination in *C. elegans* (modified from Hodgkin, 1990; Villeneuve and Meyer, 1990b). The X:A ratio is interpreted by the genes *xol*-1, *sd*-1, and *sd*-2, which regulate both sex determination and dosage compensation. Control of dosage compensation is beyond the scope of this paper (see Villeneuve and Meyer, 1990). In XX animals the *sd* genes inactivate *her*-1; therefore, *tra*-2 and *tra*-3 repress the *fem* genes so that *tra*-1 can promote female development. Although both *tra*-2 and *tra*-3 are equivalently positioned in the sex-determination pathway, *tra*-3 is postulated to be an almost dispensable positive cofactor of *tra*-2 (Hodgkin, 1980). In XO animals, *her*-1 negatively regulates *tra*-2 and *tra*-3; therefore the *fem* genes negatively regulate *tra*-1 and male development ensues. The state of *tra*-1 determines the sexual phenotype only in somatic tissue. A discussion of germline sex determination is beyond the scope of this paper (see Barton and Kimble, 1990, for discussion of germline pathway).

Genomic DNA was also sequenced using methods described above, but not all regions were sequenced on both strands.

Worm Culture

Worms were grown on petri dishes or in liquid as described (Sulston and Hodgkin, 1988). To synchronize animals, eggs were isolated by hypochlorite treatment of gravid hermaphrodites.

Descriptions of *C. elegans* genes and alleles can be found in Hodgkin *et al.* (1988). *tra-2(e1209 and e1095)* are described in Hodgkin *et al.* (1989). *tra-2(q150 and q270)* are described in Okkema and Kimble (1991).

Primer Extension and S1 Mapping

The 5' end of the 4.7-kb *tra-2* RNA was mapped by primer extension and S1 protection (Calzone *et al.*, 1987) using adult N2 poly(A+) RNA. Primer extension of the 4.7-kb *tra-2* transcript was performed using avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia) to extend 5' end-labeled oligonucleotide PK-7. S1 analysis was performed using a continuously labeled hybridization probe that was primed with PK-7 using pJK88 as a template and synthesized using Klenow DNA polymerase (identical results were obtained using an end-labeled probe). The S1 hybridization probe was digested with *EcoRI* and purified after electrophoresis on a DNA sequencing gel. Purified probe of 3×10^5 cpm was used per protection reaction. Primer extension and S1 protected products were analyzed on a DNA sequencing gel. tRNA was used as a control. The same endpoint was found when PK-7 was replaced with the oligonucleotide PK-1.

cDNA clones corresponding to the 5' end of the 4.7-kb *tra-2* RNA were synthesized using the rapid amplification of cDNA ends (RACE) protocol as described (Frohman *et al.*, 1988). cDNA that was used as a template for PCR was synthesized from 2 μ g adult N2 poly(A+) RNA using AMV reverse transcriptase (Pharmacia) and PK-1 as a primer. Excess primer was removed by centrifugation over Centricon-100 columns (Amicon, Danvers, MA). PCR reactions were set up using the conditions described (Frohman *et al.*, 1988) and included the following nested primers: RACE-1, RACE-2, and PK-7. Ends of PCR fragments generated by the RACE procedure were first made blunt using DNA polymerase I Klenow fragment and then phosphorylated with T4 kinase. These fragments were electrophoresed through acrylamide and purified and then cloned by blunt-end ligation into the *Sma* I site of pIBI76 for sequence analysis.

Computer Analysis

DNA sequences were compiled and analyzed using the WISCGCG Sequence Analysis Software Package (Devereux *et al.*, 1984). The programs Wordsearch and FASTA from the WISCGCG and BLASTP (Altschul *et al.*, 1990) were used to search the PIR-Protein and SwissProt databases for other protein sequences that might show similarities to pTra2A. The program MOTIFS was used to search the PROSITE database for amino acid motifs. Intelligenetics PC-Gene (Intelligenetics, Mountain View, CA) was used for hydropathy analyses and for scoring PEST sequences.

Sequence Analysis of *tra-2* Mutations

A PCR fragment containing the site of Tc1 insertion in *tra-2(q270)* was generated using a *tra-2* specific primer, PK-34, and a Tc1 specific primer, Tc1-498. This fragment was digested with the restriction enzymes *EcoRI* and *Sal* I and was cloned into pBSKSII(+) for sequence analysis. A DNA fragment corresponding to an intact Tc1 element flanked by *tra-2(q150)* sequence was generated using *tra-2* specific primers PK-34 and PK-55. This fragment was cloned by blunt-end ligation into the *Sma* I site of pGEM-7Z(+), and the insertion site of Tc1 was determined by sequence analysis.

No restriction-fragment-length polymorphisms were associated with *tra-2* alleles *e1095(lf)* and *e1209(lf)*, suggesting that they were caused by point mutations (data not shown). The sites of these mutations were mapped using the hydroxylamine DNA mismatch detection system (Cotton *et al.*, 1988). Both probe and mutant DNA fragments were generated by PCR using PK-36 and PK-21 as primers and were digested with either *Bgl* II or *Sal* I. PCR-generated DNA fragments containing point mutations, revealed by the detection of single-base mismatches, were cloned and sequenced from two independent PCR reactions to minimize the risk of PCR-induced artifacts.

Sequence and Location of Oligonucleotide Primers

Primers used are listed below. The nucleotide location of *tra-2* primers are enclosed by brackets and based on the numbering of the 4.7-kb *tra-2* cDNA sequence (Figure 2).

PK-7	5' TCACCACCGAAACGAGAAGT	3' (57-76)
PK-1	5' TTCCGCTAAGAGCAACC	3' (98-114)
PK-34	5' CAGCTATGCAACATTCAGAC	3' (1163-1182)
PK-55	5' ATATCAGAATTGATGTGCAAC	3' (1805-1825)
PK-36	5' TACCGCTGTCCGGGTCTA	3' (3354-3371)
PK-21	5' GTTGAGGTCGAGTGGACGAT	3' (4613-4632)
RACE-1	5' GACTCGAGTCGACATCGA(T) ₁₇	3'
RACE-2	5' GACTCGAGTCGACATCG	3'
Tc1-498	5' TCCGGAGCTACTGTTGGG	3'

RESULTS

Isolation of a cDNA Clone Corresponding to the 4.7-kb *tra-2* Transcript

A 4.7-kb *tra-2* cDNA clone, designated λ JK57, was isolated from a *C. elegans* cDNA library (see MATERIALS AND METHODS for details). This cDNA clone was detected not only with a hybridization probe that is specific for a 4.7-kb *tra-2* mRNA on Northern blots but also with a probe that detects all three *tra-2* mRNAs (Okkema and Kimble, 1991). Hence, λ JK57 corresponds to the 4.7-kb *tra-2* mRNA. Characterization of the 1.8- and 1.9-kb mRNAs will be presented elsewhere (Kuwabara, Okkema, and Kimble, unpublished data). We subcloned and sequenced the insert of λ JK57 (see MATERIALS AND METHODS). This sequence is presented in Figure 2 as part of a composite corresponding to a full-length cDNA of the 4.7-kb *tra-2* mRNA.

The 4.7-kb *tra-2* mRNA is Trans-Spliced

We mapped the 5' end of the large *tra-2* transcript by primer extension and S1 protection (Figure 3A). The primer extended product is 22-nt longer than the comparable S1 protected fragment (Figure 3A, compare lanes 1 and 2). This 22-nt difference suggested that the 4.7-kb *tra-2* transcript is trans-spliced (Krause and Hirsh, 1987; Huang and Hirsh, 1989). Because we detected no other bands of similar intensity that would correspond to additional 5' ends, most of the 4.7-kb *tra-2* mRNA must be trans-spliced.

Because the insert of λ JK57 does not represent a full-length *tra-2* cDNA, we used the RACE technique (Frohman *et al.*, 1988) to construct additional cDNA clones

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that correspond specifically to the 5' end. We sequenced seven of these clones and found that the 4.7-kb *tra-2* mRNA is trans-spliced to either SL2 (2 clones) (Huang and Hirsh, 1989) or to novel variants of SL2 (5 clones) (Figure 3B). SL2 is added to only a limited number of *C. elegans* mRNAs and hence may have regulatory significance. The types of SL2 variants that we identified are not artifacts commonly associated with the cloning of RACE products (Innis and Gelfand, 1990), and this suggests that the family of SL2 encoding genes may be larger than initially suspected.

Based on the identification of an open reading frame (ORF) (discussed below), the 5' untranslated region is predicted to be 36 nt, including the 22 nt of SL2. We also calculate that the full-length large *tra-2* mRNA is 4.7 kb without its poly(A) tail and, hence, revise the previous estimate of 5 kb based on Northern blots of poly(A⁺) mRNA (Okkema and Kimble, 1991).

Identification of a Large ORF in the 4.7-kb *tra-2* cDNA

We used computer programs developed by the University of Wisconsin Genetics Computer Group (Devreux *et al.*, 1984) to analyze the composite full-length 4.7-kb *tra-2* cDNA sequence (Figure 2). The first methionine codon, found at nucleotide 29, identifies an ORF consisting of three codons. The second methionine codon, found at nucleotide 37, marks the beginning of the longest *tra-2* ORF of 1475 codons (Figure 2), which we predict to be the *tra-2* coding region. The context of the predicted initiation codon ATG (ACAATGA) shows reasonable similarity to that of other *C. elegans* translational initiation sequences (Perry, Hertz, and Wood, personal communication). All other reading frames have multiple stop codons. Analysis of this ORF is presented in a later section.

The 3' End, Exon/Intron Structure, and Possible Promoter Region of the 4.7-kb *tra-2* mRNA

The cDNA clone, λ JK57, appears to contain the 3' end of the 4.7-kb *tra-2* mRNA. Following the large ORF is an A/T-rich region of 218 nt with multiple translational stops in all reading frames (Figure 2). This predicted 3' untranslated region (3' UTR) ends in a short poly(A) stretch, 13 nt downstream from the canonical polyad-

enylation signal AATAAA. Within the 3' UTR is a striking direct repeat composed of two perfect copies of a 28-base sequence separated by 4 bases. This repeat is located in a region associated with alterations in *tra-2(gf)* mutations (Okkema and Kimble, 1991) and therefore might be involved in regulating *tra-2* expression. Interestingly, the ATTTA motif associated with mRNA instability of *c-fos* mRNA (Shaw and Kamen, 1986) is contained within the repeated sequence in the *tra-2* 3' UTR.

Genomic DNA corresponding to the *tra-2* transcription unit (Okkema and Kimble, 1991) was sequenced and compared to the 4.7-kb *tra-2* cDNA sequence. This comparison reveals that the 4.7-kb *tra-2* mRNA is composed of 23 exons (Figure 4). We also sequenced 500 bp of genomic DNA upstream of the predicted trans-splice acceptor site (Figure 5) and 1.5 kb downstream of the polyadenylation sequence, AATAAA. Two sequences in the 5' flanking region (Figure 5), TGATAG and TGATAA, fit the consensus GATA motif, (A/T)GATA(A/G) (Kemper *et al.*, 1987), and might function as promoter elements for *tra-2*. One of these GATA sequences, CTGATAA, is identical to the *C. elegans* vitellogenin putative promoter element VPE2 (Spieth *et al.*, 1985). No additional polyadenylation motifs were detected in the 3' flanking sequence.

The 4.7-kb *tra-2* mRNA Encodes a Novel Protein

The predicted amino acid sequence encoded by the 4.7-kb *tra-2* mRNA is shown in Figure 2. This protein has 1475 amino acids and a predicted molecular weight of 170 245 Da. Computer searches of the SwissProt and PIR-Protein databases have revealed no significant sequence similarity of the *tra-2* protein with any other known protein. We call this putative *tra-2* protein pTra2A. During the course of this work, we noted that pTra2A was similar to the *Drosophila patched* protein in structure (Hooper and Scott, 1989; Nakano *et al.*, 1989) and in function (Ingham *et al.*, 1991). Therefore, we compared the two sequences using the BESTFIT program and found a 43% similarity and 19% identity that is distributed over the length of both proteins.

The carboxy-terminal domain (amino acids 1100–1475) is proline rich (11%) and hydrophilic (33% charged residues) with short highly acidic stretches and has an isoelectric point of 4.7. This domain also has

Figure 2. Sequence of a composite *tra-2* 4.7-kb cDNA. A composite sequence, compiled from cDNA clones, that corresponds to a *tra-2* 4.7-kb mRNA. The first base of the trans-spliced leader SL2 is assigned number one. Nucleotides 1–37 were derived from cDNA clone pJK216, and nucleotides 38–4693 from cDNA clone, λ JK57. The predicted amino acid sequence of the 4.7-kb *tra-2* mRNA is indicated by the single letter amino acid code positioned below the first nucleotide of each codon. The trans-spliced leader sequence SL2 is underlined. The polyadenylation signal AATAAA is marked with an overhead line and the 28-bp direct repeat is indicated by overhead arrows. The motif, ATTTA (Shaw and Kamen, 1986), contained within the direct repeat, is underlined. In the predicted amino acid sequence, hydrophobic helices are boxed (Eisenberg *et al.*, 1984) and PEST sequences are underlined (Rogers *et al.*, 1986). Potential sites of N-linked glycosylation are circled. Potential sites of cAMP-dependent phosphorylation are indicated by boxed serine or threonine residues. An asterisk is placed below each leucine repeated as in the leucine zipper motif (Landshulz *et al.*, 1988). ▼, insertion site of the transposable element Tc1 in each *tra-2(lf)* mutation; *, nonsense mutations are indicated above the nucleotide that is mutated; ^, putative signal peptide cleavage site. For additional details, see text.

Figure 3. The 5' end of the 4.7-kb *tra-2* transcript. (A) The primer extension product (arrow, right) is 22 nt longer than the comparable S1 protected fragment (arrow, left) (see MATERIALS AND METHODS for details). Lane 1, the protected fragment after digestion with S1 nuclease. Lane 2, the primer extension product. A sequencing ladder, indicated by G, A, T, and C, is included as a size reference. (B) The 4.7-kb *tra-2* mRNA is trans-spliced to SL2 or variants of SL2. SL2 and variant SL2 sequences were found by sequencing cDNA clones corresponding to the 5' end of the *tra-2* 4.7-kb mRNA. The original 22 nucleotide sequence of SL2, shown on the first line, was detected in two of seven clones. SL2 variants, listed below, were each detected at a frequency of one of seven clones. Nucleotides conserved among SL2 and variants are boxed and deletions underlined.

four strong PEST sequences that might affect protein stability (Rogers *et al.*, 1986). Potential sites for N-linked glycosylation and phosphorylation of serine/threonine residues (Figure 2) suggest that the activity of pTra2A might be regulated posttranslationally.

pTra2A is Probably a Membrane Protein

Hydropathy analyses suggest that pTra2A is an integral membrane protein with multiple membrane-spanning domains. Figure 6A shows a hydropathy

plot of pTra2A, based on Kyte and Doolittle (1982). Specific hydropathic helices are lettered and are based on the predictions of Eisenberg and colleagues (1984). The most amino-terminal hydropathic helix is contained in a potential signal sequence (von Heijne, 1986). Three separate algorithms (Eisenberg *et al.*, 1984; Klein *et al.*, 1985; Rao and Argos, 1986) predict pTra2A to be a membrane protein. Figure 6B presents one plausible prediction in which pTra2A has nine membrane-spanning helices, two major extracellular regions that are glycosylated, and two major intra-

Figure 4. Exon/intron structure of the 4.7-kb *tra-2* transcript. Above, a simplified restriction map of genomic DNA covering the *tra-2* transcription unit. Below, the structure of the 4.7-kb *tra-2* RNA: exons are boxes, introns are lines. Stippled regions designate the ORF and open regions correspond to the 5' and 3' UTRs. The 5' end is trans-spliced to SL2.

cellular regions, including the carboxy-terminus. Partially contained within the sixth predicted hydrophobic helix of pTra2A is a series of five leucine residues, repeated at intervals of seven amino acids (Figure 2). This leucine repeat is reminiscent of the leucine zipper (Landschulz *et al.*, 1988).

Molecular Localization of *tra-2* Loss-of-Function Mutations

We analyzed the molecular basis of four *tra-2* loss-of-function (*lf*) mutations (see MATERIALS AND METHODS). Two *tra-2(lf)* alleles, *q150* and *q270*, carry Tc1 transposable elements that have inserted into the pTra2A coding sequence (Figure 2). These insertions disrupt sequences that encode the 4.7-kb *tra-2* mRNA but are located outside the region that encodes the smaller *tra-2* mRNAs (Kuwabara, Okkema, and Kimble, unpublished data) and behave like null alleles of *tra-2*. Therefore, we suggest that the 4.7-kb mRNA is the principle component of *tra-2* activity.

Two other *tra-2(lf)* mutations, *e1095* and *e1209*, are not associated with any change in the *tra-2* pattern of restriction fragments. Using the hydroxylamine DNA mismatch detection method (Cotton *et al.*, 1988), we mapped each mutation to a region encoding the hydrophilic carboxy-terminal domain of pTra2A. We sequenced these mutations and found both to be nonsense mutations: *tra-2(e1095)* is a C to T transition changing Gln¹²⁹⁰ to an ochre stop codon and *tra-2(e1209)* is a G to A transition changing Trp¹³⁹⁷ to an opal stop codon (Figure 2). These nonsense mutations are predicted to affect the proteins encoded by the *tra-2* 4.7- and 1.8-kb mRNAs. The positions and nature of these two nonsense mutations support our assignment of the *tra-2* ORF.

DISCUSSION

tra-2 Encodes a Membrane Protein that Promotes Female Development

The *tra-2* gene functions in the *C. elegans* sex determination hierarchy to promote female development in XX

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-512  gaattcttct gttatcaca ttactgattt cttgaattaa aacttgaatt
-462  tatacttttc cattccagtg cctccataag caactctatt atttgtgatg
-412  gagtagaaat cggagagaat gctgacgtca ccaattgcat tgttgctaag
-362  gatcagaagg tacctgctaa aggttagttg ttttcattag tgttggtttt
-312  tttttaattt ctcgtgtttc tttataaatc aacgattttc aggcaaagtt
-262  caaaacgagg tagtagaaga tggagaagac gaagaatgga ccgatgatta
-212  acaatcttgg aatttttagga ataattggag cttttttgtg ttttctcttt
-162  tttttacgcg gttgttttat ttttgttttc tgataaattt attgaagatt
-112  aagttttttt gaaattttcg ttttcctcta tctttcactc caattgatagg
-62   ttctctaacc ctacaaatta ttttgagtaa tatttttatt tacgatatta
-12   ttttactaac ag↓

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Figure 5. Sequence of the 5' flanking region of the 4.7-kb *tra-2* mRNA. Genomic DNA sequence upstream of the 4.7-kb *tra-2* mRNA trans-splice acceptor site (vertical arrow). Numbering is based on distance away from the trans-splice acceptor site. No typical TATA sequences were found. Sequences similar to GATA motif, (A/T)-GATA(A/G), are boxed. The first GATA motif, beginning at nucleotide -133, also identifies the VPE2 consensus (see text for details).

A.

Figure 6. pTra2A is predicted to be a membrane protein. (A) Hydropathy plot of the conceptual pTra2A protein based on the algorithm of Kyte and Doolittle (1982) and using a window of nine amino acids. Twelve hydrophobic peaks, lettered A–L, are predicted to be hydrophobic helices (Eisenberg *et al.*, 1984). Helix A is part of the predicted signal sequence at the amino-terminus. See Figure 2 for amino acid sequence of each helix. (B) Schematic representation of pTra2A according to the algorithm of Klein *et al.* (1985). Not all hydrophobic helices predicted by Eisenberg *et al.* (1984) (see above) are membrane spanning according to Klein *et al.* (1985). Membrane-spanning domains are numbered 1–9; letters below these domains correspond to the hydrophobic helices in A. The original Klein *et al.* (1985) prediction merges helices H and I and does not account for the high density of charged amino acids in helix G. Therefore, we postulate that helix G (starting at amino acid 913) may be merged with helix H to form a helix containing fewer charged amino acids. Potential sites of N-linked glycosylation are marked with circles.

hermaphrodites (Figure 1) (Hodgkin, 1990; Villeneuve and Meyer, 1990b). A hermaphrodite is essentially a female that makes sperm and oocytes. The *tra-2* locus expresses three mRNAs, with sizes of 4.7 (previously 5.0 kb), 1.8, and 1.9 kb (Okkema and Kimble, 1991). All three mRNAs contain sequences encoded by the 3' half of the *tra-2* locus; however, only the 4.7-kb mRNA also contains sequences from the 5' half of the *tra-2* locus (Okkema and Kimble, 1991; Kuwabara, Okkema, and Kimble, unpublished data).

Two lines of evidence argue that the 4.7-kb mRNA contributes the essential function of *tra-2*. First, two *tra-2* mutations that appear to cause a complete loss of function map to the 5' half of the *tra-2* locus. Thus, these mutations affect the 4.7-kb *tra-2* mRNA but are located outside the region that encodes the smaller *tra-2* mRNAs (Okkema and Kimble, 1991; Kuwabara, Okkema, and Kimble, unpublished data). Second, only the 4.7-kb *tra-2* mRNA has the appropriate stage and tissue specificity: *tra-2* activity promotes female differentiation throughout XX hermaphrodite development in both somatic and germline tissues (Hodgkin and Brenner, 1977; Klass *et al.*, 1976) and only the 4.7-kb *tra-2* mRNA is similarly expressed (Okkema and Kimble, 1991). By contrast, the 1.8-kb *tra-2* mRNA is observed

only in late larval and adult stages and is restricted to the hermaphrodite germ line; the 1.9-kb *tra-2* mRNA is restricted to larval development in hermaphrodites and is male-specific in adults (Okkema and Kimble, 1991). Based on these arguments, we suggest that the 4.7-kb *tra-2* mRNA mediates the primary *tra-2* function of promoting female development.

In this paper we present the nucleotide sequence of a 4.7-kb *tra-2* mRNA. The conceptual product of this *tra-2* mRNA is likely to be a membrane protein. We call this protein pTra2A to distinguish it from other proteins that might be encoded by other *tra-2* mRNAs. Although pTra2A shares no significant sequence similarity with any known protein, it does contain a consensus signal sequence, clustered glycosylation sites, and several hydrophobic helices that are predicted to be membrane spanning. Therefore, pTra2A displays all the hallmarks of an integral membrane protein with multiple membrane-spanning domains. The computer-generated assignment of extra- and intracellular domains places potential sites of N-linked glycosylation extracellularly. The carboxy-terminus, which is predicted to be intracellular, contains PEST sequences and potential sites for phosphorylation of serine and threonine residues. This carboxy-terminal domain might be the active por-

tion of pTra2A, which is involved in regulating the *fem* genes (see below).

pTra2A May Mediate Cell Interactions in the Sex Determination Pathway

Many aspects of *C. elegans* development rely on cell-cell interactions (reviewed by Lambie and Kimble, 1991). Recently, in three independent experiments it has been found that cell-cell interactions might also influence the decision of sexual fate. First, mosaic animals that have lost *sdca-1* from an early embryonic founder cell are hermaphrodite and do not show the *sdca-1* mutant phenotype (Villeneuve and Meyer, 1990a). By contrast, XX animals that lack *sdca-1* activity in all cells are transformed from hermaphrodite to male (Villeneuve and Meyer, 1987). Therefore, in the mosaic hermaphrodites XX cells lacking *sdca-1* may be rescued by wild-type *sdca-1* in their neighbors. Second, the sexual fate of cells with an ambiguous X:A ratio is influenced by their position along the anterior/posterior axis rather than lineage (Schedin *et al.*, 1991). Third, in *her-1* mosaics some *her-1*(+) XO cells are female and some *her-1*(-) XO cells are male (Hunter and Wood, 1992). Thus, sexual fate of a cell does not always correspond to the state of the *her-1* gene, which is normally essential for male development. In contrast to these results, the *tra-1* gene, which functions at the end of the sex-determination pathway, acts cell-autonomously (Hunter and Wood, 1990). Given that the early-acting genes in the sex determination pathway appear to act nonautonomously and a late gene acts autonomously, genes in the middle of the pathway are likely to mediate signal transduction. The predicted *fem-1* (Spence *et al.*, 1990) and *fem-3* (Ahringer *et al.*, 1992) proteins are likely to be intracellular. Hence, the *tra-2* gene, which we predict encodes an integral membrane protein and which is positioned immediately downstream of *her-1* in the regulatory hierarchy (Figure 1), is the best candidate for receiving an extracellular signal.

pTra2A May Mediate Cell Communication in the C. elegans Sex Determination Pathway

The 4.7-kb *tra-2* mRNA is detected in both XX and XO animals, albeit in much lower quantity in XO animals. pTra2A might therefore be present in both sexes as well. Figure 7 suggests one possible mechanism for the inactivation of pTra2A in XO animals. The *her-1* gene, which lies immediately upstream of *tra-2* in the regulatory hierarchy (Figure 1) and which acts nonautonomously (Hunter and Wood, 1992), is predicted to encode a secreted protein of low molecular weight (Perry and Wood, personal communication). Therefore, a simple hypothesis is that in XO males, the *her-1* protein is secreted, binds to pTra2A, and interferes with *tra-2* activity (Figure 7). This proposed interaction suggests that pTra2A might function as a receptor and the *her-1* protein as a ligand. In this model, pTra2A is predicted to act cell autonomously. Although this interaction is unusual from the standpoint of ligand-binding having a repressive effect on receptor activity, a similar interaction might occur between the *Drosophila* *hedgehog* and *patched* proteins during positional signaling (Ingham *et al.*, 1991). Our comparison of the pTra2A and *patched* protein sequences indicates a marginal similarity at best (43% similar and 19% identical).

If pTra2A activity is negatively regulated in XO animals by binding *her-1*, its activity might also be positively regulated in XX animals by binding a different ligand. There are, however, no good candidates for a positively acting ligand. The *sdca* genes, which are positioned upstream of *tra-2* (Figure 1), might promote female development by activating pTra2A. However, all *sdca;her-1* double mutants are hermaphrodite, indicating that when *her-1* is absent *tra-2* activity is independent of the *sdca* genes (Villeneuve and Meyer, 1987; Nusbaum and Meyer, 1989). Furthermore, *sdca-1* is a putative zinc-finger protein (Nonet and Meyer, 1991) and hence more likely to be involved in the regulation of gene expression. Thus, pTra2A is probably either constitutively active in XX animals or activated by a

Figure 7. Model for sex determination in *C. elegans*. In XX hermaphrodites, pTra2A is depicted as a membrane protein that sequesters one or more *fem* proteins. Inhibition of *fem* activity allows *tra-1*, a putative transcription factor (Zarkower and Hodgkin, personal communication), to enter the nucleus and promote female development. In XO males the *her-1* protein is diagrammed as a ligand that binds to pTra2A and prevents it from binding the *fem* proteins. Consequently, the *fem* proteins are able to interfere with *tra-1* activity. As postulated in this model, *tra-1* protein activity is repressed by its failure to be localized to the nucleus; however, other mechanisms are possible. In this figure, gene names are used to designate protein products.

hitherto unidentified gene that is redundant or pleiotropic.

pTra2A May Function by Localizing Male-Determining Proteins to the Membrane

How might an integral membrane protein negatively regulate the male-determining *fem* genes (Hodgkin, 1986) (Figure 1)? There is no indication that pTra2A contains any catalytic domain. However, there is a clue that the carboxy-terminus is essential to *tra-2* function. Both *tra-2(e1095)* and *tra-2(e1209)* are nonsense mutations that map to the carboxy-terminus. Although *smg* suppressors appear to act by increasing the steady-state level of mutant mRNAs and, presumably, protein (Pulak and Anderson, personal communication), only the activity of *tra-2(e1209)* is partially restored by *smg* suppression (Hodgkin *et al.*, 1989). One explanation of these results is that the *tra-2(e1209)* protein is partly functional because it has a longer hydrophilic carboxy-terminus than the *tra-2(e1095)* protein. If this is true, then the additional amino acids that are present in *tra-2(e1209)* might delimit a region required for *tra-2* function (Figure 2). Alternatively, the *tra-2(e1095)* protein might be less stable than the *tra-2(e1209)* protein.

The mechanism by which pTra2A negatively regulates the *fem* genes in XX animals is open to speculation. Because each of the three *fem* genes is absolutely required for male development, only one of them needs to be inactivated to promote female development. Figure 7 presents one simple model for how pTra2A might inactivate *fem* gene products. In XX animals pTra2A might sequester one or more of the male-determining *fem* proteins to the membrane, perhaps through an interaction involving its carboxy-terminus, and thereby free the *tra-1* protein to promote female development. The *fem-1* protein contains six *cdc10/SWI6* repeats (Spence *et al.*, 1990). This motif has been implicated in mediating protein-protein interactions (Lux *et al.*, 1990; Thompson *et al.*, 1991). Therefore, the *fem-1* protein is a reasonable candidate for directly interacting with pTra2A. This, however, does not exclude the possibility of an interaction between pTra2A and the *fem-2* or *fem-3* protein. In XO animals we speculate that the same *cdc10/SWI6* repeats might repress *tra-1* protein activity (Figure 7). We raise this possibility because the transcription factor NF κ B (Bours *et al.*, 1990; Kieran *et al.*, 1990) is repressed by I κ B, a protein that also contains *cdc10/SWI6* repeats (Haskill *et al.*, 1991).

Other models exist. For example, pTra2A may have a catalytic activity that cannot be predicted from its sequence. Another potential mechanism, though implausible, is that pTra2A interacts with the *fem-3* 3' UTR and negatively regulates *fem-3* activity. It has been shown that negative regulation of *fem-3* can be mediated through a short sequence in its 3' UTR (Barton *et al.*, 1987; Ahringer and Kimble, 1991). However, because

this regulation is restricted to the germ line, it is not likely to be mediated by pTra2A. Therefore, the simplest model for *tra-2* regulation of the *fem* genes relies on protein-protein interaction.

Regulation of tra-2 is Pivotal to Sex Determination in C. elegans

Two controls of *tra-2* appear to be critical to the development of XX animals as hermaphrodite and XO animals as male. First, expression of *tra-2* is regulated by positive feedback within the sex-determination pathway (Okkema and Kimble, 1991). In wild-type adult animals, *tra-2* mRNAs are ≥ 15 -fold more abundant in XX hermaphrodites than in XO males. However, in XX males that lack *tra-1* function, *tra-2* mRNAs show male rather than hermaphrodite levels. Therefore, *tra-1* activity influences the level of *tra-2* mRNA even though it is positioned downstream of *tra-2* in the pathway (Figure 1). Given the similarity of the *tra-1* protein to known transcriptional regulators (Zarkower and Hodgkin, personal communication), a simple hypothesis is that *tra-1* itself may positively regulate *tra-2* at the transcriptional level. Because *tra-1* activity depends on *tra-2*, this feedback loop would amplify a signal for female development and lead to commitment to the female fate.

Second, the activity of pTra2A might be regulated by neighboring cells. As discussed above, *her-1* acts nonautonomously to negatively regulate *tra-2* (Hunter and Wood, 1992). Because pTra2A is predicted to be an integral membrane protein, a simple idea is that pTra2A is a receptor for the *her-1* protein. The role of cell communication in *C. elegans* sex determination may be to ensure unified sexual development throughout the animal. Because most sexual structures are formed by descendants of more than one precursor cell (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston *et al.*, 1980), it is critical that cells be coordinated to follow the same sexual fate. The discovery of *her-1* nonautonomy (Hunter and Wood, 1992) and the identification of *tra-2* as a membrane protein provide the key components for a mechanism to coordinate the sexual fate decision among neighboring cells. Figure 8 illustrates how *her-1/tra-2* mediated cell communication can be coupled to a positive feedback loop on *tra-2* expression to achieve this end. This single mechanism can drive a male cell to the female fate if it is surrounded by female cells or it can drive a female cell to the male fate if surrounded by male cells. Because cell-fate decisions are often coordinated by groups of cells during the development of both invertebrates (e.g., compartments) and vertebrates (e.g., embryonic fields), the mechanism by which *her-1* and *tra-2* coordinate cell-fate decisions in *C. elegans* may be of general significance.

Another possible role for cell communication within the sex determination pathway is to correct errors in

Figure 8. A model for the coordination of cells to adopt a single sexual fate. This model is based on several speculations about the *her-1* and *tra-2* proteins. First, we postulate that pTra2A is abundant in female cells but rare in male cells. This idea relies on the difference in abundance of *tra-2* mRNA in XX and XO animals (Okkema and Kimble, 1991). Second, we posit that the *her-1* protein is secreted and that *tra-2* is a membrane protein. These ideas are based on the nonautonomy of *her-1* (Hunter and Wood, 1992), the sequence of the predicted *her-1* product (Perry and Wood, personal communication), and the sequence of the predicted *tra-2* protein. Third, we speculate that the *her-1* protein binds and thereby inactivates pTra2A. This idea is based on genetic arguments that *her-1* negatively regulates *tra-2* (Hodgkin, 1980) and the lack of any genes identified between *her-1* and *tra-2* in the sex determination pathway. (A) One male cell in a field of female cells is recruited to the female fate by the activity of its neighbors. Left, A single male cell secretes *her-1*, which binds to the excess pTra2A on its neighbors. Middle, Because little *her-1* protein remains after it binds to neighboring cells, pTra2A on the male cell is activated. Therefore, more pTra2A is made because of the positive feedback loop on *tra-2* expression and the cell transforms from male to female. Right, All cells are committed to the female fate. (B) One female cell in a field of male cells is recruited to the male fate by the activity of its neighbors. Left, A single female cell produces abundant pTra2A, which binds and is inactivated by the excess of secreted *her-1* protein. Middle, Because pTra2A is inactivated, less pTra2A is made in the female cell, and the cell transforms from female to male. Right, All cells are committed to the male fate.

the initial reading of the X:A ratio. Such a correction mechanism might be predicted to exist within the dosage compensation pathway, which is also regulated by the X:A ratio. Alternatively, a component of the sex-determination pathway might have a role in correcting dosage compensation errors through feedback regulation. In *Drosophila*, improperly specified cells die and correctly specified cells compensate for these deaths. However, in *C. elegans* there are relatively few cells and little capacity for replacement of dying cells during development. Therefore, an incor-

rect reading of the X:A ratio would be exceptionally deleterious in *C. elegans*.

In summary we postulate that regulation of *tra-2* is pivotal to sex determination in both XX and XO animals. Our working model is that positive regulation of *tra-2* transcription, perhaps by *tra-1*, commits cells in XX animals to adopt the female fate, whereas negative regulation of pTra2A, perhaps by *her-1*, allows cells in XO animals to follow the male fate. If there was no mechanism to inactivate pTra2A and block the positive feedback loop leading to female devel-

opment, cells in an XO animal would be in danger of commitment to the wrong sexual fate. The mechanism of inactivation might be as simple as stimulation of pTra2A endocytosis or it might involve disrupting protein-protein interactions crucial to pTra2A activity. Now that *her-1*, *tra-2*, and *tra-1* are cloned, it will be possible to test some of the predictions of this model, such as binding of *her-1* protein to pTra2A, and to learn how these regulatory genes influence sex determination.

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