Regulatory Elements Required for Development of *Caenorhabditis elegans* Hermaphrodites Are Conserved in the *tra-2* Homologue of *C. remanei*, a Male/Female Sister Species

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ABSTRACT

The *Caenorhabditis elegans* hermaphrodite is essentially a female that produces sperm. In *C. elegans*, *tra-2* promotes female fates and must be repressed to achieve hermaphrodite spermatogenesis. In an effort to learn how mating systems evolve, we have cloned *tra-2* from *C. remanei*, the closest gonochoristic relative of *C. elegans*. We found its structure to be similar to that of *Ce-tra-2* but its sequence to be divergent. RNA interference demonstrates that *Cr-tra-2* promotes female fates. Two sites of *tra-2* regulation are required for the onset of hermaphrodite spermatogenesis in *C. elegans*. One, the MX region of TRA-2, is as well conserved in *C. remanei* as it is in *C. briggsae* (another male/hermaphrodite species), suggesting that this control is not unique to hermaphrodites. Another, the DRE/TGE element of the *tra-2* 3′ UTR, was not detected by sequence analysis. However, gel-shift assays demonstrate that a factor in *C. remanei* can bind specifically to the *Cr-tra-2* 3′ UTR, suggesting that this translational control is also conserved. We propose that both controls are general and do not constitute a novel "switch" that enables sexual mosaicism in hermaphrodites. However, subtle quantitative or qualitative differences in their employment may underlie differences in mating system seen in Caenorhabditis.

A particularly fruitful way to study the mechanisms underlying the evolution of phenotypic diversity has been to compare a well-studied model taxon with a close relative that differs from it in a significant way. There are two main advantages to this approach. First is the minimization of phylogenetic "noise," or molecular divergence due to separation of lineages that are not relevant to the evolving character in question. Second, by choosing well-characterized model organisms one can select a phylogenetically variable trait that has already been dissected in detail. This basic approach has been applied successfully to discover mechanisms underlying variation in larval form in ascidians (Swalla *et al.* 1993) and sea urchins (Raff 1992); vulval patterning (Eizinger *et al.* 1999), gonadal shape (Felix and Sternberg 1996), and male tail development (Fitch and Emmons 1995) in nematodes; and appendage patterning in insects (Weatherbee *et al.* 1999), among others. Recent studies on the mechanisms underlying intraspecific variation in development can be viewed as extreme examples of this methodology (*e.g.*, Mackay 1995; Doebley *et al.* 1997; de Bono and Bargmann 1998).

The evolution of mating systems is a major topic of theoretical and experimental research in evolutionary biology (*e.g.*, Darwin 1877; Charlesworth 1984; Sassaman 1989; Pannell 1997a,b), but little is known about the mechanisms that underlie transitions from one mating system to another. Species in the nematode genus Caenorhabditis produce either males and females (gonochorism or dioecy) or males and self-fertile hermaphrodites (androdioecy). Gonochorism is thought to be ancestral in the family Rhabditidae, with androdioecy evolving independently multiple times in different clades (Fitch 1997). The widely studied model *Caenorhabditis elegans* is one of the few organisms for which detailed mechanisms of sex determination are known, and it is an androdioecious species. Closely related congeners, such as *C. remanei*, are gonochoristic, relying on males and females for reproduction. Caenorhabditis therefore offers a unique opportunity to study the genetic basis of variation in mating system. We have begun a comparison of sex determination mechanisms in *C. elegans* and *C. remanei*. Because *C. elegans* hermaphrodites are essentially females whose ovotestes first produce sperm, which are stored, and thereafter make only oocytes, the variable character distinguishing these two mating systems can be conceptually narrowed to the presence or absence of spermatogenesis by the egg-producing sex.

In the *C. elegans* hermaphrodite germline, gamete sex is controlled by regulatory genes that function throughout the animal, so-called global sex-determining genes, as well as genes that control sex determination specifically in the germline (reviewed by Meyer 1997) (Figure 1). The onset of hermaphrodite spermatogenesis relies

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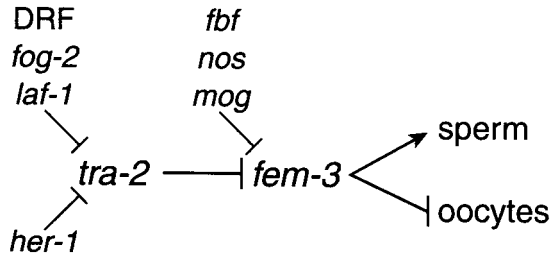

Figure 1.—Simplified model for control of germline sex determination in *C. elegans*. The *tra-2* gene promotes oogenesis by repressing *fem-3*, a negative regulation that may involve the direct binding of FEM-3 protein by TRA-2 protein. Genes or factors implicated in translational repression of the *tra-2* mRNA include DRF/GLD-1 (Jan *et al.* 1999), FOG-2 (Schedl and Kimble 1988; T. Schedl, personal communication), and *laf-1* (Goodwin *et al.* 1997). Factors that repress *fem-3* translation include FBF (Zhang *et al.* 1997), NOS (Kraemer *et al.* 1999), and perhaps the MOG proteins (Puoti and Kimble 1999). Four other genes, *fem-1*, *fem-2*, *fog-1*, and *fog-3*, act at the same position in this pathway as *fem-3* to promote spermatogenesis; these four were left out of the diagram because they are not known to be regulated to achieve the switch from spermatogenesis to oogenesis.

on the germline repression of the *tra-2* gene, which promotes female development in all tissues, whereas the switch from spermatogenesis to oogenesis relies on the germline repression of the *fem-3* gene, which promotes male development in all tissues (reviewed in Puoti *et al.* 1997). In addition to these negative controls on *tra-2* and *fem-3* mRNAs and protein, the TRA-2 protein may directly repress FEM-3 protein by binding (Mehra *et al.* 1999).

The repression of *tra-2* that is responsible for the onset of hermaphrodite spermatogenesis in *C. elegans* occurs on at least two levels. One control element resides in the *tra-2* 3′ untranslated region (UTR); this element, called the DRE for *d*irect *r*epeat *e*lement, inhibits translation of *tra-2* mRNA (Goodwin *et al.* 1993). A second control element resides in the C terminus of the TRA-2 protein; this MX (*mix*ed character of mutations in the element) regulatory region is proposed to downregulate activity of TRA-2 by an unknown mechanism (Kuwabara *et al.* 1998). *C. elegans tra-2* produces a transcript that encodes an integral membrane protein, called TRA-2A, and two smaller mRNAs encoding TRA-2B, which is essentially the cytoplasmic portion of TRA-2A (Okkema and Kimble 1991; Kuwabara *et al.* 1992). We use the general term *tra-2* or TRA-2 when referring to features shared by both of these RNAs or proteins. Thus, the transcripts encoding TRA-2A and TRA-2B share the same 3′ UTR and therefore are both controlled by DRE regulation. Similarly, the proteins TRA-2A and TRA-2B share the same C terminus and therefore are both controlled by MX regulation. Furthermore, both TRA-2A and TRA-2B share the FEM-binding site (Mehra *et al.* 1999).

Three germline-specific regulators have been identified that mediate DRE regulation by the *tra-2* 3′ UTR. These include DRFQ2/GLD-1, a protein that specifically binds the DRE (Goodwin *et al.* 1993) and controls *tra-2* translation (Jan *et al.* 1999); FOG-2, a protein that binds GLD-1 and is required for the onset of hermaphrodite spermatogenesis (Schedl and Kimble 1988; T. Schedl, personal communication); and *laf-1*, a gene that has not yet been identified at the molecular level (Goodwin *et al.* 1997) (Figure 1). The regulator acting through the MX region is not yet known.

The DRE and MX control elements were identified genetically by the isolation of dominant feminizing *tra-2* regulatory mutations that abrogate repression and eliminate hermaphrodite spermatogenesis (Doniach 1986; Schedl and Kimble 1988). The DRE and MX mutations are known as *tra-2(gf)* and *tra-2(mx)* mutations, respectively. The *fog-2* gene was identified genetically by the isolation of recessive feminizing mutations (Schedl and Kimble 1988). Of particular interest to our thinking about transitions between mating systems in evolution, we note that *tra-2(mx)*, *tra-2(gf)*, and *fog-2(null)* mutants with two XX chromosomes develop as females rather than as hermaphrodites and can be maintained with their XO counterparts as male/female strains (Doniach 1986; Schedl and Kimble 1988). It is therefore possible that hermaphroditism may have evolved from gonochorism by the creation or modification of these elements, and, conversely, that male/female strains may have evolved by regression from androdioecious strains via the loss of one or more of the elements.

We have begun our dissection of germline controls of sexual fate in *C. remanei* by analysis of its *tra-2* homologue, *Cr-tra-2*. Our choice of *tra-2* as a starting point in this study was based on the idea that the initiation of hermaphrodite spermatogenesis might be more primary in the evolution of hermaphroditism than the switch to oogenesis. However, we presume that the mechanisms controlling the initiation of spermatogenesis as well as its termination must have evolved in concert to allow continuous fertility of the ancestral form. We imagine, for example, that the *fem* male-promoting genes might have been repressed in the germline of the common female ancestor, but that a transient repression of *tra-2* might have released the *fem* genes to permit the transient production of sperm. In addition, our choice of *tra-2* was based on the practical consideration that a *tra-2* homologue had been obtained from the sister gonochoristic species *C. briggsae* (Kuwabara 1996a), but efforts to isolate a *fem-3* homologue from *C. briggsae* have been futile to date.

In this article we present evidence that *Cr-tra-2* promotes female fates in both germline and soma, as was previously shown for *Ce-tra-2* (Hodgkin and Brenner 1977) and *Cb-tra-2* (Kuwabara 1996a). By sequence analysis, the MX domain appears to be conserved in *Cr-tra-2*. Although no DRE element is detectable by sequence analysis of the *Ce-tra-2* 3′ UTR, DRF (*d*irect *r*e-

peat *f*actor)/GLD-1 binding does appear to occur, suggesting that the regulatory site is present but not recognizable. Finally, the FEM-3-binding region differs substantially between *Cr-tra-2* on the one hand and the *Ce* and *Cb-tra-2* homologs on the other. We discuss these findings with respect to the potential role of *tra-2* in the evolution of hermaphroditism.

MATERIALS AND METHODS

Nematode strains and culture: *C. remanei* strain SB146 was obtained from the Caenorhabditis Genetic Center (CGC; Univ. of Minnesota, St. Paul). *C. elegans* strain N2 was also obtained from the CGC. *C. remanei* culture conditions are essentially identical to those for *C. elegans* (Wood 1988), with the exception that growth on plates is facilitated by use of 3% agar to prevent burrowing. A note on taxonomy: the taxonomy of two strains relevant to this work has been revised recently. Strain EM464, now considered a subspecies of *C. remanei* (Sudhaus and Kiontke 1996), was initially described as *C. vulgaris* (Baird *et al.* 1994; Fitch *et al.* 1995). Strain CB5161, formerly regarded by the Caenorhabditis Genetics Center as *C. remanei*, is now known to be a distinct species and an outgroup to the *elegans*/*briggsae*/*remanei* clade (Thomas and Wilson 1991; Fitch *et al.* 1995; Baldwin *et al.* 1997).

Preparation of genomic DNA: For preparation of genomic DNA, worms from densely grown plates were used to seed a 500-ml liquid culture as described in Wood (1988). As *C. remanei* animals must mate to reproduce, the liquid culture was allowed to sit without shaking for 2 hr per day until the culture was near saturation (300 worms/ml). Worms were harvested by centrifugation and washed with M9 salts (Wood 1988). Clean, concentrated whole worms were washed once with disruption buffer (DB: 200 mm NaCl, 50 mm EDTA, 100 mm Tris, pH 8.5) and then resuspended in five volumes of DB with the addition of sodium dodecyl sulfate to 0.5% and proteinase K to 200 μg/ml. After incubation with intermittent mixing at 65° for 1 hr, the solution was diluted in DB, extracted with phenol/chloroform, and precipitated with isopropanol. The DNA was gently resuspended in 10 mm Tris, pH 8.0, 1 mm EDTA (TE) and treated with RNAse A. Genomic DNA was precipitated with ammonium acetate and isopropanol, followed by centrifugation at room temperature. Pelleted DNA was resuspended in TE overnight at 4°.

Construction of a *C. remanei* genomic library: Insert DNA was prepared by partial digestion of *C. remanei* genomic DNA with *Sau*3A, followed by size selection on an agarose gel. The gel section was frozen and spun through a spin filter (Costar, Corning, NY). The resulting DNA solution was extracted with phenol/chloroform and precipitated with sodium acetate and ethanol. The 5′ overhang resulting from *Sau*3A digestion was partially filled in with dGTP, dATP, and Klenow fragment according to a scaled-down version of the vector kit's protocol (Lambda Fix II phage vector, Stratagene, La Jolla, CA). After phenol/chloroform extraction and precipitation, the insert DNA was quantified and then ligated to phage vector that had been prepared by the vendor via digestion with *Xho*I and partial fill-in. Ligation was performed according to manufacturer's instructions, and the resulting products were packaged with Gigapack III extracts (Stratagene). A total of 167,000 independent clones were amplified.

Library screening: Basic solutions and procedures for hybridizations are described in Sambrook *et al.* (1989). A 1.1-kb *C. elegans ppp-1* genomic fragment was amplified by PCR from cosmid ZK513 (kindly provided by Dr. Alan Coulson) using the forward primer pppF (5′ TCAGCGATGCCAGTCT CATTC 3′) and reverse primer pppR (5′ GGTGACGTCAG CATTCTCTCCG 3′). This product was random-prime labeled with ^{32}P and used first to optimize the signal with a genomic Southern blot of digested *C. remanei* DNA. The conditions were determined to be hybridization overnight at 45° in 5× SSPE 1% SDS, 10× Denhardt's reagent and 100 μg/ml yeast RNA, followed by washes whose maximal stringency was at 50° in 2× SSC with 0.1% SDS. Library screening was carried out with duplicate lifts on 160,000 pfu. Clone λCRP3 was verified by Southern blotting to contain the same *ppp-1*-positive restriction fragment seen in the preliminary genomic blot analysis and was chosen for extensive analysis. Clone λCRT5 was obtained by a second high-stringency screen of the genomic library with 160,000 pfu of the genomic library with an *Eco*RI subclone of λCRP3 encoding the interval from the 3′ end of exon 7 to the 5′ end of intron 15 of *Cr-tra-2*. To obtain *Cr-tra-2* cDNA clones, 320,000 pfu from a *C. remanei* cDNA library (courtesy of David Rudel, Kimble Lab) were screened at high stringency using as probe the 3.1-kb *Xba*I fragment of λCRP3, which includes the 3′ end of the clone, in the above hybridization solution.

Sequencing and computer analysis: Sequencing of λCRP3 was accomplished by combining subcloning and vector priming with primer walking to fill gaps and resolve ambiguities. All cycle sequencing reactions used the ABI Prism FS Terminator or Big-Dye Terminator Ready Reaction Mix (PE/Applied BioSystems, Foster City, CA) and were run by the Blattner Lab sequencing service, Department of Genetics, UW-Madison. Sequence traces were inspected with the SeqMan program of the Lasergene software package (DNAstar, Madison, WI) to cull reliable data, which was then imported into a Unix workstation running the Wisconsin Package, v.9 or v.10 (Genetics Computer Group, Madison, WI) for further analysis.

RNA blot analysis: A total of 10 μg of mixed-stage *C. remanei* poly(A)$^+$ RNA (a gift of David Rudel) was electrophoresed, along with an RNA size standard, in a formaldehyde agarose gel using standard techniques (Sambrook *et al.* 1989) and blotted to Nytran (Schleicher and Schuell, Keene, NH). A ^{32}P-labeled DNA probe was prepared by random priming using the entire 1.5-kb insert of *Cr-tra-2* cDNA clone RTC8. Hybridization and washing were performed under high-stringency conditions.

RNA interference: Sense and antisense RNAs corresponding to exons 4–7B of *Cr-tra-2* were synthesized with the MEGAscript kit (Ambion, Austin, TX) from linearized templates prepared from a 1.1-kb *Eco*RI fragment of λCRP3 subcloned in the pBluescript II plasmid vector (Stratagene). Equal amounts of each RNA were mixed, denatured, and annealed by slow cooling. The resulting dsRNA was injected into the distal gonad arm or gut of *C. remanei* females using standard *C. elegans* procedures (Fire *et al.* 1998). Injectees were returned to plates with two males, allowed to lay embryos, and transferred with their mates each day to new plates to stage the progeny.

RNA gel-shift assay: PCR was used to amplify the *Cr-tra-2* 3′ UTR from cDNA clones RTC7 and RTC8, which were then cloned via terminal restriction sites in pBluescript II. *C. elegans* wild-type (N2) and *tra-2(e2020)* templates were a generous gift of Eric Jan and Elizabeth Goodwin. ^{32}P-labeled probes were synthesized by *in vitro* transcription of linearized templates and gel purified. Cold N2 3′ UTR competitor RNA was produced using the MEGAscript kit. Extracts from *C. elegans* and *C. remanei* worms, as well as all other reagents, were made according to the method of Goodwin *et al.* (1993) with the exception that no magnesium was added to the extracts. Binding reactions and native polyacrylamide gel electrophoresis were also carried out according to Goodwin *et al.* (1993).

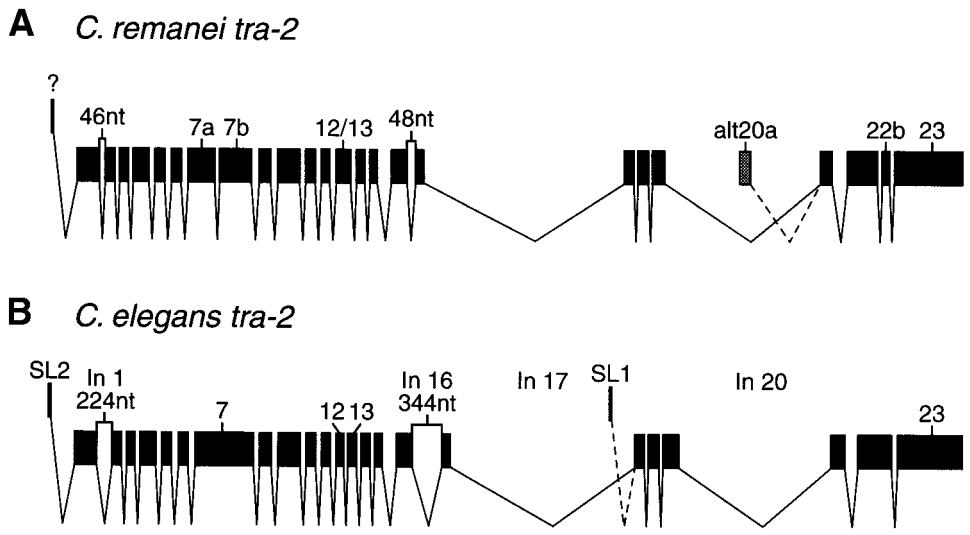

Figure 2.—Exon-intron structure of *C. remanei tra-2* compared with that of *C. elegans tra-2*. Exons, solid rectangles; introns, joining lines. *Cr-tra-2* exons and introns (A) are numbered to be consistent with their homologues in *Ce-tra-2* (B). The full-length *Cr* 4.7-kb mRNA, which encodes TRA-2A, may be *trans*-spliced at its 5′ end as is the case for *Ce-tra-2*, but no evidence for such *trans*-splicing yet exists. Alternate exon 20A is found in the *Cr* 1.6-kb mRNA, which encodes TRA-2B, where it is predicted to serve as its 5′ untranslated region. Introns with noteworthy length differences are indicated with brackets and the size in nucleotides. The genomic sequence of *Cr-tra-2* has been submitted to GenBank under accession no. AF187965.

RESULTS

Cloning *Cr-tra-2*: In both *C. elegans* and *C. briggsae*, *tra-2* is transcribed as the downstream gene in an operon; in both cases, its upstream neighbor is *ppp-1*, which encodes pyrophosphorylase. Kuwabara (1996a; Kuwabara and Shah 1994) showed that the *C. elegans* and *C. briggsae ppp-1* genes were conserved enough to allow cross-hybridization, even though their corresponding *tra-2* homologues were divergent. Furthermore, the coding sequences from *C. elegans*, *C. remanei*, and *C. briggsae* are in general conserved similarly in all pairwise combinations (Thomas and Wilson 1991; Fitch *et al.* 1995; D. Rudel, unpublished data). Therefore, we screened a *C. remanei* genomic library at low stringency with a *C. elegans ppp-1* probe (see materials and methods). We initially recovered a single clone, λCRP3, containing both a *C. remanei ppp-1* homologue (*Cr ppp-1*) and nearly all of *Cr-tra-2*, lacking only the last 115 codons and 3′ UTR. To extend this, the genomic library was again screened at high stringency with a subclone of λCRP3. This screen yielded one clone, λCRT5, that extended further 3′ than λCRP3, but it was also truncated prematurely at the stop codon. The 3′ UTR of *Cr-tra-2* was obtained by screening a *C. remanei* cDNA library (courtesy of D. Rudel) at high stringency, which yielded three overlapping clones, RTC7 (2.2 kb), RTC8 (1.5 kb), and RTC11 (1.7 kb). The three cDNAs also contain two slightly different 3′ UTR sequences (see below and in Figure 6). As *C. remanei* is an obligately outcrossing species, this may represent allelic variation that has persisted despite the frequent inbreeding imposed by routine laboratory propagation. Southern blots indicate that only one copy of the *ppp-1*/*tra-2* gene pair is present in the *C. remanei* genome (not shown).

***Cr-tra-2* gene structure and transcripts:** Figure 2 compares the predicted exon/intron structures of *Cr-tra-2* and *Ce-tra-2*. Where cDNA sequence was available (the 3′-most 2.2 kb of exonic sequence), the splicing pattern was directly inferred. For the remaining 5′ portion, it was deduced by comparing genomic DNA sequences of *Cr-tra-2* with that of *Ce-tra-2*, in conjunction with the conceptual translations of these genes and that of *Cb-tra-2*. This analysis revealed that the splicing events producing the longer full-length *tra-2* mRNA (predicted to be 4.7 kb) are generally conserved, but not identical to those of *Ce-tra-2* (Figure 2). Differences include the split of two *Ce-tra-2* exons, 7 and 22, into two smaller exons apiece (7A/7B and 22A/22B) in *Cr-tra-2* and the union of *Ce-tra-2* exons 12 and 13 into a single exon (12/13) in *Cr-tra-2*. Another noteworthy structural difference is the decreased size of introns 1 and 16 in *Cr-tra-2* relative to *Ce-tra-2*.

In addition to a full-length mRNA encoding TRA-2A, *Ce-tra-2* also produces two shorter transcripts of 1.8 kb and 1.9 kb that encode only the cytoplasmic domain, called TRA-2B (Okkema and Kimble 1991; Kuwabara *et al.* 1998). *Cb-tra-2* does not appear to produce these smaller RNAs (Kuwabara 1996a), but cDNA sequence and RNA blot analysis suggests that *Cr-tra-2* makes a TRA-2B-encoding mRNA. A unique 5′ sequence of 69 nucleotides (nt) in clone RTC11, encoded by an alternative exon lying within intron 20, suggests that a 5′-truncated transcript is produced, perhaps from an internal promoter lying inside this large intron. The transcript represented by RTC11 is predicted to encode a peptide exactly colinear with that encoded by the small transcripts produced by *Ce-tra-2* (Okkema and Kimble 1991; Kuwabara *et al.* 1998). However, the putative promoter for the *C. elegans* 1.8-kb transcript apparently resides in a different large intron, intron 17, and includes only

exons shared with the full-length 4.7-kb *Ce-tra-2* mRNA (Kuwabara *et al.* 1998). Both the 1.8-kb and 1.9-kb *Ce-tra-2* transcripts and the *Cr-tra-2* transcript corresponding to RTC11 are predicted to use a common start methionine codon that resides in exon 21, so that the *Ce-tra-2* mRNA includes exons 18–20 as a 5′ untranslated region, whereas that of *Cr-tra-2* does not (Figure 2).

The predicted minimum transcript size of the RTC11-type *Cr-tra-2* mRNA is 1639 nt, plus polyadenylation. To verify that RTC11 corresponds to a *bona fide* mRNA, we probed blots of *C. remanei* RNA with the 3′-most 1.5 kb of the *Cr-tra-2* coding sequence. This probe identified a prominent RNA of ~4.7 kb, corresponding to the full-length *Cr-tra-2*A-encoding message, as well as a weak band at ~1.6 kb (data not shown). We have not been able to verify the precise 5′ end of the alternative exon 20, but if RTC11 corresponds to the 1.6-kb transcript it is not much larger than the 69 nt contained in this cDNA.

The *Cr-tra-2* sequence and comparison with its homologues: A comparison of the amino acid sequences of *Cr-tra-2*A with both *Ce-tra-2*A and *Cb-tra-2*A reveals a conserved architecture, but a divergent sequence (Figure 3). The overall sequence similarity between *Cr-tra-2* and its homologues is quite low: 54.8% (*vs. Ce-tra-2*) and 57.4% (*vs. Cb-tra-2*) nucleotide identity for coding sequences, 43.4% (*vs. Ce-tra-2*) and 49.9% (*vs. Cb-tra-2*) identity for amino acid sequences (see Table 1). However, all three *tra-2* homologs possess a predicted signal peptide and nine predicted transmembrane domains (SP region and solid overlines in Figure 3). Of particular importance for this article, the MX domain in the cytoplasmic region of both TRA-2A and TRA-2B appears to be conserved. The *tra-2(mx)* alleles of *C. elegans* are caused by missense mutations in five different codons that are predicted to result in nonconservative amino acid changes (Kuwabara *et al.* 1998); four of these five amino acids are conserved in all three species (asterisks, Figure 3). The fifth residue is a conservative substitution shared by *Cr-tra-2* and *Cb-tra-2*. In addition to the MX domain, the alignment also revealed that *Cr-tra-2* possesses the *e*nhanced *g*ain-of-function (EG) site implicated in regulation of TRA-2A by *her-1* (Kuwabara 1996b; inverted triangle, Figure 3) and that the N-terminal putative extracellular region is more highly conserved than most of the rest of the protein (54% between *Cr-tra-2* and *Ce-tra-2*). By contrast, the FEM-3-binding region (Mehra *et al.* 1999) is poorly conserved (thick dashed overline, Figure 3; 20–25% between *Cr-tra-2* and *Ce-tra-2*, depending on alignment parameter values). In addition, this region is shortest in *Cr-tra-2*, being 8 amino acids shorter than *Ce-tra-2* and 11 shorter than *Cb-tra-2*. Although precise alignment of this region is difficult due to the lack of conservation, the plausible version shown in Figure 3 suggests that the length difference is primarily due to a 22-amino-acid deletion in *Cr-tra-2*, near the center of the domain, relative to *C. briggsae*. *Ce-tra-2* appears to possess 16 of these residues. We conclude that the *Cr-tra-2* protein shares most features with *Ce-tra-2* and *Cb-tra-2*, but that one potentially key region, the FEM-3-binding domain, is more similar in the two hermaphroditic species than in *C. remanei*.

***Cr-tra-2* promotes female development in *C. remanei*:** To ask whether the cloned *Cr-tra-2* homologue is in fact a sex-determining gene, we employed RNA interference (RNAi; Guo and Kemphues 1995; Fire *et al.* 1998) to disrupt its activity in *C. remanei*. Using double-stranded (ds) RNA, we observed a range of masculinization among progeny of the injected mother (Figure 4). Thus, weakly masculinized animals were vulvaless (Vul) with gonads containing only sperm; moderately affected animals were Vul with a fully developed testis but whip-like tails characteristic of females; and severely transformed animals were pseudomales similar to *tra-2* null mutants in *C. elegans* (Figure 5A). These pseudomales did not exhibit mating behavior, were unusually small (compare Figure 5, A and B), and were lethargic and uncoordinated. The near absence of wild-type females, a graded distribution of partially masculinized animals, and the large fraction of normal males led us to conclude that all non-wild-type male progeny were genetically female.

In addition to sexual transformation, dsRNAi with *Cr-tra-2* also significantly reduced the number and viability of embryos laid by injected mothers relative to Tris-EDTA buffer (TE) mock-injected controls (Figure 4; $P < 0.01$ for both 22-hr laying windows, on the basis of binomial confidence limits as described in Sokal and Rohlf 1995). Although only data from the first two laying windows is shown, by the fourth window, broods dropped in both size and viability until injected mothers were laying only a few inviable embryos. Therefore, the *Cr-tra-2* RNA may have interfered with germline function of the mother. In contrast, mothers mock-injected with TE actually increased their brood size in the second day after injection, with the viability of their progeny always being >97%. Many embryos of RNAi-treated mothers that failed to develop were obviously abnormal under the dissecting scope (not shown). In addition to the general decrease in viability of progeny, a sex-biased lethality was also seen. Only 87/236 (37%) of viable progeny laid in the first 22 hr and 76/179 (42%) in the second 22 hr were genetically female. These numbers are significantly different from the null hypothesis of equal sex ratios at the level of 0.01 for the first laying window and 0.05 for the second (χ^2 test).

As no lethality had been observed in *C. elegans tra-2* null mutants (Hodgkin and Brenner 1977; E. Haag, unpublished observations), we also investigated whether RNAi directed against *Ce-tra-2* resulted in such effects in *C. elegans*. Using dsRNA corresponding to the 5′-most 1.5 kb of the *Ce-tra-2* coding sequence, we found that viability was comparable to that of uninjected animals over two 22-hr laying windows (98%, $N = 877$). Furthermore, *Ce-tra-2(RNAi)* mothers did not suffer the same

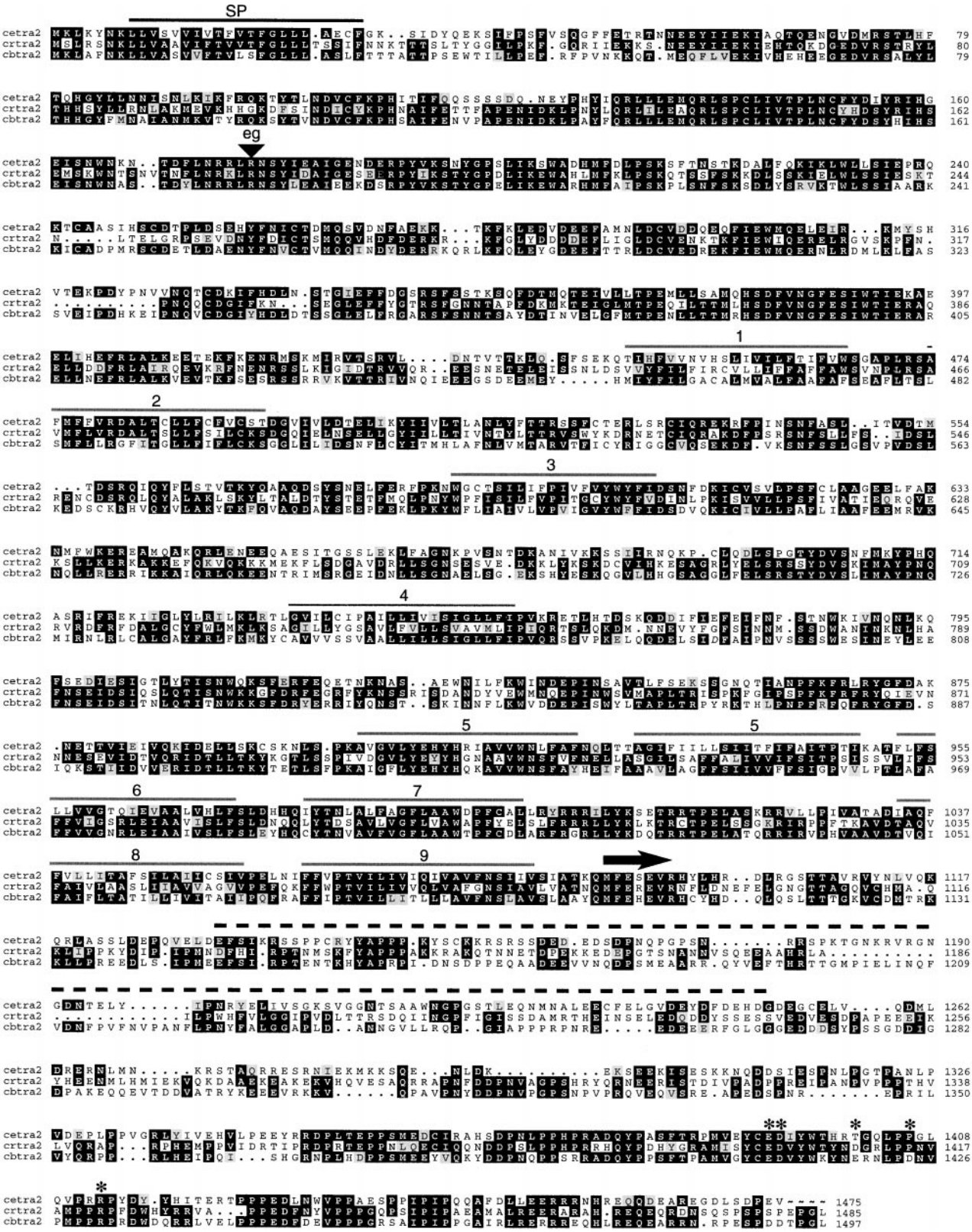

Figure 3.—Alignment of TRA-2A homologues from *C. elegans*, *C. remanei*, and *C. briggsae*. Black boxes indicate amino acid identity; gray boxes indicate similarity, and unshaded residues are divergent. The conserved signal peptide (SP) is indicated at the N terminus; the inverted triangle denotes the EG amino acid at position 177 of *Ce-tra-2*A, which is thought to be required for negative regulation of TRA-2A by HER-1 (Kuwabara 1996b). Numbered solid overlines delineate the nine hydrophobic regions predicted to encode membrane-spanning domains by Kuwabara *et al.* (1992). Their model of TRA-2A structure proposed that the hydrophobic amino acids 496–516 of *Ce-tra-2*A are not a transmembrane domain, and the alignment supports this proposal: *Cb-tra-2* and *Cr-tra-2* have two and three fewer hydrophobic residues, respectively, in this region. The arrow at position 1089 of the *C. elegans* sequence indicates the initiating methionine for TRA-2B, the cytoplasmic protein product of the smaller transcripts produced by both *Ce-tra-2* and *Cr-tra-2* (see text for details). The heavy dashed line overline denotes the FEM-3-binding region (Mehra *et al.* 1999). Asterisks near the C terminus indicate amino acids required for the MX domain (Kuwabara *et al.* 1998).

TABLE 1

Sequence similarities between *tra-2* homologues

Sequence pair	Amino acid identity (%)	Coding sequence nucleotide identity (%)	3′ UTR identity[a] (%)
elegans / briggsae	43.7	55.2	45.5
elegans / remanei	43.4	54.8	44.7
briggsae / remanei	49.9	57.4	48.8

[a] These values are only slightly higher than those obtained via aligning randomized versions of the UTR sequences, which ranged from 38 to 42% identity over three trials. A + U content was exceptionally high, ranging from 70.0 to 74.2%, with U being especially abundant in the sense strand. Of the 97 identities in the alignment of the 3′ UTRs from *Ce-tra-2* and *Cr-tra-2* (using the GAP program of the GCG Wisconsin Package), 63 were U residues. Similarly, 51 of 92 matches in *Ce-tra-2 vs. Cr-tra-2* were U. Only the alignment of *Cb-tra-2* and *Cr-tra-2* 3′ UTR sequences produces stretches of identities longer than 6 nt.

decrease in brood size seen in the *Cr-tra-2(RNAi)* experiment and actually increased their progeny from 321 in the first laying window to 556 in the second, a recovery effect similar to that seen in TE-injected control animals. The Tra phenotype of *Ce-tra-2(RNAi)* progeny was 100% penetrant in the 22–44-hr laying window, though the extent of transformation, especially in the tail, was somewhat variable. Although no lethality was observed, 14% (78/556) of the pseudomales were unusually small. Closer inspection of these animals revealed that they had defects in defecation that resulted in a blocked posterior gut and gradual necrosis. The extent to which these animals were sexually transformed was not distinguishably different from that of other sibling pseudomales.

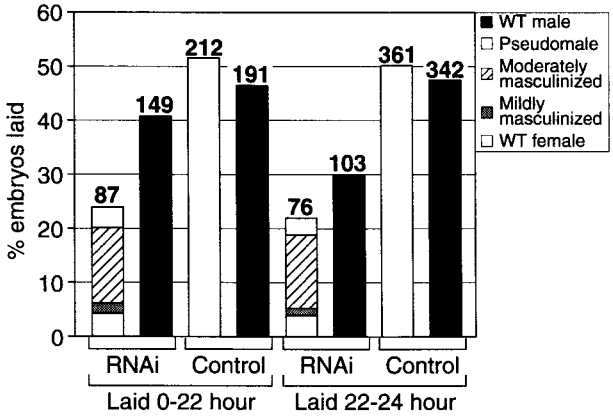

Figure 4.—*Cr-tra-2(RNAi)* masculinizes both somatic and germline tissues. Mildly affected animals lacked a vulva, had an armless gonad containing sperm, and were of normal size. Moderately affected worms had a single-armed testis, a nearly normal female whip-like tail, and were smaller than normal. Severely affected animals had a well-formed testis, an imperfectly masculinized tail, and were very small. Data combine totals from 13 different RNAi and 8 mock-injected control injectees. All RNAi progeny that were not wild-type males were assumed to be genetically female (see text for rationale) and are grouped together. The numbers above each column indicate the total number of animals scored for each presumed genetic sex. The trends of decreasing brood size and viability among RNAi progeny seen in the two collection windows shown here continued until all injected animals were sterile by day 4 (not shown).

The *Cr-tra-2* 3′ UTR binds a potential repressor *in vitro*: In *C. elegans*, the DRE control elements of the *tra-2* 3′ UTR bind DRF and repress translation (see Introduction). In the *Cb-tra-2* 3′ UTR, DRE elements were not detected by sequence comparison (Kuwabara 1996a), but binding by DRF was observed using gel-shift assays (Jan *et al.* 1997). As a result, Jan and colleagues defined a smaller motif, called the tra-gli element (TGE), as the repressor binding site. We inspected the *Cr-tra-2* 3′ UTR sequence for either a DRE or TGE, but found neither (see Figure 6 and its legend). As a more stringent test than sequence comparison, which had also failed for *Cb-tra-2*, we used the gel mobility shift method of Goodwin *et al.* (1993) to assay for DRF binding to the *Cr-tra-2* 3′ UTR. We found that a factor present in *C. remanei* extract can bind either the *Ce-tra-2* 3′ UTR (Figure 7, lane 4) or the *Cr-tra-2* 3′ UTR (Figure 7, lane 8) *in vitro* under conditions used to demonstrate DRF binding in *C. elegans.* The specificity of this binding was explored in two ways. First, we asked if a mutant *Ce-tra-2* 3′ UTR that lacks the DREs can bind factor in the *C.*

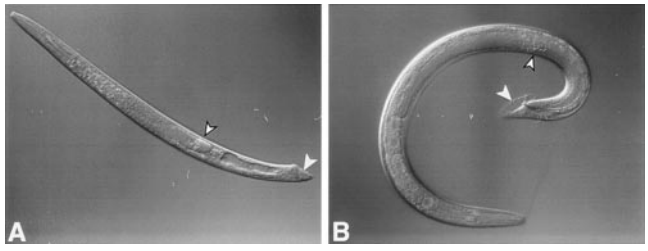

Figure 5.—Sexual transformation of *Cr-tra-2(RNAi)* progeny. Nomarski differential interference contrast optics. (A) Pseudomale has a well-formed testis, sperm (outlined arrowhead), and an imperfectly transformed tail (white arrowhead). This animal is 5 days postpartum, but pseudomales never reached normal size. (B) Adult wild-type male (roughly 4 days postpartum). This male is ~50% larger in volume than the pseudomale in A. Both A and B are shown at identical magnification. Outlined arrowhead, sperm; white arrowhead, male tail.

```
RTC7      1 GAUCGCCCCUUUUAUAUUGAUCUAAUGUAUUUUCAUGAAAAAACUUUCAU  50
            |||||||||||||||| ||||||||||||||||||||||||| ||||||
RTC11     1 GAUCGCCCCUUUUAUACUGAUCUAAUGUAUUUUCAUGAAAAAAUUUUCAU  50

RTC7     51 AUCAUUCCUUGCAUGUUCUAAACAUUUCAUUCGAUAUCAGCCUCUACUUG 100
            |||||||||||||||||||||||||||||||||||||||||||||||||||
RTC11    51 AUCAUUCCUUGCAUGUUCUAAACAUUUCAUUCGAUAUCAGCCUCUACUUG 100

RTC7    101 CACUCAUUUUCUUCUUAAACUUAGAUCUCCUAGACUCAUUUACUUCUUUG 150
            |||||||||||| ||||||||||||||||||||||||||||||||||||||
RTC11   101 CACUCAUUUUCUU.UUAAACUUAGAUCUCCUAGACUCAUUUACUUCUUUG 149

RTC7    151 UAUCAAUUUUUAUGAUCUUGUAACAAAACGGACUUCUUUUUUCAUUGUUU 200
            |||||||||||||||||||||||||||| ||||||||||||||||||||||
RTC11   150 UAUCAAUUUUUAUGAUCUUGUAACAAAACCGACUUCUUUUUUCAUUGUUU 199

RTC7    201 UUUUAUCUUCAAAGAUUCAAUAAAUUUAUUAAUUUUU 237
            |||| |||||||||| ||||||||||||||||||||||
RTC11   200 UUUUUUCUUCAAAGUUUCAAUAAAUUUAUUAAUUUUU 236
```

Figure 6.—Aligned sequences of two putative allelic variants of the *Cr-tra-2* 3′ UTR. The sequences are from cDNA clones RTC7 and RTC11 (RTC8 is identical to RTC11). They differ at 6 positions out of 237 and are thus 97.5% identical. The consensus TGE sequence for DRF binding proposed by Jan *et al.* (1997) is 5′ CUCA [n] CC/AA UUUC C/U U [n] UUUCU 3′. The two occurrences of CUCA in *Cr-tra-2* are indicated in bold. Neither is associated with the complete consensus, although the sequence UUUCUU occurs immediately 3′ from the CUCA at position 103. The putative polyadenylation sequence AAUAAA is shown in outlined text.

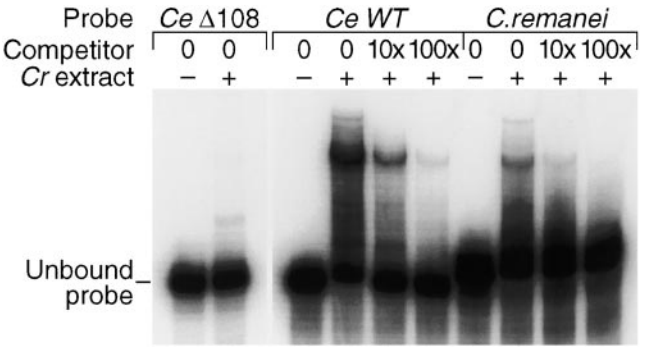

Figure 7.—Binding of a DRF-like factor to the *Cr-tra-2* 3′ UTR. After incubation of 5 fmol labeled RNA probe with 2 μg *C. remanei* crude extract, free and bound forms were separated by gel electrophoresis (see materials and methods). In certain incubations, unlabeled wild-type *C. elegans tra-2* 3′ UTR RNA was added at 10- or 100-fold molar excess to the binding reactions as competitor. Both variants of the *Cr-tra-2* 3′UTR gave similar results; only data from the RTC7 probe are shown here. The CeΔ108 probe deletes both DRE elements and was derived from *C. elegans tra-2(e2020 gf)*; this probe does not bind DRF and when serving as a 3′ UTR does not support translational repression. The CeΔ108 lanes were aligned with the others for convenience.

remanei extract. The mutant used, *tra-2(e2020)*, deletes 108 nucleotides from the 3′ UTR, including both DREs. We found that this mutant probe did not shift after incubation with *C. remanei* extract. Second, we asked if binding of the factor to the *Cr-tra-2* 3′ UTR could be inhibited by addition of unlabeled wild-type *Ce-tra-2* 3′ UTR. To this end, we added either 10-fold or 100-fold wild-type competitor and found that binding by either the *Ce-tra-2* 3′ UTR (Figure 7, lanes 5 and 6) or the *Cr-tra-2* 3′ UTR (Figure 7, lanes 9 and 10) was inhibited. We conclude that the *Ce-tra-2* and *Cr-tra-2* 3′ UTRs are likely to compete for the same factor. Identical results were obtained when these same probes were incubated with extracts from wild-type *C. elegans* worms (not shown). We note that the *C. elegans* 3′ UTR consistently produced a somewhat stronger shift than that of *C. remanei* (2.1-fold average over three experiments). Although this difference was reproducible in several experiments and observed using either *C. elegans* or *C. remanei* extract, its significance is not known.

DISCUSSION

Role of *Cr-tra-2* in sex determination is conserved: Several lines of evidence suggest that we have isolated the true *Cr-tra-2* homologue and that its role in sex determination has been conserved. Like the *C. elegans* and *C. briggsae tra-2* homologs, *Cr-tra-2* lies in a putative operon just 3′ of the *Cr-ppp-1* gene; it encodes a large integral membrane protein with multiple predicted transmembrane domains; and it promotes female development in both somatic and germline tissues. In the simplest scenario, *Cr-tra-2* would be predicted to interact with homologues of the same upstream and downstream genes as found in the sex determination pathway of *C. elegans*. This idea is supported by conservation of the EG site in the N terminus of TRA-2A, which is thought to mediate regulation by *her-1* (Figure 6; Kuwabara 1996a), the conservation of *fem-2* and *tra-1* genes in *C. briggsae* (de Bono and Hodgkin 1996; Hansen and Pilgrim 1998), and the recent identification of a *fem-1* homologue in both *C. briggsae* and *C. remanei* (J. Gaudet and A. Spence, personal communication). Therefore, sex determination in all three Caenorhabditis species appears to be controlled by a homologous regulatory pathway.

In addition to the expected masculinization of *Cr-tra-2(RNAi)* animals, severely transformed animals were much smaller than normal and *Cr-tra-2(RNAi)* embryos were less viable than controls. The lethal phenotype of *Cr-tra-2(RNAi)* embryos, furthermore, is biased toward, but not restricted to, females, which is reminiscent of *C. elegans* defects in dosage compensation (Meyer 1997). However, neither the effects on brood size nor on viability of progeny were seen in a comparable RNAi experiment with *Ce-tra-2*. In *C. elegans* the only known maternal function of *tra-2* is to decrease sperm number (Kuwabara *et al.* 1998). Genetically female (XX) *C. elegans tra-2* null mutants do not suffer lethality, but are transformed with complete penetrance into vigorous but nonmating pseudomales (Hodgkin and Brenner 1977). Therefore *C. elegans tra-2(RNAi)* animals closely resemble *Ce-tra-2* null mutants, and thus if *Cr-tra-2(RNAi)* also reflects the null phenotype, *tra-2* may perform a novel role in *C. remanei* in maintaining the viability of embryos, especially in genetically female animals. The presence of unusually small and feeble pseudomales among the *Ce-tra-2(RNAi)* animals, apparently

rendered thus by a defecation defect, raises the possibility that the smallness of *Cr-tra-2(RNAi)* pseudomales is due to a similar defect and thus is distinct from embryonic lethality. While the small *Cr-tra-2(RNAi)* pseudomales did not show the same symptoms of a blocked gut (a large impacted mass of *Escherichia coli* dilating the posterior gut, prone to rupture upon handling of the animals), they did appear to be partially paralyzed posteriorly and thus we cannot rule out a common underlying defect for both phenotypes.

The gradual loss of fertility of injected *C. remanei* mothers indicates that continual zygotic *tra-2* function may be required to maintain fertility. Temperature-shift experiments using a *tra-2(ts)* allele (Klass *et al.* 1976) indicate that young adult *C. elegans* hermaphrodites also require *tra-2* function for maximal fertility, but that after 70 hr posthatching (~36 hr into adulthood at 25°) brood size is not reduced by shifting to restrictive temperature. This suggests that in *C. elegans*, unlike in *C. remanei*, *tra-2* function is needed only transiently for normal fertility. Our results with *Ce-tra-2(RNAi)* support this scenario, as no decrease in brood size is observed in hermaphrodites injected ~24 hr after attaining adulthood. The reduced fertility of *Cr-tra-2(RNAi)* mothers is unlikely to be a general effect of the RNAi technique, as RNAi with two other *C. remanei* genes, *Cr-lag-1* and *Cr-glp-1*, produced highly penetrant phenotypes of the expected sort in progeny, but did not adversely affect maternal fecundity in the first 48 hr after injection (E. Haag and D. Rudel, unpublished data).

Conservation of *tra-2* is extensive among Caenorhabditis species and largely independent of mating system: This work was motivated by our desire to find differences in the molecular regulation of sex determination in androdioecious species such as *C. elegans* and gonochoristic species such as *C. remanei*. We surmised that close gonochoristic relatives of *C. elegans* might not possess negative controls of *tra-2* necessary for the onset of hermaphrodite spermatogenesis. Consistent with this idea, the germline of genetically female *C. remanei* animals was masculinized by reduction of *Cr-tra-2* activity via RNAi. Despite these expectations and in spite of the large amount of sequence divergence in the three known *tra-2* homologues, we provide evidence that *Cr-tra-2* possesses both DRE/TGE and MX regulatory sites used for repression of its *Ce-tra-2* homologue. While the *Cr-tra-2* 3′ UTR binding factor has not been shown to be a translational repressor, the ability of the *Ce-tra-2* 3′ UTR to bind this factor and the complementary ability of the *Cr-tra-2* 3′ UTR to bind the *C. elegans* DRF suggests that these are homologous factors. Given that the *Cr-tra-2* 3′ UTR does not contain a canonical TGE element (Jan *et al.* 1997; legend to Figure 6), we suggest that the more broadly conserved feature may be a structural motif in the 3′ UTR that is difficult to recognize from sequence gazing alone.

Given that the protein TRA-1 has recently been shown to regulate *tra-2* mRNA localization by binding to its 3′ UTR (Graves *et al.* 1999), the concern exists that perhaps the factor binding the *Cr-tra-2* 3′ UTR in these experiments is actually not DRF, but TRA-1. However, the *tra-2(e2020)* RNA that served as our negative control is known to bind TRA-1 (Graves *et al.* 1999) and undergoes little or no shift upon incubation with crude extracts, both in our hands (Figure 7, lane 2) and in studies published by others (Goodwin *et al.* 1993; Jan *et al.* 1997). As RNA binding by TRA-1 was demonstrated with purified protein, the endogenous protein may simply not be abundant enough in crude extracts to produce a detectable shift.

The *tra-2* gene acts genetically as a repressor of *fem-3* (Hodgkin 1986). Furthermore, the cytoplasmic domain shared by TRA-2A and TRA-2B binds FEM-3 protein (Mehra *et al.* 1999). The inference is that the binding of FEM-3 by TRA-2 may inhibit FEM-3 activity, which might explain why a balance between *tra-2* and *fem-3* activity controls sperm number in hermaphrodites (Schedl and Kimble 1988). Intriguingly, the most poorly conserved domain among the three cloned *tra-2* homologues is the FEM-3-binding site in the cytoplasmic domain of *Ce-tra-2* (Figure 4). *Cr-tra-2*, perhaps importantly, appears to have a deletion in this region relative to *Ce-tra-2* and *Cb-tra-2*. Such divergence in a domain that mediates a potentially critical protein-protein contact suggests that this region may have undergone selection. Positive selection on protein-protein interactions has been most clearly demonstrated for gamete recognition components in marine invertebrates, in which rapid evolution of proteins facilitating the interaction of sperm and egg facilitates reproductive isolation of congeneric sympatric species (Metz and Palumbi 1996; Swanson and Vacquier 1998). Homologues of *fem-3* have yet to be isolated, but a reasonable prediction is that the region of FEM-3 that binds *tra-2* protein products will also be unusually divergent in sequence. Such modification of the TRA-2A/B-FEM-3 interaction might allow the number of sperm made by hermaphrodites to be optimized to prevailing ecological conditions. The actual number of self-progeny produced by *C. elegans* and *C. briggsae* hermaphrodites does in fact differ (Fodor *et al.* 1983; Hodgkin and Barnes 1991).

Our data also suggest that *Cr-tra-2* produces a small transcript of ~1.6 kb encoding the cytoplasmic domain, similar to the 1.8-kb and 1.9-kb *C. elegans* mRNAs known to encode TRA-2B. It is unclear if these smaller transcripts are homologous in the strict sense, however. A striking similarity is the exact colinearity of the proteins encoded by the 1.6-kb *Cr-tra-2* mRNA and the two smaller *Ce-tra-2* transcripts. But the *Cr-tra-2* transcript appears to be transcribed from a different intron than either smaller *Ce-tra-2* mRNA, suggesting that TRA-2B-encoding transcripts may have evolved independently. We have, however, named the protein *Cr-tra-2*B by analogy. TRA-2B is oocyte specific in *C. elegans* and is thought

to promote oocyte differentiation and limit sperm production in hermaphrodites (Kuwabara *et al.* 1998), perhaps by binding and downregulating FEM-3 (Mehra *et al.* 1999). Interestingly, no TRA-2B-encoding transcript was detected in *C. briggsae* (Kuwabara 1996a), suggesting that hermaphroditic development can occur in its absence. This notion is supported by the relatively small effect produced by removing maternal *tra-2* function in *C. elegans* (Kuwabara *et al.* 1998). Since *C. remanei* females make no sperm, we surmise that the ancestral function of TRA-2B was either to promote oocyte development or to act maternally to promote female development in XX progeny.

Potential roles for *tra-2* in female *vs.* hermaphrodite development: Given the overall similarity of *Cr-tra-2* structure and function to its homologues in androdioecious species, three possible scenarios can be envisioned for its role in the evolution of germline sex determination in Caenorhabditis. One is that *tra-2* in fact is regulated in the same way regardless of mating system. If true, this implies that the MX and translational controls on *tra-2* do not exist primarily to control hermaphrodite germ cell differentiation as suggested previously (Kuwabara 1996a; Jan *et al.* 1997). Instead, both controls may instead be more general—necessary for sexual mosaicism in the hermaphrodite germ line, but not the factors that actually drive it. This idea is also supported by the fact that *tra-2* and *fem-3* translational controls are exerted in the soma of both males and hermaphrodites (Goodwin *et al.* 1993; Jan *et al.* 1997; Gallegos *et al.* 1998), even though in somatic tissues their removal causes little or no sexual transformation. We suggest that the hermaphrodite germline may simply be more sensitive to their loss.

A second possibility is that the regulatory interactions required for hermaphrodite spermatogenesis are present in *C. remanei*, but differ quantitatively in their effects. One datum consistent with this model is the apparently weaker affinity of the DRF-like factor for the *Cr-tra-2* 3′ UTR than for the *Ce-tra-2* 3′ UTR (Figure 7). The *Cb-tra-2* 3′ UTR is known to bind a DRF homologue and repress translation *in vivo* to an extent similar to that seen in *Ce-tra-2* (Jan *et al.* 1997). This raises the possibility that the translational control may have originally evolved as a redundant mechanism in male sex determination and was later coopted for use in hermaphrodite germline development by strengthening its effect.

The third possibility is that a qualitative difference in *tra-2* regulation exists between hermaphrodites and females. For example, the factor that binds the *Cr-tra-2* 3′ UTR may not be capable of repressing translation. Or there may be additional factors in hermaphrodites that act in concert with a conserved RNA-binding protein to enhance translational repression. Indeed, a well-conserved homologue of GLD-1, which binds the *tra-2* DRE and represses translation in *C. elegans* (Jan *et al.* 1999), has been identified in *C. remanei* (Jones and Schedl 1995). In addition, the germline-specific *C. elegans* protein FOG-2 is required for the onset of spermatogenesis and binds GLD-1 (Schedl and Kimble 1988; T. Schedl, personal communication). FOG-2 is thus a good candidate for a factor that might distinguish germline sex determination of hermaphrodites and females, as previously suggested (Schedl and Kimble 1988). Regulation of the MX domain might also be distinct, but little is currently known about its role in controlling hermaphrodite spermatogenesis.

***tra-2*, Caenorhabditis phylogeny, and the evolution of mating system:** Of the seven very closely related Caenorhabditis species that form the Elegans group (see Sudhaus and Kiontke 1996), only *elegans* and *briggsae* are androdioecious. The sister group to the Elegans group, composed of *perrieri* and *craspedocerca*, also produces hermaphrodites, but all other described Caenorhabditis species are gonochoristic. The Elegans group species are nearly identical in morphology, and molecular sequences thus far exist for only four of them. Further, at least two of the species may be synonymous with others in the group (Sudhaus and Kiontke 1996; S. Baird, personal communication). Consequently, most relationships within the group are not yet known and it is therefore not clear for an individual Elegans group species whether its mating system is a derived or ancestral condition. A clade of the two androdioecious species would suggest that hermaphroditism is a shared derived character. However, if a gonochoristic species such as *C. remanei* were instead grouped with one of the androdioecious species, with the other as an outgroup, then it would be equally parsimonious to postulate either independent gains of hermaphroditism by *elegans* and *briggsae* or a single gain in the common ancestor of all three species, followed by the reversion to dioecy in *remanei*. Neither of the two previous attempts to determine the exact phylogeny of *elegans*, *briggsae*, and *remanei* have suggested an all-hermaphrodite clade. Both a *briggsae*/*remanei* clade (Fitch *et al.* 1995) and a *remanei*/*elegans* clade (Baldwin *et al.* 1997) have been proposed, albeit with weak statistical support in both cases. On the basis of the latter result, Sudhaus and Kiontke (1996) suggested that androdioecy may have evolved three times in Caenorhabditis, once in the *perrieri*/*craspedocerca* clade and twice in the Elegans group, with *elegans* and *briggsae* thus representing independent acquisitions. However, the hypothesis of Fitch *et al.* (1995) would fit this scenario just as well.

As shown in Table 1, all analyzed features of the *tra-2* sequences indicate that *Cr-tra-2* and *Cb-tra-2* are more similar to each other than either is to *Ce-tra-2*. This would be most consistent with a clade of *C. briggsae* plus *C. remanei*, the proposal of Fitch *et al.* (1995). This pairing is also supported by the unique ability of *briggsae* and *remanei* to produce viable hybrid progeny (Baird *et al.* 1992). But in the absence of a fourth outgroup sequence, a rigorous phylogenetic analysis using charac-

ter-state methods (parsimony or maximum likelihood) cannot be performed. Simplified distance methods using the assumption of midpoint rooting produced trees from the *tra-2* amino acid sequence with all three possible topologies, depending upon the correction for multiple hits and the tree-building algorithm employed (not shown). Thus until an outgroup sequence is available, *tra-2* is of little utility for resolving Caenorhabditis phylogeny.

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