Evolution of Sex Determination in *Caenorhabditis*: Unusually High Divergence of *tra-1* and Its Functional Consequences

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ABSTRACT

The *tra-1* gene is a terminal regulator of somatic sex in *Caenorhabditis elegans*: high *tra-1* activity elicits female development, low *tra-1* activity elicits male development. To investigate the function and evolution of *tra-1*, we examined the *tra-1* gene from the closely related nematode *C. briggsae*. *Ce-tra-1* and *Cb-tra-1* are unusually divergent. Each gene generates two transcripts, but only one of these is present in both species. This common transcript encodes TRA-1A, which shows only 44% amino acid identity between the species, a figure much lower than that for previously compared genes. A *Cb-tra-1* transgene rescues many tissues of *tra-1*(null) mutants of *C. elegans* but not the somatic gonad or germ line. This transgene also causes nongonadal feminization of XO animals, indicating incorrect sexual regulation. Alignment of Ce-TRA-1A and Cb-TRA-1A defines several conserved regions likely to be important for *tra-1* function. The phenotypic differences between *Ce-tra-1*(null) mutants rescued by *Cb-tra-1* transgenes and wild-type *C. elegans* indicate significant divergence of regulatory regions. These molecular and functional studies suggest that evolution of sex determination in nematodes is rapid and genetically complex.

THE sex-determining mechanisms of mammals, *Drosophila* and *Caenorhabditis* are among the best understood developmental processes (VILLENEUVE and MEYER 1990; STEINMANN-ZWICKY *et al.* 1990; GOODFELLOW and LOVELL-BADGE 1992; KUWABARA and KIMBLE 1992; MCKEOWN and MADIGAN 1992). Although sex determination is an almost universal feature of animals, these mechanisms appear to bear no similarity at the molecular level (HODGKIN 1990, 1992; PARKHURST and MENEELY 1994). In contrast, important features of pattern formation are strikingly conserved across these three animal groups (MANAK and SCOTT 1994), suggesting that sex determination has undergone considerable divergence (HODGKIN 1992). Interspecific comparison of the sex-determining gene *SRY* from different mammals (TUCKER and LUNDRIGAN 1993; WHITFIELD *et al.* 1993) and of *transformer* from various *Drosophila* species (O'NEILL and BELOTE 1992) support this notion: both these genes show unusually high evolutionary divergence.

Classical studies also provide evidence for rapid changes in sex-determining mechanisms (BULL 1983). For example different insect species, sometimes closely related, may use XY systems, monofactorial "autosomal" systems that do not involve heteromorphic chromosomes, maternal effects, X chromosome dosage, or haplo-diploidy to determine sex. Among Rhabditidae, the family of nematodes to which *C. elegans* belongs, sex determination in the germ line is clearly evolving.

Within the genus *Caenorhabditis*, for example, *C. elegans* and *C. briggsae* have male and hermaphrodite sexes, while *C. vulgaris* and *C. remanei* have male and female sexes (SUDHAUS 1974; BAIRD *et al.* 1992, 1994).

Studies of the evolution of sex determination in *Caenorhabditis* have only just begun (KUWABARA and SHAH 1994). However, the extensive genetic studies of sex determination in *C. elegans*, and the observed differences in germ-line sexual development in different species of *Caenorhabditis* make this process well suited to address questions about the evolution of development. What is the genetic complexity underlying evolutionary changes in development? What are the molecular mechanisms by which developmental processes evolve? To begin to answer these questions, we have functionally compared the terminal sex determining gene *tra-1* from two species of *Caenorhabditis*. As well as addressing evolutionary questions, this analysis was expected to reveal conserved and therefore important features of the *tra-1* gene.

The sexes of *C. elegans* are the male (XO) and the hermaphrodite (XX). The hermaphrodite is essentially a female with a germ line that produces sperm before oogenesis: the sperm can be used for self-fertilization but not for cross insemination. By contrast true females in male/female species of *Caenorhabditis* do not make sperm. Sexual dimorphism in *C. elegans* is extensive: at least 30% of somatic cells in hermaphrodites (XX), and 40% of somatic cells in males (XO) are sexually specialized (HODGKIN 1988; VILLENEUVE and MEYER 1990). In each of these cells, choice of sexual fate is regulated by *tra-1*: high *tra-1* activity elicits female development, while low *tra-1* activity elicits male development (HODG-

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KIN 1987). In addition, *tra-1* is an important regulator of germ line sex (HODGKIN 1987; SCHEDL *et al.* 1989; DE BONO *et al.* 1995). Mosaic analysis has shown that *tra-1* acts cell-autonomously to determine sexual fate in all somatic tissues examined, with the expected exception of the vulva (HUNTER and WOOD 1990).

The *tra-1* gene has been extensively studied at the molecular level (ZARKOWER and HODGKIN 1992, 1993; HODGKIN 1993; ZARKOWER *et al.* 1994; DE BONO *et al.* 1995). It produces two mRNAs of 5 and 1.5 kb by alternative RNA processing (ZARKOWER and HODGKIN 1992). The 5-kb RNA encodes TRA-1A, a protein with five zinc fingers, which binds DNA *in vitro* and is likely to act as a transcriptional regulator (ZARKOWER and HODGKIN 1993). The 1.5-kb RNA encodes TRA-1B, a carboxyl terminally truncated version of TRA-1A with only two zinc fingers. TRA-1B has no appreciable DNA binding activity *in vitro* (ZARKOWER and HODGKIN 1993). Molecular analyses of *tra-1* mutants indicate that TRA-1A is necessary for *tra-1* function but have not established a role for TRA-1B (ZARKOWER and HODGKIN 1992).

Although sex-determining mechanisms are not conserved across phyla, *tra-1* has homologues in both flies and mammals. More specifically, the five zinc fingers encoded by *tra-1* are unusually highly conserved in the GLI family of proteins. This family includes the segment polarity gene *cubitus interruptus Dominant* (ORENIC *et al.* 1990) and the pair-rule gene *odd-paired* (BENEDYK *et al.* 1994), both from *Drosophila*, the human oncogene GLI (KINZLER *et al.* 1988), and the developmental regulator GLI3 (RUPPERT *et al.* 1990), which is implicated in human limb and craniofacial development (VORTKAMP *et al.* 1991). With the exception of *tra-1*, none of these genes is thought to play a role in sex determination, and their homology to *tra-1* does not appear to extend outside the zinc fingers.

In this work, we describe a functional homologue of *Ce-tra-1* from *C. briggsae*. Although *Ce-tra-1* and *Cb-tra-1* show unusually high evolutionary divergence, a *Cb-tra-1* transgene restores female somatic development to many tissues in *Ce-tra-1(null)* mutants. However, the *Cb-tra-1* transgene shows important differences from endogenous *Ce-tra-1*, which suggest divergence of regulatory regions of the two genes. Comparison of *Ce-tra-1* and *Cb-tra-1* reveals that only the transcript encoding TRA-1A is present in both species. This protein is therefore likely to mediate *tra-1* function. Alignment of *Ce-TRA-1A* and *Cb-TRA-1A* delineates several conserved regions likely to be important for *tra-1* to direct female somatic development.

MATERIALS AND METHODS

Nematode handling: *C. elegans* (N2, Bristol) and *C. briggsae* (var. Gujarat; FODOR *et al.* 1983) were grown and maintained using standard techniques (SULSTON and HODGKIN 1988). Genetic nomenclature is according to HORVITZ *et al.* (1979).

Southern analysis: *C. briggsae* genomic DNA was isolated following protocols devised for *C. elegans* (SULSTON and HODGKIN 1988). Molecular biological techniques were as described in SAMBROOK *et al.* (1989). Southern blotting of *C. briggsae* DNA was carried out at 50° in aqueous solution (6× SSC, 5× Denhardt's, 0.5% SDS, 100 µg/ml denatured salmon sperm DNA) with washing at the same temperature in 2× SSC, 0.1% SDS. Two probes were used: both were generated from *Ce-tra-1* cDNA. The GF region probe extends from 180 to 830 bp, and the zinc finger probe from 855 to 1500 bp of the *Ce-tra-1* cDNA sequence (ZARKOWER and HODGKIN 1992).

***C. briggsae* genomic libraries:** The *C. briggsae* genomic DNA phage library was a gift of T. SNUTCH and D. BAILLIE and was propagated on a C600 *Escherichia coli* host. The *C. briggsae* genomic DNA cosmid library used was a gift of J. COUCH and R. WATERSTON and was grown on XL1BlueMR. Protocols used to make plaque and colony lifts are described in SAMBROOK *et al.* (1989). Probes and hybridization conditions used for purifying phage and cosmid clones were identical to those used for Southern blotting *C. briggsae* genomic DNA.

Phage and cosmid analyses: Five recombinant phage carrying *tra-1* homologous DNA were purified and analysed by restriction digestions (SAMBROOK *et al.* 1989). All five phage isolates represented the same clone, designated CB#MDB1. The restriction digests were examined further by Southern blotting, using a probe extending from 180 to 1023 bp of the *Ce-tra-1* cDNA sequence. Hybridization was in aqueous solution at 62°; washings were in 2× SSC, 0.1% SDS at 62°. A 1.8-kb *XhoI* fragment was identified that contained most of the cross-hybridizing *tra-1* sequence and used to make a *Sau3A* library in pBSSK⁺ (Stratagene). Twenty clones from this library were sequenced.

Seventeen recombinant cosmids containing *tra-1* homologous sequences were purified. Cosmid DNA from each isolate was prepared using the Wizard miniprep kit (Promega). Restriction analyses showed that the 17 cosmid isolates represent two clones, designated CB#MDB2 (three isolates) and CB#MDB3 (14 isolates).

DNA sequencing: DNA sequencing followed established protocols, either using a Sequenase Version 2.0 kit (USB) or DyeDeoxy termination reactions (ABI) with base-specific fluorescent dyes as labels. Data analysis used Sequencer 2.1 software and the programs of R. STADEN (1987). The primers used for both types of sequencing reactions were M13 forward and reverse oligonucleotides, or custom made oligonucleotides.

Reverse transcription (RT) and RT-PCR analyses: *C. briggsae* cDNA was prepared from ~5 µg of either total RNA or poly(A)⁺ RNA isolated from a mixed stage *C. briggsae* population. RT reactions were in 20 µl and used AMV reverse transcriptase (HT) and random hexamers (Pharmacia). The 5' ends of *Cb-tra-1* cDNAs were generated by nested PCR of total *C. briggsae* cDNA using an SL1 trans-spliced leader oligonucleotide (MDB11) as the forward primer, and MDB9 and MDB10 as the reverse primers. This reaction generated two products that differ at their 5' end by 300 bp but are otherwise colinear. The longer cDNA encodes Cb-TRA-1A, the shorter cDNA Cb-TRA-1B. Sequence between zinc fingers 2 and 4 was amplified by PCR of total *C. briggsae* cDNA using primers MDB12 and DZ31. Annealing temperature for these reactions was 55°. The 3' end of *Cb-tra-1* cDNA, comprising 3.5 kb of sequence, was amplified using a modified 3' RACE protocol (INNIS *et al.* 1988). Poly(A)⁺ RNA from mixed stage *C. briggsae* was reverse transcribed using MDB35, a hybrid primer consisting of oligo dT and an adaptor sequence. The cDNA generated was amplified by PCR using MDB36, a primer corresponding to the adaptor sequence, and MDB34, a *Cb-tra-1* forward primer beginning at 1136 bp. These primers were designed to be unusu-

ally long to allow high annealing temperatures and consequently high specificity. Amplification was carried out using the TaKaRa LA PCR kit (Takara Shuzo Co. Ltd.). The PCR profile was 94°, 2 min, one cycle; 94°, 0.5 min, 68°, 4.5 min, 35 cycles. In all reactions described above, PCR products were cloned into Bluescript SK⁺ using restriction sites engineered in the primers and sequenced. Primers are listed below.

Primers: DZ31: AAACTCGAGAATGTGTACGATTCTGATG (*C. elegans* cDNA antisense strand beginning at 1154 bp); M13 forward primer: GTAAACGACGGCCAGT; M13 reverse primer: CAGGAAACAGCTATGAC; MDB9: CTCGAGCTCTGTAGATTGGACATGTTTCTC (*C. briggsae* cDNA antisense strand beginning at 972 bp); MDB10: TGCGAATTC-CAGCATACAATCTTTCCAACG (*C. briggsae* cDNA antisense strand beginning at 912 bp); MDB11(SL1): CGCTCGAGGGT-TTAATTACCCAAGTTTGAG; MDB12: GAGCTCGAGCATCATGCGCTGGCGCTGTGAGTG (*C. briggsae* cDNA sense strand beginning at 973 bp); MDB34: ATCTCGAGACGCATC-GTAGAACCCATACTGGCGAG (*C. briggsae* cDNA sense strand beginning at 1136 bp); MDB35: AAGAGCTCTGCA-GGGCCCGGATCCTCTAGATTTTTTTTTTTTTTTT (adaptor/oligo dT hybrid primer); MDB36: AAGAGCTCTGCAGGGCCCG-GATCCTCTAG (adaptor primer).

Restriction enzyme sites in the primers are underlined. The *C. briggsae* cDNA sequences encoding Cb-TRA-1A and Cb-TRA-1B can be obtained from GenBank (The *Cb-tra-1* sequence is available in GenBank, accession number U60649.). The location of primers and probes given is that in the cDNA sequence encoding Cb-TRA-1A.

Transcript analysis: Large quantities of various *C. briggsae* stages were collected as previously described (SULSTON and HODGKIN 1988). RNA preparation and blotting was as described by ZARKOWER and HODGKIN (1992), except that hybridization was at 42°, and washes were in 0.2× SSC, 0.1% SDS at 67°. Three probes were used in different Northern blot experiments to detect *Cb-tra-1* RNA. The first probe corresponded to 300 bp at the 5' end of the Cb-TRA-1A cDNA that is absent in the Cb-TRA-1B cDNA. This probe detected only the longer *Cb-tra-1* RNA. The second probe extended from 325 to 905 bp and the third probe from 1138 bp to the poly A tail of the cDNA encoding Cb-TRA-1A. The second and third probes both detected two RNA species of ~5 kb. To compare loadings, the Northern blot illustrated in Figure 1 was rehybridized at reduced stringency with a heterologous probe, *Ce-myosin-1* (MILLER *et al.* 1986); this indicated that the L1 lane contained substantially more poly(A)⁺ RNA than the other lanes (data not shown). Probes were made using the oligonucleotide labeling kit made by Amersham.

Transgenic analyses: To examine rescue by *Cb-tra-1* of *tra-1*(null) mutants, XX hermaphrodites of genotype *unc-32(e189) tra-1(e1834)/qC1* were coinjected with CB#MDB2 and the dominant transformation marker *rol-6(su1006)* using the method of MELLO *et al.* (1991). Four independent lines that carried *rol-6* on a heritable extra-chromosomal array were established: these lines were designated *eEx54*, *eEx55*, *eEx56* and *eEx57*. Rolling larvae of genotype *unc-32(e189) tra-1(e1834)/qC1*, which carried each of these arrays, were picked, and the sexual phenotype of their uncoordinated roller (Unc Rol) self-progeny was examined by Nomarski microscopy (SULSTON and HODGKIN 1988). The *qC1* chromosome suppresses recombination between *unc-32* and *tra-1*, so that all Unc animals should be homozygous for *tra-1(e1834)*.

To examine the phenotype of *tra-1*(+) XX animals containing *Cb-tra-1*, rolling L4 larvae carrying each of the four arrays were picked and their rolling self-progeny examined as young adults by Nomarski microscopy. These animals, which should almost entirely be XX, exhibited wild-type female somatic development. To examine the phenotypes of XO ani-

mals expressing *Cb-tra-1*, XX hermaphrodites carrying each of the four transgenic arrays were mated with N2 males under conditions that favored out-crossing. Rolling progeny, half of which should be XO, were inspected as young adults by Nomarski microscopy.

RESULTS

A homologue of *tra-1* in *C. briggsae*: To identify homologues of *C. elegans tra-1*, we performed low stringency Southern blotting on genomic DNA from *C. briggsae* with two adjacent nonoverlapping cDNA probes from *Ce-tra-1*. Both probes detected strongly hybridizing fragments, suggesting that *C. briggsae* contains a homologue of *tra-1* (designated *Cb-tra-1*). To isolate this putative homologue, phage and cosmid libraries of *C. briggsae* genomic DNA were screened with the same probes and under the same conditions used in the Southern blotting experiments (see MATERIALS AND METHODS). Clones that hybridized to both *Ce-tra-1* probes were purified, and their restriction patterns compared. All the phage isolates represent one clone, designated CB#MDB1, while the cosmid isolates represented two different but overlapping clones designated CB#MDB2 and CB#MDB3. The phage and cosmid clones derived from the same genomic region because they shared common restriction fragments. Southern blotting experiments of CB#MDB1 DNA identified a 1.8-kb *XhoI* fragment containing most of the sequences cross-hybridizing to a *Ce-tra-1* probe (see MATERIALS AND METHODS). Partial sequencing of this fragment identified a stretch of DNA that could encode a zinc finger similar to finger 1 encoded by *C. elegans tra-1* (see below).

The *tra-1* gene of *C. elegans* extends over more than 20 kb (HODGKIN 1993), however most of this sequence is intronic (ZARKOWER and HODGKIN 1992). To facilitate sequence analysis, *Cb-tra-1* cDNAs were isolated using reverse transcription PCR (RT-PCR) and sequenced (see MATERIALS AND METHODS). Primers designed from the cDNA sequence were then used to sequence genomic subclones of CB#MDB2. All of the *Cb-tra-1* genomic region was sequenced, with the exception of the large introns 2 and 4. The sizes of these introns were determined by PCR.

***C. elegans tra-1* and *C. briggsae tra-1* generate different sets of mRNAs:** The two RNAs generated from *Ce-tra-1* share a common 5' end that is spliced to the trans-spliced leader SL1 (ZARKOWER and HODGKIN 1992). RT-PCR studies using SL1 as the forward primer suggested that *Cb-tra-1* also generates two transcripts that are trans-spliced to SL1, but these differ at their 5' end by 300 bp (see MATERIALS AND METHODS). To investigate the transcript profile of *Cb-tra-1*, we performed Northern blot analysis of *C. briggsae* poly(A)⁺ mRNA prepared from embryos, the four larval stages and adults. Probes used to detect *Cb-tra-1* RNAs were made from various *Cb-tra-1* cDNA fragments (see MATERIALS AND METHODS). All probes except one detected two transcripts of

FIGURE 1.—Developmental profile of *tra-1* RNA expression in *C. elegans* and *C. briggsae*. (A) *C. elegans*. (B) *C. briggsae*. E, poly(A)+ RNA from embryos; L1, L2, L3, L4, poly(A)+ RNA from the first to fourth larval stages; A, poly(A)+ RNA from adults. Arrows indicate transcript size. Lanes in A are loaded with approximately equal amounts of RNA, but loading is uneven in B (see MATERIALS AND METHODS). Data for A is taken from ZARKOWER and HODGKIN (1992).

~5 kb that differ in size by ~300 bp (Figure 1). The exception was a probe made from the 5' 300 bp found only in the longer cDNA product generated by RT-PCR (MATERIALS AND METHODS, and data not shown). This probe detected the longer but not the shorter transcript. Thus the two RNAs detected by Northern analysis differ at the 5' end and probably correspond to the two cDNA types detected by RT-PCR. The predicted product of the longer RNA is designated Cb-TRA-1A, while the predicted product of the shorter transcript is designated Cb-TRA-1B. Cb-TRA-1B is an amino-terminally truncated version of Cb-TRA-1A (Figure 2).

Comparison of the transcription profiles of *Ce-tra-1* and *Cb-tra-1* revealed significant differences (Figure 1). First, the 1.5-kb *Ce-tra-1* mRNA, which encodes Ce-TRA-1B and is present in relative abundance during L1, L2, and L3 larval stages (ZARKOWER and HODGKIN 1992), has not been detected in *C. briggsae*. Second, *C. briggsae*

has two large transcripts of ~5 kb; *C. elegans* has only one transcript of this size. The smaller *Cb-tra-1* transcript, which encodes Cb-TRA-1B is only found in *C. briggsae*. This transcript appears to be enriched in L3s, L4s and adults (Figure 1). The longer transcripts from *Cb-tra-1* and *Ce-tra-1* have the same 5' end and are similar in length: the proteins they encode, Ce-TRA-1A and Cb-TRA-1A, are colinear (see below). This transcript can be detected at all developmental stages in each species.

The two *Ce-tra-1* RNAs result from alternative processing of intron 8 (ZARKOWER and HODGKIN 1992). Splicing across this intron generates the 5-kb RNA. The 1.5-kb RNA is produced if a polyadenylation signal present in this intron is used before the intron has been spliced out (Figure 2). To investigate the absence of the 1.5-kb *Ce-tra-1* RNA in *C. briggsae*, we compared *Ce-tra-1* intron 8 to the corresponding intron in *Cb-tra-1*, which is intron 6. Like other introns for which such comparisons have been made, this intron is highly diverged between *C. elegans* and *C. briggsae* (data not shown). Inspection of *Cb* intron 6 for the polyadenylation signal AAUAAA reveals two perfect matches (data not shown). The splice donor and acceptor sites of the two introns show no significant differences. Thus, the data available do not explain why the polyadenylation signal is used in *Ce-tra-1* but not in *Cb-tra-1*.

***Ce-tra-1* and *Cb-tra-1* are highly diverged:** The transcribed region of *Cb-tra-1* is substantially smaller than that of *Ce-tra-1*: the exons of *Ce-tra-1* span 23 kb, while the exons of *Cb-tra-1* span less than 15 kb (Figure 2). This disparity is due to differences in intron size and number: *Cb-tra-1* lacks five of the 17 introns present in *Ce-tra-1*, and with one exception, the introns in *Cb-tra-1* are smaller than their counterparts in *Ce-tra-1*. Previous interspecific comparisons (e.g., *ges-1*, KENNEDY *et al.* 1993; *mec-3*, XUE

FIGURE 2.—Comparison of the structure of the *Cb-tra-1* and *Ce-tra-1* transcripts. (A) *Cb-tra-1*. (B) *Ce-tra-1*. The larger transcript from *Ce-tra-1* is colinear with the larger transcript from *Cb-tra-1*: these transcripts encode Ce-TRA-1A and Cb-TRA-1A, respectively. The smaller *tra-1* transcript in each species is species-specific and is generated by distinct mechanisms in *C. elegans* and *C. briggsae* (see text). Cb-TRA-1B and Ce-TRA-1B are consequently different: Cb-TRA-1B is an amino-terminally truncated version of Cb-TRA-1A, while Ce-TRA-1B is a carboxy-terminally truncated version of Ce-TRA1A. The transcribed region of *Cb-tra-1* spans ~15 kb, while the same region of *Ce-tra-1* extends over more than 23 kb. Boxes represent *tra-1* exons, with open blocks for untranslated regions. The bottom line in each panel represents the *tra-1* genomic region, with *Eco*RI sites shown for *Ce-tra-1*, and *Xho*I sites shown for *Cb-tra-1*. For *Cb-tra-1* the bottom line also represents the extent of the cosmid clone CB#MDB2, which was used in transgenic studies (see Figure 5 and text). The *Ce-tra-1* data are from ZARKOWER and HODGKIN (1992).

FIGURE 3.—The 5' untranslated region of the longer *Ce-tra-1* transcript is conserved in its *C. briggsae* counterpart. Comparison of genomic sequence encoding the 5' end of the *tra-1* transcript that is present in both *C. elegans* and *C. briggsae*. The trans-splice site of the RNA is conserved in the two species. The 5' UTR contains a conserved stretch of sequence that could encode a short open reading frame of 10 amino acids. This open reading frame begins with the first initiation codon in the RNA. The initiation and termination codons of this short open reading frame are boxed. The complete nucleic acid sequence is shown only for *C. briggsae*. For *C. elegans* only those bases that differ from the *C. briggsae* sequence are given: residues identical in the two species are indicated by dashes. Dots represent deletions in the sequence relative to the other species. The splice acceptor site that accepts SL1 in the trans-splicing reaction is underlined. The trans-splice site itself is indicated by an arrowhead. Two tandem initiation codons at the start of the TRA-1A open reading frame are in bold.

et al. 1992) have also noted a smaller number of introns in *C. briggsae* genes relative to their *C. elegans* counterparts, suggesting that this may be generally true.

As described above, only one *tra-1* transcript appears to be conserved between *C. elegans* and *C. briggsae*. This transcript encodes Ce-TRA-1A in *C. elegans* and Cb-TRA-1A in *C. briggsae*. Figure 3 shows an alignment of genomic sequences that generate the 5' end of this transcript. The trans-splice site for the RNA is conserved between *Ce-tra-1* and *Cb-tra-1*, and the 5' untranslated region (5' UTR) shows 85% identity in the first 40 nucleotides following the trans-splice site. Within this conserved stretch is a short open reading frame of 10 amino acids that begins with the 5'-most AUG in both RNAs. The regulatory significance of this sequence, if any, is unknown. The two consecutive AUG triplets that are thought to represent the start site of translation for Ce-TRA-1A are conserved in *C. briggsae*. The 3' UTR of the conserved transcript has also been compared between *C. elegans* and *C. briggsae*. The alignment reveals several short stretches of conserved sequence (data not shown), but there is no extensive sequence identity.

The amino acid sequences of Ce-TRA-1A (1110 amino acids) and Cb-TRA-1A (1165 amino acids) are aligned in Figure 4. The two proteins are colinear and show 44% amino acid identity. The conservation predominantly lies in seven discrete blocks of high amino acid identity separated by highly diverged stretches that show less than 21% amino acid identity.

A *Cb-tra-1* transgene rescues many somatic tissues of *Ce-tra*(null) mutants, but does not rescue gonadal development: XX animals homozygous for *tra-1(e1834)*, a null mutation of *tra-1*, are invariably transformed into males and develop the anatomically complex tail used by males to copulate (Figure 5). To examine whether *Cb-tra-1* can function in *C. elegans* to direct female somatic development, we attempted to rescue the sex reversal of XX animals homozygous for *tra-1(e1834)* using the cosmid CB#MDB2. This genomic clone extends from 12 kb 5' of the trans-splice site for the RNA encoding Cb-TRA-1A to 4 kb 3' of the polyadenylation site of

both major *Cb-tra-1* RNAs (Figure 2). Four extrachromosomal arrays containing CB#MDB2, designated *eEx54*, *eEx55*, *eEx56* and *eEx57*, were generated, and the phenotypes of young adult *tra-1(e1834)* XX animals were transgenic for each array examined. Each array restored extensive female development to these animals, including a simple tail spike characteristic of hermaphrodites and yolk that is produced by the adult female intestine but is absent in males. However, none of the transgenic *tra-1(e1834)* animals showed signs of female gonadal development: the somatic gonads of such animals were indistinguishable from the somatic gonads of non-transgenic *tra-1(e1834)* animals (data not shown). In addition, the transgenic animals displayed no signs of vulval development, which requires induction by a female somatic gonad (KIMBLE 1981).

Wild-type *C. elegans* XO animals are feminized by transgenic *Cb-tra-1*: To investigate the effect of *Cb-tra-1* on the development of wild-type *C. elegans*, we examined the phenotypes of *tra-1(+)* XX and XO animals carrying each of the four *Cb-tra-1* extrachromosomal arrays generated. None of the arrays appeared to affect the sexual development of XX animals; however, each array feminized XO animals. The degree of XO feminization varied: three of the arrays, *eEx55*, *eEx56* and *eEx57*, almost completely transformed the male tail into a hermaphrodite tail spike (Figure 5), while *eEx54* caused more variable feminization, with some animals lacking the male-specific V-rays and fan, and others having some but not all the rays and a reduced fan. The intestine of XO animals was also feminized by the arrays and produced yolk. However none of the arrays feminized the somatic gonad of N2 XO animals, which developed into apparently wild-type one-armed male gonads producing large amounts of sperm and no signs of oogenesis. Consistent with the lack of gonadal feminization, the XO animals carrying the arrays did not exhibit vulval induction.

DISCUSSION

The overall conservation of gene organization and sequence shows that we have cloned the *C. briggsae* ho-

A

B

Conserved region	1	2	3	4	5	6	7
Position (amino acids)	1-35	79-105	162-208	227-385	386-455	473-537	724-800
% Amino Acid Identity	83 (29/35)	67 (18/27)	96 (45/47)	91 (144/159)	77 (54/70)	55 (36/65)	62 (48/77)
Function	Nuclear localization signal (?)	GF region: Sexual regulation of <i>tra-1</i>	Involved in DNA binding	zinc fingers; DNA binding	?	?	?

C

[illegible]

FIGURE 4.—Ce-TRA-1A and Cb-TRA-1A show only 44% amino acid identity. (A) Schematic diagram of TRA-1A that shows the location and extent of regions that are highly conserved between *C. elegans* and *C. briggsae*. (B) Description of the conserved regions in A. Position refers to the sequence alignment in C. Functions were assigned to regions 2, 3, and 4 from previous data (ZARKOWER and HODGKIN 1992, 1993; DE BONO *et al.* 1995). The proposed function of region 1 is based only on the presence of positively charged residues that are hallmarks of nuclear localization signals. (C) Alignment of Cb-TRA-1A and Ce-TRA-1A. The complete amino acid sequence is shown only for *C. briggsae*. For *C. elegans* only those amino acids that differ from the *C. briggsae* sequence are given: residues identical in the two species are indicated by dashes. Dots represent deletions in the sequence relative to the other species. The highly conserved regions described in A and B are boxed.

mologue of the *C. elegans* sex-determining gene *tra-1*. Transgene experiments also demonstrate that *Cb-tra-1* can partly substitute for *Ce-tra-1*, indicating conservation of function. Therefore, it is likely that *tra-1* plays a similar role in directing female development in both species, though this has not yet been directly demonstrated in *C. briggsae*.

The results described here shed light on two related questions concerning this nematode sex-determining gene. The first question is directed at analyzing in greater detail how *tra-1* regulates sex determination in *Caenorhabditis*. The second question is evolutionary, concerned with understanding how a major developmental switch gene like *tra-1* changes with time and speciation.

The absence of the 1.5-kb *Ce-tra-1* RNA in *C. briggsae* suggests that this short RNA may not have much functional importance, because it is not a conserved feature of the gene. The only RNA present in both *Caenorhabditis* species is that encoding TRA-1A, so it is possible that this protein is sufficient for *tra-1* activity. If the other RNAs generated from *Cb-tra-1* and *Ce-tra-1* do have a functional role, it is likely to be in regulating TRA-1A activity. Alignment of Ce-TRA-1A with Cb-TRA-1A delineates seven highly conserved regions interspersed with widely diverged stretches (Figure 4). Highly conserved regions are likely to have functional significance, particularly since *Cb-tra-1* restores female development to many tissues in *Ce-tra-1(null)* animals. This assumption is supported by func-

FIGURE 5.—The activity of *Cb-tra-1* in transgenic *C. elegans*. A–E show the tail region of *C. elegans* animals of various genotypes in the presence and absence of the *Cb-tra-1* transgene *eEx55*. (A) Wild-type XO animal. (B) Wild-type XX animal. (C) XX animal homozygous for a null mutation of *tra-1*, *tra-1* (*e1834*). (D) XX animal homozygous for *tra-1*(*e1834*) but transgenic for *Cb-tra-1*. (E) Wild-type XO animal transgenic for *Cb-tra-1*. The *Cb-tra-1* transgene restores female development to the tail region of XX animals mutant for *tra-1* and strongly feminizes the tail of wild-type XO animals. However *Cb-tra-1* appears to have no effect on the development of the somatic gonad and germ-line of wild-type XO animals and *tra-1*(null) mutant XX animals (data not shown).

tional data for the second, third and fourth conserved regions.

The second conserved region comprises the GF region (for Gain-of-Function region), which previous work has shown to be crucial for correct sexual regulation of *Ce-tra-1* (DE BONO *et al.* 1995). Nonconservative amino acid substitutions of any of eight residues in this region results in dominant feminization of *C. elegans* XO animals. These eight residues are conserved in *Cb-tra-1*, suggesting that the GF region retains its regulatory role in *C. briggsae*. However, many other residues in and around the GF region are not conserved. The significance of these differences is unclear: perhaps the unconserved residues do not affect sexual regulation of *tra-1* and are free to diverge. Alternatively the divergence could result from selective pressure acting on the sexual regulation of *tra-1* (see below).

The fourth conserved region corresponds to the five zinc fingers of TRA-1A, which are highly conserved in the GLI family of DNA-binding proteins. The pattern of conservation is characteristic of the GLI family: fingers 2–5 are almost identical, but finger 1 is diverged (ZARKOWER and HODGKIN 1993). This pattern agrees well with structural data of the five zinc fingers of GLI

bound to their DNA target site: in the GLI cocrystal, finger 1 does not contact DNA (PAVLETICH and PABO 1993). The divergence of finger 1 between *Ce-tra-1* and *Cb-tra-1* suggests that finger 1 is also unlikely to contribute to DNA-binding specificity *in vivo*.

In vitro DNA-binding studies have implicated a region upstream of the zinc fingers in mediating specific DNA contacts (ZARKOWER and HODGKIN 1993). This region lies at the position of the third block of sequence conserved between *Ce-tra-1* and *Cb-tra-1*. This correlation suggests that conserved region 3, which detects no homology in the database, may define a novel DNA-binding domain.

The significance for *tra-1* function of the first, fifth, sixth and seventh blocks of conserved sequence is unclear. However, their function can now potentially be investigated by combining *in vitro* mutagenesis with transgenic studies. The first block of homology that extends over 30 amino acids includes a stretch of four or five basic residues in an eight-amino acid span. Such basic stretches are characteristic of nuclear localization signals (SILVER 1991), so this sequence may be part or all of a nuclear localization signal for TRA-1. This conserved region would be absent in Cb-TRA-1B. Blocks 5, 6, and 7 do not detect homologies in the database, although region 6 is glutamine rich, so it is conceivably involved in transcriptional activation. At least part of the carboxy-terminal half of TRA-1A downstream of conserved region 5 is important for *tra-1* function, because stop codons that truncate *Ce-TRA-1A* in conserved region 6 have strong *tra-1* loss-of-function phenotypes (ZARKOWER and HODGKIN 1992).

Despite the conserved regions, the overall amino acid sequence identity between *Ce-TRA-1A* and *Cb-TRA-1A* is only 44%. This is a far lower level of conservation than that observed for other proteins compared between *C. elegans* and *C. briggsae* (Table 1). The high divergence raises the question of the role of unconserved sequences in *tra-1* function. Could expression of other members of the GLI family (which only share the zinc fingers with TRA-1A) under the *tra-1* promoter direct female somatic development in *C. elegans tra-1*(null) mutants? This question and further studies of the structure/function relationship of *tra-1* will require transgenic studies of *in vitro* modified *tra-1* genes. No genomic clone that contains *tra-1* rescuing activity has previously been reported, partly due to the presence of sequences in *Ce-tra-1* that are very hard to maintain in *E. coli* (HODGKIN 1993). The cosmid CB#MDB2 should now permit these studies, by providing a backbone around which to construct a fully functional *tra-1* transgene.

The *Cb-tra-1* transgene restores female development to many lineages of *Ce-tra-1*(null) mutants, but it also displays important differences from endogenous wild-type *Ce-tra-1*. These differences may be due to regulatory changes and could provide insights into the ways

TABLE 1

Amino acid sequence identity between genes of *C. elegans* and their homologues in other nematode species

Gene	Species	Identity (%)	Homology
<i>cal-1</i>	<i>C. briggsae</i>	100	Calmodulin
<i>hsp-3</i>	<i>C. briggsae</i>	98	70-kD HS protein
<i>gpd-3</i>	<i>C. briggsae</i>	97	GAPDH
<i>unc-69</i>	<i>C. briggsae</i>	97	Unknown
<i>unc-50</i>	<i>C. briggsae</i>	96	Unknown
<i>gpd-2</i>	<i>C. briggsae</i>	95	GAPDH
<i>mec-3</i>	<i>C. vulgaris</i>	91	LIM, homeobox
<i>cyt-1</i>	<i>C. briggsae</i>	89	Cytochrome b ₅₆₀
<i>let-60</i>	<i>P. pacificus</i>	85	ras
<i>ced-3</i>	<i>C. briggsae</i>	85	ICE cysteine protease
<i>ges-1</i>	<i>C. briggsae</i>	83	Gut esterase
<i>lin-3</i>	<i>C. briggsae</i>	78	EGF growth factor
<i>ced-9</i>	<i>C. briggsae</i>	66	bcl-2
<i>tra-1</i>	<i>C. briggsae</i>	44	Zinc finger protein

References for these gene comparisons are as follows: *cal-1*, THOMAS and WILSON (1991); *hsp-3*, HESCHL and BAILLIE (1990); *gpd-2* and *gpd-3*, LEE *et al.* (1992); *unc-69* and *unc-50*, M. O. HENGARTNER, N. TSUNG and H. R. HORVITZ, personal communication; *mec-3*, WAY *et al.* (1991); *cyt-1* and *ced-9*, M. O. HENGARTNER and H. R. HORVITZ (1994); *let-60*, SOMMER *et al.* (1996); *ced-3*, YUAN *et al.* (1993); *lin-3*, P. TZOU and P. STERNBERG, personal communication; *tra-1*, this paper.

in which major developmental switch genes like *tra-1* evolve. First, the *Cb-tra-1* transgene feminizes wild-type *C. elegans* XO animals. Activity of *tra-1* is thought to be negatively regulated in XO animals by a protein-protein interaction (DE BONO *et al.* 1995). One explanation for why *Cb-tra-1* feminizes *C. elegans* XO animals is that high levels of expression of the *Cb-tra-1* transgene outcompetes the inhibitory protein. A second possibility is that *Cb-tra-1* is not efficiently repressed by the *Ce-tra-1* inhibitor due to divergence of its docking site on Cb-TRA-1. The GF region is thought to form part or all of this site in Ce-TRA-1, so changes in the GF region could explain why *Cb-tra-1* is not correctly sexually regulated. The two explanations given are not mutually exclusive. Evidence against the first hypothesis is that neither multiple copies of a *Ce-tra-1* transgene construct nor four copies of the endogenous *tra-1* gene feminize the soma of XO animals, suggesting that sexual differentiation is relatively insensitive to alterations in *tra-1* dose (D. ZARKOWER, M. DE BONO and J. HODGKIN, unpublished results; HODGKIN 1987 and unpublished data). Further studies of chimaeras between *Ce-tra-1* and *Cb-tra-1* should identify the sequence differences that underlie lack of sexual regulation of *Cb-tra-1* in *C. elegans*.

The second important functional difference between *Cb-tra-1* and *Ce-tra-1* is that *Cb-tra-1* does not restore any visible sign of female development to the somatic gonad in *C. elegans tra-1(null)* mutants. In contrast, a *Ce-tra-1* transgene construct, consisting of 7.5 kb of upstream sequence joined to cDNA sequences, restores female

somatic development and self-fertility to such animals (D. ZARKOWER, M. DE BONO and J. HODGKIN, unpublished data). The simplest explanation for this difference is that the *Cb-tra-1* transgene is expressed at levels that are too low to direct development of a female somatic gonad in *C. elegans*, perhaps due to changes in the *tra-1* promoter. The unusually high divergence of *tra-1* sequence, the differences in *tra-1* RNA profiles and the observation that transgenic *Ce-tra-1* and *Cb-tra-1* exhibit functional differences suggest that *tra-1* is evolving unusually rapidly. The *SRY* gene in mammals (WHITFIELD *et al.* 1993; TUCKER and LUNDRIGAN 1993) and *transformer* in *Drosophila* (O'NEILL and BELOTE 1992) also diverge unusually rapidly, suggesting that rapid evolution may be characteristic of sex-determining genes in many animal groups. The evolutionary changes in *tra-1* could reflect either a lack of functional constraint or species-specific adaptive divergence by positive selection. In mammals, the rapid evolution of the sex-determining gene *SRY* appears to be due to positive selection (TUCKER and LUNDRIGAN 1993; WHITFIELD *et al.* 1993), but there is as yet insufficient data to draw the same conclusion for *tra-1*. In conclusion, the observations described here together with those described in the accompanying paper (KUWABARA 1996) suggest that sex determination in *Caenorhabditis* may provide a good model system to study how major developmental switch genes evolve.

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