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The *tra-3* sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family

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The *Caenorhabditis elegans* sex determination gene *tra-3* is required for the correct sexual development of the soma and germ line in hermaphrodites, while being fully dispensable in males. Genetic analysis of *tra-3* has suggested that its product may act as a potentiator of another sex determination gene, *tra-2*. Molecular analysis reported here reveals that the predicted *tra-3* gene product is a member of the calpain family of calcium-regulated cytosolic proteases, though it lacks the calcium binding regulatory domain. Calpains are regulatory processing proteases, exhibiting marked substrate specificity, and mutations in the p94 isoform underlie the human hereditary condition limb-girdle muscular dystrophy type 2A. The molecular identity of TRA-3 is consistent with previous genetic analysis which suggested that *tra-3* plays a very selective modulatory role and is required in very small amounts. Based on these observations and new genetic data, we suggest a refinement of the position of *tra-3* within the sex determination cascade and discuss possible mechanisms of action for the TRA-3 protein.

Keywords: calpain/*C.elegans*/proteolysis/sex determination/*tra-3*

Introduction

The two naturally occurring sexes in *Caenorhabditis elegans* are the hermaphrodite, which has two X chromosomes, and the male, which has one X chromosome. The choice between these alternative developmental fates is normally governed by the ratio of the X chromosome dose to the number of sets of autosomes (the X:A ratio) (Madl and Herman, 1979). Many mutants defective in implementing the correct mode of sexual development have been isolated, as well as mutants affected in both sex determination and the related process of sex chromosome dosage compensation, and these have been ordered into a pathway of epistatic relationships (for reviews, see Hodgkin, 1988; Villeneuve and Meyer, 1990). This pathway is one of the most extensively analysed regulatory pathways in eukaryotic development, and serves as a model for the regulation of a developmental switch. Seven genes define the main sex determination pathway, a cascade of negative interactions, which acts to control the sexual fate of both the soma and the germ line (Hodgkin, 1988; Villeneuve and Meyer, 1990). Molecular and genetic analyses have suggested that this cascade begins and ends with transcriptional control by the products of the genes *sdc-1*, *sdc-3* and *tra-1* (Nonet and Meyer, 1991; Trent *et al.*, 1991; Zarkower and Hodgkin, 1992; Klein and Meyer, 1993), but has steps in the middle that entail protein–protein interactions and communication between cells, involving the gene products of *her-1*, *tra-2*, *fem-1*, *fem-2* and *fem-3* (Spence *et al.*, 1990; Ahringer *et al.*, 1992; Hunter and Wood, 1992; Kuwabara *et al.*, 1992; Perry *et al.*, 1993; Kuwabara and Kimble, 1995; Pilgrim *et al.*, 1995) (see Figure 4) Specifically, HER-1 has been proposed to be a small, secreted paracrine or endocrine factor whose candidate receptor would be TRA-2A, the larger product of the *tra-2* locus (Kuwabara and Kimble, 1992; Kuwabara *et al.*, 1992; Perry *et al.*, 1993). The carboxy-terminal domain of TRA-2A is proposed to be cytosolic and contain an effector domain, whose function would be to sequester or inhibit at least one of the products of the three *fem* genes (the FEM proteins) (Kuwabara and Kimble, 1992, 1995; Kuwabara *et al.*, 1992), one of which (*fem-2*) encodes a protein phosphatase (Pilgrim *et al.*, 1995).

tra-3 appears to act at the same step in the sex determination cascade as *tra-2* and, from its genetic properties, it has been postulated that *tra-3* is a potentiator of *tra-2* (Hodgkin and Brenner, 1977; Hodgkin, 1980) (see Figure 4). Furthermore, it appears that very little TRA-3 protein is required in hermaphrodites for wild-type function (Hodgkin and Brenner, 1977; Kimble *et al.*, 1982; Hodgkin, 1985; Kondo *et al.*, 1988). The only requirement for the *tra-3* gene is in hermaphrodite sex determination, since *tra-3(null) XO* males are perfectly normal, and because viable male/female lines can be established which are homozygous for *tra-3(null)* mutations (Hodgkin, 1986). In *XX tra-3* mutants, however, both the soma and germ line are abnormal, and these animals are intersexual and sterile (Hodgkin and Brenner, 1977; Hodgkin, 1985). It has also been proposed that *tra-3* could have a role in fine-tuning the hermaphrodite brood size (Hodgkin and Barnes, 1991). To characterize *tra-3* function further, we have undertaken molecular and genetic analysis of this gene, which we report below.

Results

Cloning the tra-3 gene

We positioned *tra-3* within a region encompassing >3000 kb by positional cloning. Since this was begun when the physical map of the *C.elegans* genome (Coulson *et al.*, 1986, 1988, 1991) was much more fragmentary than at present, this first required assembling the physical map in this region (Figure 1A). A series of restriction fragment length polymorphisms (RFLPs) were mapped

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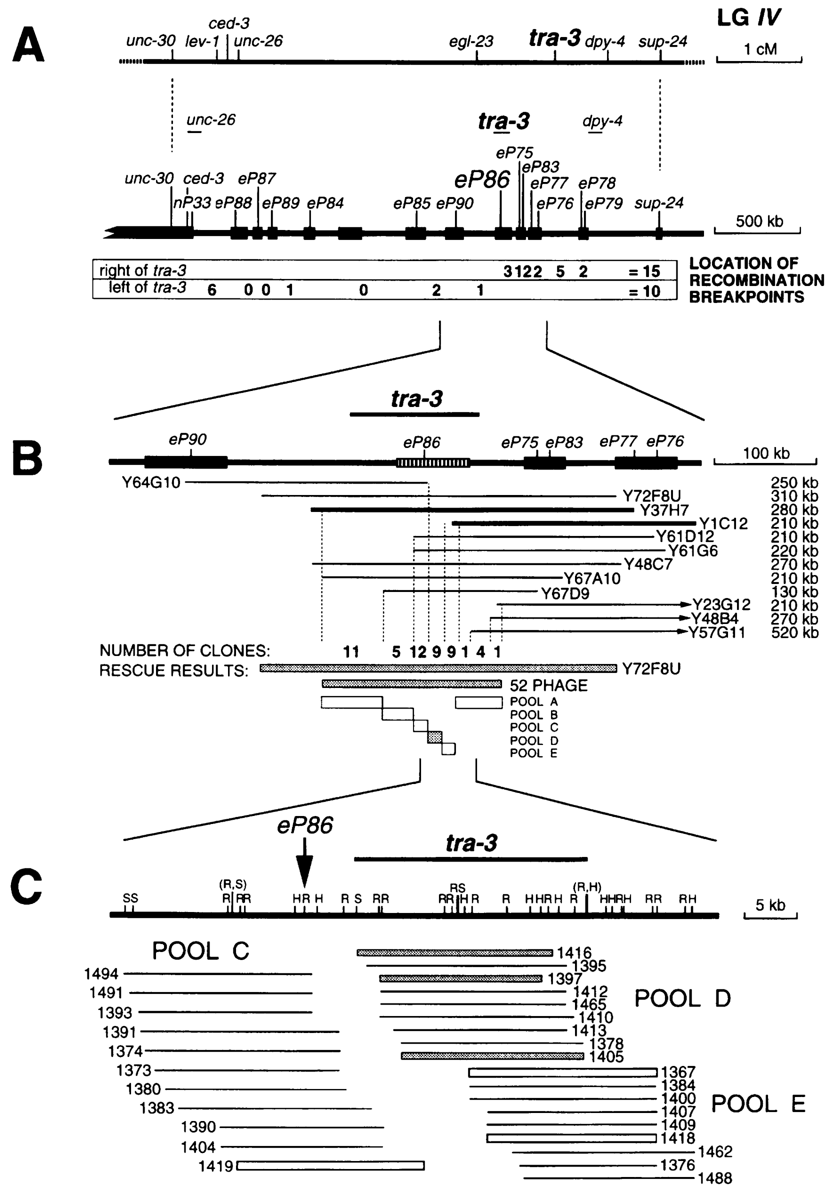

Fig. 1. Positional cloning of *tra-3*. (**A**) The upper part shows the genetic map in the region of *tra-3* (Barnes, 1991) on linkage group IV. The lower part shows the assembled physical map around *tra-3* (Barnes, 1991) and the results of RFLP analysis. The thicker line indicates regions covered by cosmids and YACs; the thinner line indicates regions covered only by YACs. RFLPs (with respect to the N2 background) were identified in the strain RW7000 (see Materials and methods), and recombinants between N2 and RW7000 were selected in the *unc-26* to *tra-3* and *tra-3* to *dpy-4* intervals and scored for these polymorphisms (see Materials and methods). The results are shown under the physical map. All 10 recombination breakpoints in the *unc-26* to *tra-3* interval occurred between *nP33* and *eP86*, and all 15 breakpoints in the *tra-3* to *dpy-4* interval occurred between *eP86* and *eP79*. The inferred locations of *unc-26*, *dpy-4* and *tra-3* are thus shown above the physical map. (**B**) Above the physical map [drawn as in (A)] is shown the estimated location of *tra-3* from the RFLP analysis. Underneath, the YACs that bridge this region are shown (extents are approximate). Based on the estimated location of *tra-3*, we screened for λ clones from the region covered by the left half of Y37H7 (see Materials and methods). One such clone, CB#1380, was used to find *eP86*. We obtained a final tally of 52 clones, which were sorted into classes by hybridization to each of the YACs shown. Fingerprint analysis (Coulson *et al.*, 1986) of these λ clones revealed five unpositioned cosmids covering the same region, which could be merged into one contig (striped box). At the bottom of (B) the location of *tra-3(+)* activity, as determined by transformation rescue (see Materials and methods), is shown by shading. Rescuing activity resided only in pool D. CB#1380 (detecting *eP86*) is located in pool C. (**C**) The position of *tra-3* inferred from (B) is shown. The restriction map was derived from the phage and checked by genomic Southern (R = *Eco*RI; H = *Hin*dIII; S = *Sac*I). *eP86* was found to be the loss of the indicated *Eco*RI site. Individual phage clones tested for rescue are drawn as boxes. Those which did rescue are shaded. In particular, injections with CB#1405 (lacking the last two exons of *tra-3*) resulted in one line which fully complemented *e1107am*.

relative to *tra-3*, and one of these, *eP86*, remained unseparated from this gene (Figure 1B; and see Materials and methods). To confirm this location, the 310 kb yeast artificial chromosome (YAC) Y72F8U was shown by microinjection (Fire, 1985; Mello *et al.*, 1991; see Materials and methods) to be able to complement a *tra-3* amber mutant. At the time, there were no known cosmids in the region likely to contain *tra-3*, so two YACs were used to select clones encompassing the predicted *tra-3* location from a λ2001 genomic library (see Figure 1B). These clones were sorted into groups based on their positions within the *tra-3* region, and one group able to rescue *tra-3* mutants was identified (Figure 1B). Restriction mapping and injection of individual clones further

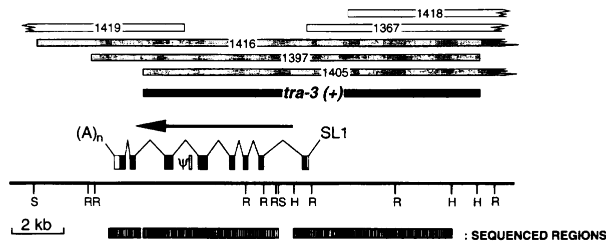

Fig. 2. Genomic organization of *tra-3*. At the top is shown the 13.5 kb *tra-3(+)* region defined by phage rescue (drawn as in Figure 1C). The gene organization inferred from genomic and cDNA sequencing is shown (see Materials and methods). The *tra-3* sequence has been submitted to GenBank under the accession nos U12516, U12920 and U12921. The arrow indicates the direction of transcription, and the boxes indicate exons. The 3′ end of the sequence reported here matches the 3′ sequence of a random *C.elegans* cDNA clone, yk15g5 (accession no. D27625). Unfilled regions are untranslated. The positions of SL1 and poly(A) addition are shown. The small shaded box indicates a 'pseudoexon' (marked Ψ). This is a near-perfect direct repeat of the last 58 bp of exon 5 in intron 5, 236 bp further downstream. Three of the differences would be silent changes in the pseudoexon, while another has GAG (Glu) in the pseudoexon for GATGATCGAGCT (AspAspArgAla$_{387}$) in exon 5, nominally preserving the frame. The repeat also partially extends into the splice donor sequence, but then the similarity breaks down so that this splice site is unlikely to be efficient. Furthermore, there is no appropriate splice acceptor sequence upstream of the pseudoexon, and RT–PCR experiments indicate that it is not found in wild-type hermaphrodite mRNA (data not shown).

restricted *tra-3(+)* activity to the 13.5 kb overlap between CB#1405 and CB#1397 (Figure 1C). Subsequent analysis revealed that the *tra-3* gene extended outside this minimal rescuing region. To assist the identification of the *tra-3* gene, we sequenced most of the 13.5 kb rescuing region, as well as an additional 1.2 kb adjacent to the rescuing region (Figure 2).

Organization of the tra-3 gene

DNA sequence analysis predicted one gene that would occupy the majority of this sequence. The predicted translation product of this gene shows extensive similarity to the large subunit of a family of vertebrate and invertebrate calcium-regulated proteases, the calpains (Figure 3). The identity of this gene was confirmed by identifying TGG→TAG(amber) mutations in the DNA from two of the three known amber *tra-3* alleles (Figure 3). The predicted intron–exon structure of the gene was confirmed by sequencing PCR-generated cDNA (Frohman *et al.*, 1988). No evidence for alternative splicing was found. Upstream of the proposed initiator methionine, there is a sequence resembling an unpaired splice acceptor, typical of *trans*-spliced genes (Blumenthal, 1995). *trans*-splicing to SL1, but not SL2 (Blumenthal, 1995), was confirmed by RT–PCR (our unpublished data). The *tra-3* gene spans some 8 kb, and is predicted to encode an mRNA of 2242 bases [excluding the poly(A) tail] in eight exons (Figure 2). This gene lacks 50 bp size class introns (Blumenthal and Thomas, 1988) typical of most other *C.elegans* genes, although the significance of this is unclear. The assignment of the initiator methionine codon is straightforward, as there is only one candidate towards the 5′ end of the mRNA in any reading frame and this is exactly preceded by an in-frame stop codon. The 3′-untranslated region (UTR) contains a consensus polyadenylation signal 14 bases upstream of the site of poly(A) addition.

tra-3 encodes a member of the calpain protein family

The predicted TRA-3 protein is a moderately diverged member of the calpain large subunit family (Figure 3), with a predicted M_r of 73.6 kDa, and a predicted pI of 7.5, consistent with a cytosolic location and typical of calpains (Ohno *et al.*, 1984; Sorimachi and Suzuki, 1992). TRA-3 has similarity to calpain large subunits throughout calpain domains I–III (Figure 3). In particular, the three residues in the proteolytic domain II implicated in catalysis (Arthur *et al.*, 1995; Berti and Storer, 1995) are conserved (Figure 3). This similarity is likely to reflect functional conservation for several reasons. First, the percentage of residues of TRA-3 identical or similar to rat calpain p94 is comparable with the same values comparing the biochemically active *Schistosoma* calpain and rat p94 (see Figure 3 legend). This similarity is concentrated in residues which tend to be conserved in all calpains. For example, 63% of the residues identical between TRA-3 and rat p94 in domain II are common to all calpains. Secondly, the proteolytic domain of calpain exhibits sequence similarity to members of the papain family of cysteine proteases near the three residues implicated in catalysis (Berti and Storer, 1995). These similarities involve highly conserved tryptophan residues, which from crystallographic analyses of actinidin, cathepsin B and papain are important for establishing the hydrophobic core of the protease (Berti and Storer, 1995). These similarities are also found in TRA-3. Finally, a number of calpain genes first identified only on the basis of DNA similarity were subsequently shown to encode proteolytically active enzymes (Emori *et al.*, 1986; Sorimachi *et al.*, 1989, 1993). In addition to these similarities, TRA-3 contains a number of distinct features (Figure 3): domain I, of unknown function, is the shortest so far reported in TRA-3; there are various small insertions in domains II and III, with one prominent insertion of ~14 residues between the His and Asn residues of the active site; the strongly favoured sequence –GCSI(D,E)–, found just before the His active site residue, is divergent in TRA-3; and an 'acid blob' found in other calpains in domain III, is not present in TRA-3. The significance of these distinctions is currently unclear, but the extended protein similarity and similar general organization strongly suggest that TRA-3 is a biologically active protease with properties in common with calpains.

At the beginning of the calmodulin-like calcium binding domain IV, the TRA-3 sequence diverges from other calpains, while extending for another 147 residues, which we propose naming domain T. Domain T is encoded by the last two exons of *tra-3*, perhaps suggesting that these exons have been substituted for a canonical calpain domain IV-coding gene segment (Miyake *et al.*, 1986). Alternatively, domain T may have diverged by drift from a canonical domain IV, because it is compositionally similar and because the carboxy-terminus of domain T resembles calpain carboxy-termini (Figure 3), which are implicated in enzymatic activity (Imajoh *et al.*, 1987). In either case, since the last two exons lie outside the minimal rescuing region (Figure 2), they are not critical for function in TRA-3. Furthermore, PROSITE (release 13.1) does not predict any EF hand-type calcium binding domain in domain T. On the other hand, a peptide spanning the boundary of domains II and III from *Schistosoma* calpain has been shown to bind

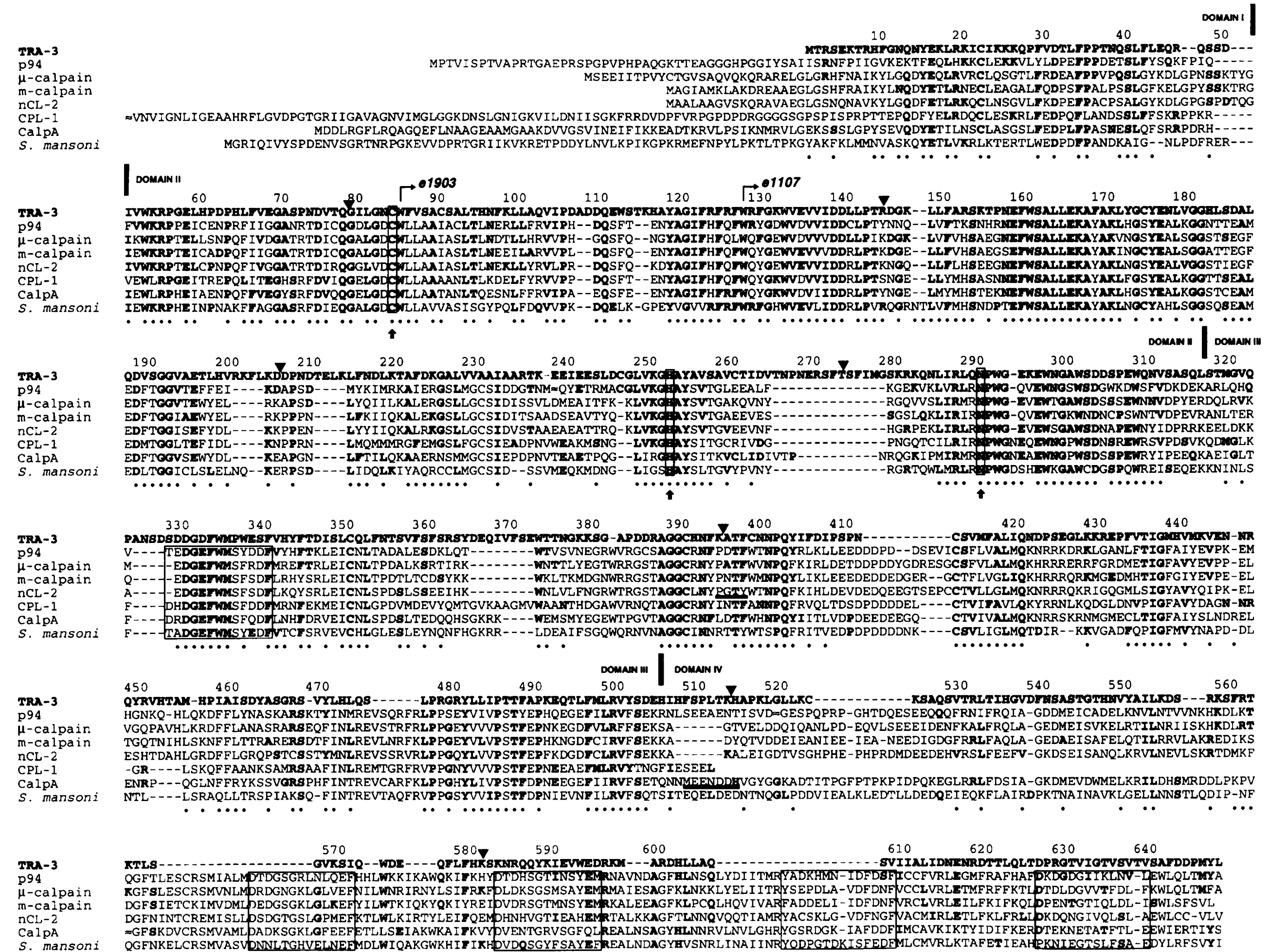

Fig. 3. TRA-3 is a member of the calpain large subunit family. TRA-3 has been aligned with representatives of the four different classes of rat large subunit, and three invertebrate calpains, from *Schistosoma*, *Drosophila* and CPL-1 (for calpain-like large subunit) from *C.elegans*. The sequence of CPL-1 corresponds to C06G4.2 (accession no. L25598) but has been corrected based on matching cDNA sequences for the carboxy-terminus and on protein similarity for a missed exon. In particular, the sequences of random cDNAs from CPL-1 (accession Nos D32353 and D34510) confirm the absence of a domain IV in this protein. nCL-2 and CalpA have alternatively spliced forms; in nCL-2, instead of ...PGTY... (underlined) in domain III, there is ...PGSSZ (Sorimachi *et al.*, 1993), while in CalpA, instead of ...MEENDDH... (underlined) at the end of domain III, there is ...MERTSRQZ (Theopold *et al.*, 1995). Residues identical to TRA-3 are in bold, and dots underneath the alignment indicate positions where the majority of residues are similar to TRA-3 residues. Unique insertions in some sequences have been removed (marked by ≈) for compactness. These constitute 110 residues from the amino-terminus of CPL-1, 77 residues from domain IV of CalpA and 48 and 47 residues respectively for IS1 and IS2 (Sorimachi and Suzuki, 1992) of p94. The positions of the domain boundaries are indicated. The residues of the catalytic triad (C, H, N) in domain II are boxed and marked with an upward arrow. The proposed EF hand (CaM-like) sequences in domain IV (Ohno *et al.*, 1984) and the additional proposed EF hand sequence in domain III (Andresen *et al.*, 1991) are also boxed. Not all of these boxed sequences are expected to bind calcium and, indeed, no EF hand is predicted in TRA-3 by PROSITE release 13.1. The residues affected in *e1903am* and *e1107am* are indicated. Each is a transition changing a TGG (Trp) to TAG (amber). Arrowheads show *tra-3* intron positions. The similarity of domains I, II and III of TRA-3 to those of rat p94 are 78, 56 and 56% respectively (30, 39 and 27% identical respectively). For the *Schistosoma* calpain and rat p94, the corresponding values are 50, 48 and 65% similarity and 22, 35 and 40% identity respectively. The alignment of the last domain of TRA-3 with domain IV is not intended to imply significance. The accession numbers of the sequences used in the alignment are: p94, J05121; μ-calpain, X04366; m-calpain, L09120; nCL-2, D14478; *S.mansoni*, M74233; and CalpA, Z46891.

calcium (Andresen *et al.*, 1991), and this region is well conserved in TRA-3 and other calpains (Figure 3). Thus not all calcium regulation may be lost in TRA-3.

tra-3 expression may not be regulated by the sex determination pathway

In the current biochemical model for sex determination in *C.elegans* (see Figure 4), HER-1 and TRA-2A proteins physically interact outside of the cell in males (Kuwabara and Kimble, 1992; Kuwabara *et al.*, 1992; Perry *et al.*, 1993). In this model, it seems unlikely that there would be any regulation of *tra-3* by *her-1*. Instead, it seems more likely that *tra-3* activity is present in both *XX* and *XO* animals, although serving no function in the latter. However, the possibility remains that *tra-3* activity might respond to X chromosome dosage in some other way, so that it would be down-regulated in *XO* animals. In order to test this possibility, genetic experiments were carried out to ask whether *tra-3* is expressed in an *XO* germ line and can provide the same degree of maternal rescue as is afforded by an *XX* germ line.

Three different kinds of *tra-3/+ XO* hermaphrodite or female animals were generated, using either *her-1* loss-of-function *(lf)*, *fem-1(lf)* or *tra-1* gain-of-function *(gf)*

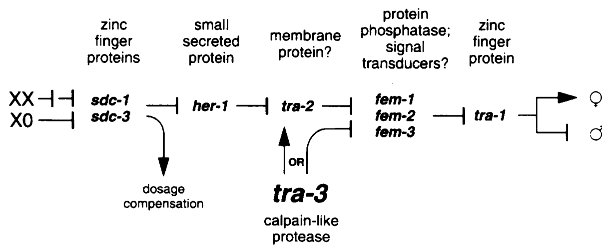

Fig. 4. Models for the action of TRA-3 in the *C.elegans* sex determination pathway. Some of the genes in the *C.elegans* sex determination and dosage compensation epistasis pathway and the nature of their products are shown (Spence *et al.*, 1990; Nonet and Meyer, 1991; Ahringer *et al.*, 1992; Kuwabara *et al.*, 1992; Zarkower and Hodgkin, 1992; Klein and Meyer, 1993; Perry *et al.*, 1993; Pilgrim *et al.*, 1995). Arrows and bars denote positive and negative regulation, respectively. *tra-3* has traditionally been drawn at the same step as *tra-2*, although it has been argued to be a potentiator of *tra-2* function (Hodgkin and Brenner, 1977; Hodgkin, 1980, 1988). However, from the data presented here, and the molecular and genetic data concerning other genes in the pathway, we propose that either TRA-3 activates *tra-2* function directly, or it inactivates the FEM function in a *tra-2*-dependent fashion (see text). In neither of these models does *her-1* directly regulate *tra-3*.

mutations (see Materials and methods). These were crossed with *tra-3 XO* males, to generate *tra-3/tra-3 XX* progeny. In each of the three cases, normal self-fertile hermaphrodites of this genotype were obtained, which showed no sign of masculinization, showing that the *tra-3(+)* allele in the *XO* maternal parent had been active, and had contributed enough *tra-3* product to the *XX* progeny to support normal hermaphrodite development. *her-1* is believed to act upstream of *tra-2* and *tra-3*, while *fem-1* and *tra-1* appear to act downstream of these genes, but in all cases it appears that *tra-3* has remained active in an *XO* female germ line. This suggests that *tra-3* activity is not subject to regulation by the sex determination system. One caveat to this conclusion is that feedback effects might lead to higher *tra-3* activity during oogenesis, independently of X chromosome dosage, as may be the case with *tra-2* (Okkema and Kimble, 1991), but the simpler interpretation is that *tra-3* activity is not sexually regulated (Figure 4).

Discussion

Here we have shown that the *C.elegans tra-3* gene encodes a member of the calpain gene family, but lacks the regulatory calcium binding domain. Furthermore, we suggest a refinement of the standard *C.elegans* sex determination cascade which would place *tra-3* outside of direct *her-1* control.

How do these observations help us understand calpain function? Vertebrate calpains are biochemically defined calcium-activated cytosolic cysteine proteases, comprising an 80–94 kDa catalytic subunit (Melloni and Pontremoli, 1989; Goll *et al.*, 1992; Sorimachi *et al.*, 1994). Four isoforms are known, two ubiquitously expressed (μ- and m-calpain) and two tissue-specific (p94 and nCL-2) (Kawashima *et al.*, 1988; Sorimachi *et al.*, 1989, 1993). The two ubiquitous forms associate non-covalently with a common 30 kDa subunit (Suzuki *et al.*, 1987), which is encoded by a separate gene (Emori *et al.*, 1986). This small subunit has a carboxy-terminal calcium binding domain related to calmodulin (CaM) that is highly similar to the corresponding domain of the large subunit, both by sequence (Emori *et al.*, 1986) and intron placement (Miyake *et al.*, 1986), and the two subunits associate through this region (Nishimura and Goll, 1991). This association prevents calpain activation until calcium binding releases the subunits (Goll *et al.*, 1992). Small subunit association has not been detected for the tissue-specific isoforms (Sorimachi *et al.*, 1989, 1993), but a *Schistosoma* calpain co-purifies with a 28 kDa protein (Siddiqui *et al.*, 1993). TRA-3 lacks domain IV, which suggests that it would be unable to interact with a small subunit, and hence could be monomeric and potentially constitutively active. Loss of domain IV has been noted in some other calpains: alternative splicing of a human stomach-specific calpain gene produces a transcript lacking domain IV and most of domain III (Sorimachi *et al.*, 1993) and, similarly, in *Drosophila*, alternative splicing of the *CalpA* gene produces both full-length and domain IV-lacking transcripts (Theopold *et al.*, 1995) (Figure 3). These may also be monomeric and constitutive. Furthermore, in *C.elegans*, several other calpain-like large subunit genes have been identified by genomic and cDNA sequencing (Waterston *et al.*, 1992; Wilson *et al.*, 1994), at least one of which lacks any CaM-like domain at its carboxy-termini (see Figure 3 and legend). In summary, the structure of the human stomach, the insect and the nematode proteins suggests that calpain large subunits can function independently of a major regulatory domain.

What effect might this have on the enzyme? Many calpain targets defined *in vitro* contain CaM binding sites, and it has been suggested recently that calpains associate with these substrates via domain IV binding to the CaM binding site of the substrate (Molinari *et al.*, 1995). Thus the potential problem posed to the cell by such unregulated proteases may be limited by the narrowing of substrate specificity. Indeed, in the case of TRA-3, there are not likely to be many *in vivo* substrates, as strong *lf* alleles do not reveal any defects other than in hermaphrodite sex determination (Hodgkin, 1988; Villeneuve and Meyer, 1990). A similar specificity may thus exist for other calpains lacking domain IV. Other calpain regulators have been described, including activator proteins (Michetti *et al.*, 1991; Shiba *et al.*, 1992) and, most notably, calpastatin, a specific, endogenous calcium-dependent inhibitor (Goll *et al.*, 1992). The interaction with calpastatin has been studied in detail, but reports conflict over which domain (III or IV) the interaction is specific to (Nishimura and Goll, 1991; Croall and McGrody, 1994). Thus it remains currently unclear whether calpastatin regulation would be intact in isoforms such as TRA-3 that lack domain IV.

How do the results presented in this paper help us understand *tra-3* function? Calpains are processing proteases, cleaving a substrate at only a very limited number of sites, capable of causing either activation or inactivation (for review, see Goll *et al.*, 1992). Possible substrates include transmembrane and nuclear hormone receptors, cytoskeletal components, and key regulatory enzymes; however, most substrates have only been defined *in vitro* (Goll *et al.*, 1992). Calpains have been found in all mammalian tissues examined and, in addition to the involvement of p94 in human limb-girdle muscular

dystrophy type 2A (Richard *et al.*, 1995), other isoforms may be involved in medically important processes as diverse as neuronal death in Alzheimer's disease, muscle wastage in Duchenne muscular dystrophy, acquisition of long-term potentiation, and others (Kay, 1984; Denny *et al.*, 1990; Saito *et al.*, 1993). Given this diversity, what might the TRA-3 substrate(s) be? The small requirement for TRA-3 is consistent with both a catalytic role and action on one of the other regulatory proteins in the sex determination cascade. Both TRA-2 and FEM-1 have features of known calpain substrates. Specifically, the TRA-2A carboxy-terminal domain is rich in protein degradation (PEST) sequences (Kuwabara *et al.*, 1992), thought to be a target of calpains (Rechsteiner, 1990), while FEM-1 contains ANK repeats (Spence *et al.*, 1990), at the boundary of which μ-calpain cleaves ankyrin (Boivin *et al.*, 1990). Two basic models can be imagined. Since calpain cleavage can activate or inactivate a substrate (Goll *et al.*, 1992), in one model TRA-3 would activate TRA-2A, and thus promote the sequestration or inactivation of FEM proteins, while in another model TRA-3 would inactivate a FEM protein (Figure 4). In the latter model, since *tra-2* and *tra-3* are not genetically additive in their effects on sex determination (Hodgkin and Brenner, 1977; Hodgkin, 1980, 1986), TRA-2 and TRA-3 would not act independently on the FEMs. In both models, TRA-2A is proposed to be active and able to bind and inactivate the FEM proteins without TRA-3, but less efficiently than in its presence. This would be consistent with *tra-2(gf)* mutants, which overexpress TRA-2, being largely *tra-3* independent (Doniach, 1986). Both models are consistent with TRA-3 acting at or near the membrane, which is believed to be a major site of action of vertebrate calpains (Goll *et al.*, 1992). In neither model would *her-1* directly regulate *tra-3* in males, consistent with the genetic data presented here.

It is also possible that *tra-3* affects the activity of other sex determination genes indirectly. Goodwin *et al.* (1993) have presented evidence that a region of the *tra-2* 3′-UTR is a target for negative regulation at the translational level, by some unknown regulator. Conceivably, TRA-3 might act to increase levels of TRA-2A by inactivating this translational inhibitor. However, there is some evidence against this possibility. The strongest *tra-2(gf)* mutation, *e2020*, carries a deletion of the whole 3′ regulatory region, yet does not suppress *tra-3* mutations (Doniach, 1986) completely, which is contrary to the expectations of a simple translational model.

A role for proteolysis in sex determination in *C.elegans* had not been suspected previously. Intracellular proteolytic events, however, are increasing in prominence as important regulators of cell fate, e.g. the role of the interleukin-1β converting enzyme (ICE) and its relatives in activating programmed cell death (Kumar, 1995). We show that TRA-3, a new member of the calpain protease family, is implicated in another major cell fate decision, that of sex. TRA-3 appears to be a strikingly specialized molecule, as its genetic function is entirely dispensable in males and is needed only for correct hermaphrodite sex determination. It will be of interest to see whether such specialization *in vivo* will be a hallmark of calpains in general.

Materials and methods

General methods and libraries

Standard cloning procedures (Ausubel *et al.*, 1992) and nematode culture methods (Brenner, 1974; Sulston and Hodgkin, 1988) were used unless specified. The YAC, cosmid and λ genomic libraries and their screening have been described previously (Coulson *et al.*, 1986, 1988, 1991). For primary λ genomic library screening, phage were plated on CES200 (*recBCsbcB*) (Wyman and Wertman, 1987; Hodgkin, 1993). Samples of individual YAC DNAs were obtained from the LMB archive (Coulson *et al.*, 1988). λ minilibraries from Y48B4 and Y23G12 were prepared by J.Sulston from material excised from pulsed field gels (PFGs): *Sau*3A I-partially digested YAC DNA was cloned into the *Bam*HI site of λ2001.

RFLP mapping

The wild strain RW7000 [Bergerac] is known to contain many RFLPs with respect to the reference wild strain, N2 [Bristol] (Emmons *et al.*, 1979). From *unc-26 tra-3 dpy-4* [Bristol]/ + + + [Bergerac] animals, 10 recombinants between *unc-26* and *tra-3* and 15 recombinants between *tra-3* and *dpy-4* were selected, and DNA was isolated from homozygous descendants. RFLPs were identified using whole cosmids from the *tra-3* region as probes on Southerns of N2 and RW7000 genomic DNA pairs digested with various enzymes. For the polymorphisms identified in this work, the probe–enzyme combinations that detect them, and the fragment sizes (kb) in N2/RW7000 are, respectively: *eP75* T27E7–*Bgl*II, 13.7–18; *eP76*, F13G11–*Eco*RI, 2–4; *eP77*, F13G11–*Bgl*II, 4.5–8.8; *eP78*, F52D11–*Eco*RI, 5.5–5.2; *eP79*, F52D11–*Xba*I, 13 and 6–19 and 4.5; *eP83*, pRA1 (a *Hin*dIII subclone of T27E7)–*Hin*dIII, 3.5–3.4; *eP84*, F56F12–*Hin*dIII, 4.7–5.2; *eP85*, F52B11–*Hin*dIII, 3.1–3.3; *eP86*, CB#1380–*Eco*RI, 6.0 and 3.4–9; *eP87*, F52G2–*Hin*dIII, 15 and 9–12; *eP88*, W02A2–*Eco*RI, 2.4–2.3; *eP89*, T04H5–*Eco*RI, 3.6–3.7; *eP90*, K01G12–*Eco*RI, 6.0–6.0 and 5.8. *nP33* is known to lie close to *unc-26* (Yuan *et al.*, 1993). The *sup-24* clone, which also detects a polymorphism (Barnes, 1991), lies 0.6 cM right of *dpy-4*.

Obtaining genomic clones under the YAC Y72F8U

Since at the time there were no known cosmid clones in the *tra-3* region, we employed a general strategy to saturate with λ clones an area bridged only by YACs (Barnes, 1991). Phage minilibraries made from YAC DNA excised from PFGs were problematic in that there was a high degree of chimaerism, plus contamination from yeast genomic 2 μm circular DNA (Barnes, 1990). Thus we screened a 9-fold redundant primary λ genomic library with Y37H7 and, at the same time, negatively screened with a Y1C12 which covered the right half of Y37H7 (these YACs are drawn as thicker lines). The YAC and λ library vectors do not cross-hybridize (Coulson *et al.*, 1988). We obtained 52 clones that survived rescreening.

Transformation rescue of tra-3

Microinjection of worms was as described (Fire, 1985; Mello *et al.*, 1991), using 20 μg/ml pRF4 [carrying *rol-6(su1006dm)*] as a co-injection marker in some cases. Injected animals were maternally rescued (i.e. phenotypically wild-type) *tra-3(e1107am)* homozygotes derived from either *e1107am/nT1 unc(n754dm) let (IV); +/nT1 (V)* (Fire, 1985) or the *e1107am; eEx24* generated in this work (see below). Each λ clone was injected at 2 μg/ml whether used singly or in a pool. YAC DNA for rescue was excised from a PFG of low melting temperature agarose and then purified by extraction with phenol using standard methods (Ausubel *et al.*, 1992). This procedure however had <10% efficiency. YAC DNA was injected at a concentration of 3 μg/ml. Transformation rescue had not been attempted previously with a clone the size of Y72F8U (310 kb), but worked adequately in this case. We obtained three partially rescued individuals. A permanent line was obtained from the pool of all 52 phage clones. Five subpools of the 52 clones were prepared and tested. The extrachromosomal array from a permanent line from pool D (see Figure 1C) was dubbed *eEx24*.

DNA sequencing

Two subclones of the rescuing region were shotgun sequenced (Bankier *et al.*, 1987): the 6.1 kb *Hin*dIII fragment, and a 5.4 kb fragment from the left end of CB#1405 to the first *Sal*I site. Sonicated shotgun libraries in M13mp18 were kindly prepared by J.Sulston. All of the sequence was determined on both strands, except for some intronic regions which were determined on one strand only. For the last two exons, both strands of a PCR-generated cDNA (Frohman *et al.*, 1988) were sequenced by thermal cycling (Craxton, 1991). For the corresponding genomic

sequence, DNA was amplified by PCR from CB#1416 and sequenced. There were no discrepancies between any cDNA and genomic sequences. To find the lesions in *e1903* and *e1107*, selected regions encompassing Trp codons were amplified by PCR from homozygous DNA, and then sequenced by thermal cycling (Craxton, 1991).

Tests for tra-3 expression in XO females

Three kinds of fertile *XO* hermaphrodites and females were generated, using either homozygous *her-1(lf)*, homozygous *fem-1(lf)* or heterozygous *tra-1(gf)*. In each case, *tra-3/+ XO* mothers were crossed with *tra-3 XO* fathers, to test whether the maternal *tra-3(+)* allele is able to rescue homozygous *tra-3 XX* progeny. If rescue occurs, normal *XX* hermaphrodites with a *tra-3/tra-3* genotype should be produced. In the absence of maternal rescue, *XX* progeny with the distinctive Tra-3 pseudomale phenotype would have been seen. For all crosses, the strong non-amber *tra-3* allele *e1767* was used. For the *her-1* and *fem-1* crosses, the sex-linked recessive marker *unc-7(e5)* was used to distinguish *XO* progeny from *XX* progeny.

For *her-1* (using the strong *lf* allele *e1518*), *dpy-1; her-1; unc-7 XX* hermaphrodites were crossed with *tra-3; her-1/+ XO* males, to yield *dpy-1/+; tra-3/+; her-1/her-1; unc-7/O XO* Unc non-Dpy hermaphrodite progeny. These were crossed with *tra-3 XO* males. No Tra-3 *XX* progeny were seen, but two out of six self-fertile *XX* hermaphrodite progeny produced pure Tra-3 broods, indicating homozygosity for *tra-3*.

For *fem-1* (using the strong *lf* allele *e1965*), *fem-1 tra-3 dpy-4; unc-7 XX* females were crossed with *fem-1/+ XO* males, to generate *fem-1 tra-3 dpy-4/fem-1 + +; unc-7/O XO* Unc non-Dpy females. These were crossed with *tra-3 XO* males. No Tra-3 *XX* progeny were seen, but two out of four self-fertile *XX* hermaphrodite progeny from this cross produced pure Tra-3 broods.

For *tra-1(gf)* (using the strong dominant *gf* allele *e1575*), *tra-1(gf)/+; tra-3 dpy-4 XX* females were crossed with wild-type males, to yield *tra-1(gf)/+; tra-3 dpy-4/+ + XO* females (distinguishable from *XX* sisters by a truncated tail spike). These animals were crossed with *tra-3 dpy-4/tra-3 + XO* males. No Tra-3 *XX* progeny were seen, but five self-fertile *XX* hermaphrodites (one Dpy, four non-Dpy) were obtained, all of which produced pure Tra-3 broods.

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