ABSTRACT

Title of Document: ISOLATION AND CHARACTERIZATION OF CAENORHABDITIS BRIGGSAE TRA MUTANTS.

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In order to aid in the reconstruction of the *Caenorhabditis briggsae* sex determination pathway, for the purposes of studying the convergent evolution of hermaphroditism and mating system change in nematodes, *Cb-tra* mutants have been isolated through forward mutagenesis. Based on phenotype, genetic linkage, molecular linkage, and in some cases sequence analysis, the *C. briggsae* homologs of *tra-1*, *tra-2*, and possibly *tra-3* have been identified. Upon further characterization, the function of the *tra* genes during sex determination between *C. elegans* and *C. briggsae* appears to be largely conserved. However, notable differences, with respect to the role of *tra-1* and potentially the role of *tra-3*, have been observed between these two species. In addition, intra-species suppression of the putative *tra-3* mutant (AF16 background) by genomic variants in the wild-type strain HK104 suggests that molecular interactions underlying sex determination are changing between these two *C. briggsae* populations.

ISOLATION AND CHARACTERIZATION OF CAENORHABDITIS BRIGGSAE TRA MUTANTS.

By

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CHAPTER 1: INTRODUCTION

1.1. Diversity of Sex Determination Mechanisms Within the Metazoa.

Unlike many other developmental processes, such as anterior/posterior patterning and limb formation, sex determination varies greatly between different phyla (GILBERT 2003; reviewed by PANGANIBAN and RUBENSTEIN 2002; ZARKOWER 2001). Two forms of sex determination exist: environmental sex determination (ESD) and genetic sex determination (GSD). Although a great deal could be written describing the various examples of ESD and GSD, in the interest of brevity, only a few well-studied cases will be discussed.

ESD has been studied most extensively in reptiles and fish. In many cases, temperature is the environmental trigger; however, its effect on sex ratios varies between species (reviewed by BAROILLER and D'COTTA 2001; DEVLIN 2002; GILBERT 2003; WESTERN and SINCLAIR 2001). Several other environmental triggers, such as pH, density, and social factors, have also been shown to influence sex ratios in various fish species (reviewed by BAROILLER and D'COTTA 2001). For instance, in the coral reef fish, *Thalassoma duperrey,* the phenomenon of female-to-male sex reversal is visually stimulated and occurs in response to the ratio of large fish (males) to small fish (females) (ROSS *et al.* 1983).

In contrast to ESD, mammals and *Drosophila* employ a form of GSD in which females are XX and males are XY. However, the molecular mechanism underlying sex determination in these two groups is not identical. In mammals, the male dominant Y chromosome determines sex through the action of SRY (GRAVES 2002; KOOPMAN *et al.* 1991; SINCLAIR *et al.* 1990), while in *Drosophila*, the X to autosome

(X:A) ratio determines sex through the differential splicing of *dsx* (the Y chromosome is only needed for male fertility) (reviewed by SCHUTT and NOTHIGER 2000). In contrast to the XX/XY system, nematodes employ an XX/XO system in which hermaphrodites are XX and males are XO. Like *Drosophila*, sex determination in nematodes proceeds through an X:A ratio. However, unlike *Drosophila*, the nematode responds to the X:A ratio through a negative regulatory cascade which acts on *tra-1*, rendering it off in males and on in hermaphrodites (reviewed by GOODWIN and ELLIS 2002). In addition to the examples presented above, the sex determination mechanisms employed by hymenoptera and birds provides further diversity within GSD. In hymenoptera, one sex is haploid and the other sex is diploid (BEYE *et al.* 2003; reviewed by COOK 1993), while in birds, females are the heterogametic sex (ZW) and males are the homogametic sex (ZZ) (HORI *et al.* 2000).

1.2. Sex Determination Appears to be Homologous, but Rapidly Evolving.

Given the variety of mechanisms that have been shown to determine sex, it is no wonder that sex determination was once thought by many to have arisen multiple times during animal evolution. It has since been shown that the sex determination genes *dsx* in *D. melanogaster*, *mab-3* in *C. elegans*, and *DMRTI* in humans are all homologs of the DM (*doublesex-mab-3)* domain gene family (RAYMOND *et al.* 1998). The conservation of the DM domain proteins in male sex determination across the metazoa has fueled the notion that the various sex determination methods may be derived from a common ancestor, but rapidly evolving (MATSUDA *et al.* 2002; MILLER *et al.* 2003; ZARKOWER 2001). Therefore, sex determination is an ideal process in which to study the evolution of development. However, given the rapidity

with which sex determination evolves, comparisons between different phyla would not be as informative, with regards to evolutionary process, as comparisons between related species. Since nematodes are closely related species, and because they utilize different mating systems, these animals were considered in the hunt for suitable organisms in which to study the evolution of sex determination.

1.3. Phylogeny of Nematodes and Mating System Arrangement.

Androdioecy (males and hermaphrodites) and gonochorism (males and females) are the two mating system types employed in the genus *Caenorhabditis*. Recent phylogenetic analysis (CHO *et al.* 2004; KIONTKE *et al.* 2004) has clarified the relationships between several of these taxa (Fig. 1). The results clearly indicate that

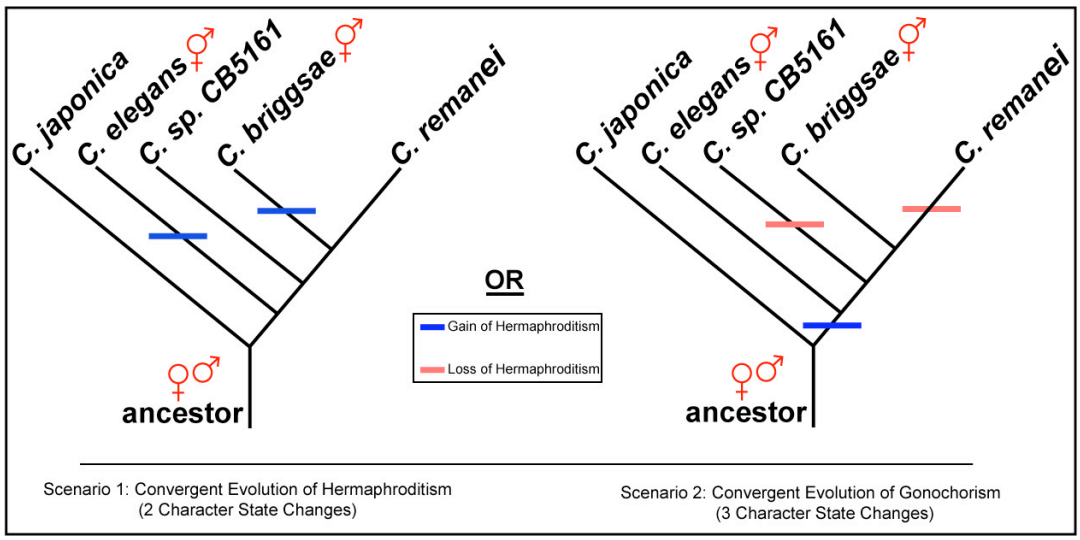

Figure 1. Phylogenetic relationships within the Elegans group of the genus *Caenorhabditis*. The tree above is based on sequence analysis of the following nuclear genes: large and small subunit rRNA-encoding DNA (rDNA), RNA polymerase II (a.k.a. *ama-1*), *par-6*, and *pkc-3* (KIONTKE *et al.* 2004) and the proteins FOG-1 and FOG-3 (CHO *et al.* 2004). The branch positions of *C. elegans* and *C. briggsae* suggests convergent evolution of hermaphroditism in these two lineages, based on parsimony.

C. briggsae and *C. remanei* are the two closest related species that utilize different mating systems. Based on parsimony, the tree also suggests that the evolution of

hermaphroditism in *C. elegans* and *C. briggsae* could be convergent. Therefore, the study of the evolution of sex determination using this group of organisms is ideal, due to the presence of different mating systems and their phylogenetic arrangement within the clade. Fortunately, hermaphroditism has been extensively explored in *C. elegans*.

1.4. The Sex Determination Pathway of *C. elegans*.

As mentioned previously, sex determination in *C. elegans* is a negative regulatory cascade of various interactions induced by the X:A ratio (Fig. 2). In the

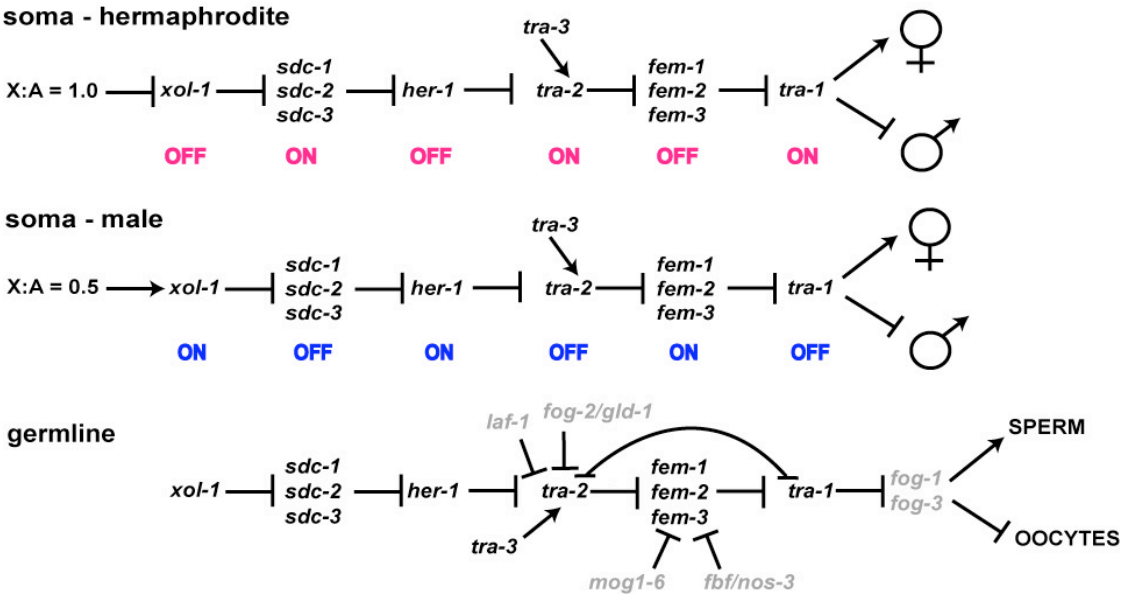

Figure 2. The sex determination pathway of *C. elegans*. The first two panels represent somatic sex determination in hermaphrodites and males, while the lower panel shows hermaphrodite germline modifications necessary to produce sperm and eggs. Genes required only for germline sex determination are shown in grey, and all but *fog-1* and *fog-3* are hermaphrodite specific.

soma, XOL-1 is transcriptionally repressed in response to an X:A ratio of 1, while an X:A ratio of 0.5 promotes the transcription of XOL-1 (reviewed by MEYER 2000). Low levels of XOL-1 indirectly promotes the production of hermaphrodites through the transcriptional inhibition of the ligand HER-1, while high levels of XOL-1 indirectly promotes the production of males by allowing expression of HER-1

(HODGKIN 1980; reviewed by MEYER 2000; RHIND *et al.* 1995; TRENT *et al.* 1991). In the absence HER-1, TRA-2, in conjunction with TRA-3, is capable of repressing the FEM proteins, which allows the transcription factor TRA-1 to directly activate female development and repress male development (reviewed by GOODWIN and ELLIS 2002). In contrast, following HER-1 binding, TRA-2 is incapable of inhibiting the FEM proteins, which leads to the repression of TRA-1 and activation of male fates (reviewed by GOODWIN and ELLIS 2002; HAMAOKA *et al.* 2004; KUWABARA 1996b).

Since *C. elegans* hermaphrodites are essentially females that undergo spermatogenesis prior to oogenesis, the germline must transiently down-regulate TRA-1 in order to produce a limited quantity of sperm before oogenesis (reviewed in GOODWIN and ELLIS 2002). This is essentially accomplished through the post-transcriptional regulation of the *tra-2* and *fem-3* mRNAs via their 3' UTR sequences and a TRA-2/TRA-1 protein interaction (Fig. 2). In order to produce sperm, the *tra-2* message is translationally repressed, through the action of *laf-1* and FOG-2/GLD-1, in order to prevent the production of the TRA-2 protein (CLIFFORD *et al.* 2000; JAN *et al.* 1999). In addition a TRA-2/TRA-1 protein interaction is necessary for sperm production in hermaphrodites (LUM *et al.* 2000; WANG and KIMBLE 2001). Following spermatogenesis, the translation of *fem-3* mRNA is repressed through the action of MOG 1-6 and through the binding of FBF/NOS-3 to the *fem-3* 3' UTR (GALLEGOS *et al.* 1998; KRAEMER *et al.* 1999; ZHANG *et al.* 1997). Consequently, oogenesis begins.

1.5. Comparison of Nematode Sex Determination Pathways.

In addition to the nematode phylogeny presented above (Fig. 1) (CHO *et al.* 2004; KIONTKE *et al.* 2004), several lines of evidence support the hypothesis that *C. elegans* and *C. briggsae* have evolved hermaphroditism independently. These include the lack of a *C. briggsae fog-2* ortholog (NAYAK *et al.* 2005), the female promoting role of *Cb-gld-1*, and the self-fertility of *Cb-fem* mutants (R. Hill, pers. comm.). However, in order to fully investigate this hypothesis, reconstruction of the *C. briggsae* sex determination pathway must be performed in order to accurately compare the two pathways. In addition to providing insight into the evolutionary mechanisms underlying convergent evolution, discovery of the sex determination mechanism in *C. briggsae* will create a foundation for comparison with its sister species, *C. remanei*, in the eventual study of mating system evolution. Therefore, the isolation and characterization of *Cb-tra* mutants is the focus of this thesis, due to the necessity of such mutants in the eventual reconstruction of the *C. briggsae* sex determination pathway.

CHAPTER 2: METHODS

2.1 Nematode Husbandry

General nematode propagation and storage procedures outlined by Brenner (1974) were employed to maintain all wild type (AF16 and HK104) and mutant *C. briggsae* strains utilized in this study. Incubations were carried out between 15°C and 25°C, unless the temperature is specifically stated.

2.2 F_2 EMS Tra Screens

Late L4 – early adult *C. briggsae* AF16 hermaphrodites were placed in a 0.05M ethylmethanesulfonate (EMS) M9 solution and incubated at room temperature (RT) for 4 hours. Following mutagenesis, worms were washed with M9 and transferred to small NGM plates (20-40 worms per plate) for selfing (incubation at 25°C was used for temperature sensitive screens). The resultant F_1 L4 hermaphrodites were transferred to new NGM plates and allowed to self (250 plates with 2 worms per plate = 1000 haploid genomes). Each plate was subsequently scored for the presence of F_2 Tra animals. Positive *Cb-tra* alleles were maintained through sib-selection. In addition, most *Cb-tra* alleles have been outcrossed at least two times. Note: Several *Cb-tra* alleles (*nm25* and lower) were isolated prior to this study (M. Layton, pers. comm.).

2.3 *Cb-tra(nm1)* Genetic Linkage Mapping

Cb-cby-15 hermaphrodites were mated with AF16 males to produce *cby-15* heterozygous males in the F_1. The F_1 males were crossed with trans heterozygous *Cb-tra(nm1) Cb-dpy(nm4)* hermaphrodites. Many F_2 hermaphrodites, consisting of four genotypic classes, were singled for selfing. The four possible genotypic classes

of the F2 hermaphrodites were: 1/4 *nm1/+*, 1/4 *nm1/+; cby-15/+*, 1/4 *nm4/+*, and 1/4 *nm4/+; cby-15/+*. The F_3 *cby-15* progeny were only scored from plates containing both *Cb-tra(nm1)*and *Cb-cby-15*. A 3:1 phenotypic ratio of Cby-15 to Cby-15/Tra would indicate non-linkage, while a marked decrease in the number of expected Cby-15/Tra animals would indicate linkage.

2.4 *Cb-tra(nm2)* Genetic Linkage Mapping

Cb-cby-1 hermaphrodites were mated with AF16 males to produce *cby-1* heterozygous males in the F_1. One-to-one matings were set up between F_1 males and potential *Cb-tra(nm2)* heterozygous hermaphrodites. Many F_2 hermaphrodites from each plate were singled for selfing. The four possible F_2 hermaphrodite genotypic classes, if the hermaphrodite from the previous cross was *nm2/+*, were: 1/4 *nm2/+*, 1/4 *nm2/+; cby-1/+*, 1/4 *+/+*, and 1/4 *cby-1/+*. The F_3 *cby-1* progeny were only scored from plates containing both *Cb-tra(nm2)*and *Cb-cby-1*. A 3:1 phenotypic ratio of Cby-1 to Cby-1/Tra would indicate non-linkage, while the marked decrease in the number of expected Cby-1/Tra animals would indicate linkage.

2.5 Single Worm Lysis

In all applications calling for this procedure, single worm (SW) lysis was performed using the standard protocol set forth by Barstead *et. al.* (1991).

2.6 AF16/HK104 DNA Indel Mapping

In most cases, *Cb-tra* hermaphrodites (heterozygotes and temperature sensitive rescued homozygotes) were mated with wild type HK104 males one time (see figure 11A for crossing scheme deviation). Successfully mated hermaphrodites were transferred to individual plates and allowed to self. Several of the resultant F_1

hermaphrodites from each plate were subsequently singled and selfed (temperature sensitive *tra* alleles were incubated at 25°C). Many of the available F_2 Tra animals were isolated for SW-PCR, using one of the three *Cb-tra*-specific PCR conditions described below. In addition, to verify that the initial HK104 mating was successful, one of the *Cb-tra* indel PCR conditions was performed on either the F_1 hermaphrodites or the non-Tra F_2 siblings following SW lysis. Wild type AF16 and HK104 animals were also used in the SW-PCR assays below as positive controls.

For the *Cb-tra-1* DNA indel assay, the PCR cocktail was prepared as follows: 0.25 mM dNTP, 1X PCR buffer, 2 mM Mg^{2+}, 0.5 μM primers (EH15/EH16), and 1-5 units of Taq DNA polymerase. The cycling conditions were as follows: 95°C denature (2 min) [95°C denature (30 sec), 55°C annealing (35 sec), 72°C extension (45 sec)] 32 cycles, 72°C extension (4-7 min), 4°C hold. The PCR products were separated using a 1.8% TBE agarose gel.

For the *Cb-tra-2* DNA indel assay, the PCR cocktail was prepared as follows: 0.25 mM dNTP, 1X PCR buffer, 2.5 mM Mg^{2+}, 0.5 μM primers (EB2/EB3), and 1-5 units of Taq DNA polymerase. The cycling conditions were as follows: 95°C denature (2 min) [95°C denature (30 sec), 57°C annealing (35 sec), 72°C extension (1 min)] 32 cycles, 72°C extension (4-7 min), 4°C hold. The PCR products were restriction digested with BstB I (NEB) and separated using a 1.8% TBE agarose gel.

For the *Cb-tra-3* DNA indel assay, the PCR cocktail was prepared as follows: 0.25 mM dNTP, 1X PCR buffer, 2 mM Mg^{2+}, 0.5 μM primers (DK48/DK49), and 1-5 units of Taq DNA polymerase. The cycling conditions were as follows: 95°C denature (2 min) [95°C denature (30 sec), 62°C annealing (35 sec), 72°C extension (1

min)] 32 cycles, 72°C extension (4-7 min), 4°C hold. The PCR products were restriction digested with Spe I (NEB) and separated using a 0.8% TBE agarose gel.

2.7 Sequencing

Cb-tra-2: SW-PCR reactions, using *Cb-tra-2* primers (see below) flanking the *Cb-tra-2* exons, were performed on several *Cb-tra(nm1)* animals (3-5 animals per fragment typically). Similar PCR products from each *tra* animal were pooled during gel extraction (Qiagen) and resuspended to a final DNA concentration of 5-30 ng/µl. Smaller PCR fragments (<500 bp) were direct-sequenced using 15-20 ng of DNA (Big Dye Sequencing Reactions). Larger PCR fragments were cloned using the Original TA Cloning Kit (Invitrogen). Several positive colonies were minipreped (Promega Wizard *Plus* Minipreps) and digested with EcoR I to verify proper ligation of the *Cb-tra-2* PCR insert. Correctly cloned plasmids were subjected to the GPS-1 Genome Priming System (NEB) in order to randomly insert transposons for high-throughput sequencing. Ten random GPS-positive colonies from each *Cb-tra-2* were minipreped and sequenced using a GPS-specific primer.

Cb-tra-1: SW-PCR reactions, using overlapping *Cb-tra-1* primers (see below) flanking the *Cb-tra-1* exons, were performed on several *Cb-tra(nm2)* and AF16 animals (3-5 animals per fragment typically). To minimize the production of polymerase-induced mutations, the high fidelity polymerase, optimase polymerase (Transgenomic) was utilized for all PCR reactions. Similar PCR products from both the *tra* and AF16 animals were pooled independently during gel extraction (Qiagen) and resuspended to a final DNA concentration of 20-100 ng/µl. To determine which PCR fragment contained the *Cb-tra-1* lesion, the samples were tested using the

Surveyor Mutation Detection Kit (Transgenomic). The *Cb-tra-1* PCR fragment identified as containing the lesion was then sequenced.

2.8 RT-PCR

Successive centrifigations of 5 similarly aged adult worms picked into 5 µl drops of nuclease-free water (located in the lid of a microcentrifuge tube) were performed until a total of 10 *Cb-tra(nm2)*, 25 AF16 hermaphrodites, and 25 AF16 males were obtained. Each sample was incubated at -80°C for at least 2 hours following the addition of 200 µl of TRI-Reagent (Molecular Research Center, Inc.). After freezing, the samples were thawed and lysed by sonication for 1 minute. Following the addition of 800 µl of TRI-Reagent and 4 µl of Polyacryl Carrier, the RNA was extracted according to the manufacturer's protocol (Molecular Research Center, Inc.). Samples containing 10 worms and 25 worms were resuspended in 20 µl (1X RNA) and 25 µl (2X RNA) of nuclease-free water, respectively. However, for the subsequent RT-PCR steps, equal concentrations of RNA template from each sample were utilized in the AccessQuick™ RT-PCR System (Promega). R. Ellis provided the primers used for the *Cb-act-3* loading control, while DK52 and DK57 were the primers used to determine the level of *Cb-tra-1* mRNA. Negative control reactions contained RNA template and both *Cb-tra-1* primers, but did not include reverse transcriptase.

2.9 *Cb-tra-1* RNAi

Inseminated AF16 hermaphrodites and *Cb-tra(nm2)/Cb-let(nm28)* hermaphrodites were microinjected in either the rachis or gut with *Cb-tra-1* dsRNA provided by E. Haag. Successfully injected worms were rescued and transferred to

new NGM plates to recover. For several days, the injected worms were moved to new NGM plates in order to distribute their progeny over several 24 hour laying windows. The F_1 progeny from each laying window were scored for the following phenotypic characters: presence of sperm/ooids/oocytes, tail morphology, presence of a vulva (AF16 XX animals only), and presence of a one-armed male gonad. The observation of a complete male tail was used to identify XX *tra* homozygotes produced from *Cb-tra(nm2)/Cb-let(nm28)* hermaphrodites and XO males produced from inseminated AF16 hermaphrodites.

2.10 BAC Rescue

BAC clone DNA from *Cb-tra-1* (cb25.fpc2976_RPCI94_08N16) and *Cb-tra-2* (cb25.fpc2454_RPCI94_10F17) was isolated using the BAC PAC Resources protocol. Following isolation the samples were phenol-chloroform extracted, RNase A treated, phenol-chloroform extracted again, and digested with Not I to remove the *C. briggsae*-specific insert. Following digestion, the samples were phenol-chloroform extracted one final time. BAC DNA was resuspended to a final concentration of 100-150 ng/µl.

AF16 genomic DNA was isolated, digested with Pvu II, and phenol-chloroform extracted. To column purify the samples, a modified Wizard *Plus* Miniprep kit protocol was employed. One volume of resuspension buffer was added to the AF16/Pvu II sample. Following centrifugation at 15,700 x g for 5 minutes, a one volume mixture of lysis/neutralization buffer was added to the resuspended AF16/Pvu II sample to create the "lysate". Preparation of the column, addition of the lysate, washing of the column, and recovery of the sample were performed following

the manufacture's protocol (Promega). Following this procedure, the AF16/Pvu II DNA was phenol-chloroform extracted one more time and resuspended to a final concentration of ~150 ng/µl.

The *myo-2::GFP* marker was isolated using the Wizard *Plus* Miniprep kit. Following isolation, restriction digestion was performed using Sac I in order to linearize the plasmid. Further purification steps, including phenol-chloroform extraction, were performed by E. Haag. The *myo-2::GFP* was resuspended to a final concentration of ~130 ng/µl.

Two separate complex array microinjection conditions were used to inject ~20 *Cb-tra(nm1)/Cb-dpy(nm4)* and ~20 *Cb-tra-2(nm2)/Cb-let(nm28)* heterozygous hermaphrodites. The first complex array cocktail was prepared as follows: AF16/Pvu II (100 ng/µl), BAC DNA/Not I (5 ng/µl), and *myo-2::GFP*/Sac I (2 ng/µl). The second complex array cocktail was prepared as follows: AF16/Pvu II (100 ng/µl), BAC DNA/Not I (4 ng/µl), and *myo-2::GFP*/Sac I (8 ng/µl). Repetitive array cocktails were injected into 5 AF16, 4 N2 (*C. elegans* wild-type strain), and 6 HK104 hermaphrodites and were prepared as follows: BAC DNA/Not I (40 ng/µl), and *myo-2::GFP*/Sac I (20 ng/µl). BAC DNA (80 ng/µl) only was also injected into 6 *Cb-tra(ed23ts)* hermaphrodites (progeny incubated at 25°C).

2.11 Mating Test

Potentially fertile *Cb-tra* pseudo-males were mated with *Cb-dpy(nm3)X* hermaphrodites. After the attempted mating, the males were removed. F1 progeny were scored for the presence of non-dpy hermaphrodites and dpy males. If an XX

Tra pseudomale were capable of mating, the F_1 would be characterized by the presence of non-dpy hermaphrodites and the absence of dpy males.

2.12 *Cb-tra-1* XO Assay

The genetic scheme necessary to produce *Cb-tra(nm2)* XO homozygotes is presented in Figure 9A. Based on this scheme, following SW lysis, a *Cb-tra(nm2)* allele-specific PCR will be necessary to identify both the F_1 *nm2* carrying males and the F_2 *nm2* homozygous progeny. To perform the *nm2* genotyping assay, the samples were subjected to three separate PCR conditions following SW lysis.

To prepare the *Cb-tra(nm2)* outer PCR reaction, the PCR cocktail was set up as follows: 50 μl reaction volume, 0.25 mM dNTP, 1X PCR buffer, 2 mM Mg^{2+}, 0.5 μM primers (DK52/DK57), and 2.5 units of Taq DNA polymerase. The cycling conditions were as follows: 95°C denature (2 min) [95°C denature (30 sec), 65°C annealing (35 sec), 72°C extension (30 sec)] 32 cycles, 72°C extension (7 min), 4°C hold. The PCR products (~350 bp) were separated using a 1.8% TBE agarose gel.

For genotyping, two separate PCR reactions [DK52/DK58 (WT) and DK52/DK59 (*nm2*)] per sample were performed. To prepare the *Cb-tra(nm2)* ASPCR reactions, the PCR cocktails were set up as follows: 20 μl reaction volume, 0.5 μl of a 1:10 diluted outer reaction as template, 0.1 mM dNTP, 1X PCR buffer, 2 mM Mg^{2+}, 0.05 μM primers (DK52/DK58 or DK52/DK59), and 1 unit of Taq DNA polymerase. The cycling conditions were as follows: 95°C denature (2 min) [95°C denature (30 sec), 68°C annealing (35 sec), 72°C extension (15 sec)] 18 cycles, 72°C extension (4 min), 4°C hold. The PCR

products (~170 bp - Note: faint outer band is also made from residual DK57 primer in template) were separated using a 1.8% TBE agarose gel.

2.13 *Cb-tra-2* XO Assay

Several WT AF16 males and *Cb-tra(ed23ts)* hermaphrodites were mated at 15°C. From this cross, successfully plugged *Cb-tra(ed23ts)* hermaphrodites were grouped on a new plate and incubated at 15°C until *Cb-tra(ed23ts)* heterozygous F_1 males were observed. Next, several *Cb-tra(ed23ts)* carrier males and *Cb-tra(ed23ts)* hermaphrodites were mated at 15°C (O/N) and later shifted to 25°C until obvious plugs were observed. Successfully mated hermaphrodites were then moved to individual plates and the F_1 progeny were reared at 25°C. Crossing a heterozygous *Cb-tra(ed23ts)* male with a *Cb-tra(ed23ts)* hermaphrodite should produce four genotypic classes in equal frequencies: 1/4 XX *Cb-tra(ed23ts)* heterozygotes, 1/4 XX *Cb-tra(ed23ts)* homozygotes, 1/4 XO *Cb-tra(ed23ts)* heterozygotes, and 1/4 XO *Cb-tra(ed23ts)* homozygotes. All the F_1 progeny on plates containing hermaphrodites, XO males, and Tra males were scored and the phenotypic ratios observed. In addition, a subset of the obviously XO animals were observed with DIC.

2.14 *tra-2* Complementation Testing: Strain Construction

In order to construct the *Cb-tra(ed23ts); Cb-dpy(nm4)* strain necessary for this procedure, *Cb-tra(ed23ts)* hermaphrodites were mated with AF16 males at 15°C to produce *Cb-tra(ed23ts)* heterozygous males. The resultant F1 males were then mated with *Cb-dpy(nm4)* hermaphrodites. Several non-Dpy F_2 hermaphrodites were singled for selfing at 25°C. F_3 Dpy hermaphrodites from plates containing both Dpy and Tra progeny were singled for selfing at 25°C to identify those Dpy mutants that were

heterozygous for the *ed23ts* allele. The resultant *Cb-tra(ed23ts)/+; Cb-dpy(nm4)* strain was maintained by sib-selection at 25°C. In order to construct the double homozygote, 16 *dpy(nm4)* hermaphrodites from a recently sib-selected *Cb-tra(ed23ts)/+; Cb-dpy(nm4)* strain were singled and selfed for several generations at 15°C. L4 Dpy hermaphrodites (5-10) from all 16 plates were transferred to new NGM plates and allowed to self at 25°C (the original plates remained at 15°C). Plates at 15°C were identified as *Cb-tra(ed23ts); Cb-dpy(mn4)* strains when the corresponding plate at 25°C was observed to possess Dpy/Tra progeny exclusively.

2.15 Primer List

Cb-tra-2 Sequencing/DNA polymorphism:

DK1 – 5' GACTGATCTTCTGGAGGTTAACGG 3'

DK2 – 5' TTCGGAATATCGGAATCTCAGGA 3'

DK3 – 5' TGCTTGCGGGCAGAACTACTTCCA 3'

DK4 – 5' GTCATTCCGACTTCGTCAACGGTT 3'

DK5 – 5' GGCCTTTGAATTCTCAGGTTCTTG 3'

DK6 – 5' GGAGATCAAGACTCTGAAGTTTGG 3'

DK7 – 5' ACACTTTCAGATGATGATGGCTGC 3'

DK8 – 5' CAGGCCTTTTCTCTAGGTCAAGTT 3'

DK9 – 5' CTAGGCCATTTTGACATGGTAGCC 3'

DK10 – 5' TCCTCCCTATTAGGCCTTGGTGGT 3'

DK11 – 5' TCGCAGTCTTCAATTCCCTCGCCG 3'

DK12 – 5' TACGAAGCACTATGCTCCACGGCC 3'

DK13 – 5' GATTCCATCACAGACCTGATTCGG 3'

DK14 – 5’ CCGAATCAGGTCTGTGATGGAATC 3’

DK15 – 5’ CGAGAAGGAGACGCTGAACTGTCC 3’

DK16 – 5’ GGACAGTTCAGCGTCTCCTTCTCG 3’

DK17 – 5’ CGCCTCTGGACATGATCCTTGTGT 3’

DK18 – 5’ ACACAAGGATCATGTCCAGAGGCG 3’

DK19 – 5’ GTCGTGTCAGAGGAGCTGTCAGGT 3’

DK20 – 5’ ACCTGACAGCTCCTCTGACACGAC 3’

DK21 – 5’ CATCATCCTGGGAGAGCATCAACG 3’

DK22 – 5’ CGTTGATGCTCTCCCAGGATGATG 3’

DK23 – 5’ GAAGCTCACCCGTGCCGTCAACTT 3’

DK24 – 5’ AAGTTGACGGCACGGGTGAGCTTC 3’

DK25 – 5’ GAAGGGCCCCCTTCCAAATATGTA 3’

Cb-tra-1 Sequencing/DNA polymorphism/RT-PCR:

DK26 – 5’ CGACAGCGGTGTGCCGATTCCACC 3’

DK27 – 5’ CGTCTTCTGACTGGTCTACGTGAG 3’

DK28 – 5’ TGGTTCCGATGTGACGCGGAGCCG 3’

DK29 – 5’ GAGCTCTCGGGCATCTGAAAGCAC 3’

DK30 – 5’GCCAGTCGGCTGGCGCCTTTGTTT 3’

DK31 – 5’ TGTGTGTGCGTTGGCTCCGCCCAC 3’

DK32 – 5’ GCGGCCGTCGGATTGCATACATAA 3’

DK33 – 5’GGAGGAGGTGTGAGTGTGAGAGGG 3’

DK34 – 5’ TTGCCGCACCAGGTGTTCATCAGC 3’

DK35 – 5’ GCTGCAGGAACGGATGTCGGGATG 3’

DK36 – 5’ CGCCGCTTACACCAATGGGAGCAA 3’

DK37 – 5’ TCATCGTCGAAGGCTTCAGCAGCC 3’

DK38 – 5’ GGTGGACGCCCGATCCTGTCGGTT 3’

DK39 – 5’ GCGGCACGAACAATGCGATGATGA 3’

DK40 – 5’ TGCTGAAGCAGTTCCCGGAACATC 3’

DK41 – 5’ GAAGGAGGGGGCGAGAGCCGTTGA 3’

RE883 – 5’ GGACGTACCACCGGAGTCGTCC 3’

RE884 – 5’ GCATACGATCAGCAATTCCTGGG 3’

Cb-tra-3 DNA Polymorphism:

DK48 – 5’ CACTGACAGCTGTGAGCTACACGG 3’

DK49 – 5’ CAGAGCTGTCCCGACCATTTGTCG 3’

Cb-tra-2 XO ASPCR:

DK52 – 5’ CGCTGCTCTGATGGATCCGAATGG 3’

DK57 – 5’ GGTGTGATCGGAGTATGCGGAGTC 3’

DK58 – 5’ TTGAGCTTGCTGAAGCGCAGCTGG 3’

DK59 – 5’ TTGAGCTTGCTGAAGCGCAGCTGA 3’

CHAPTER 3: RESULTS

3.1 Isolation of *Cb-tra* Mutants

Forward mutagenic screens, the historical method of choice in *C. elegans*, have been employed in the hunt for *Cb-tra* mutants. Since males are produced through meiotic nondisjunction of the X chromosome at a low frequency (~0.2%), the transformation of XX hermaphrodites into males due to a *tra* mutation is easily scored (Fig. 3). Also, the large amount of genomic DNA encompassed by the known

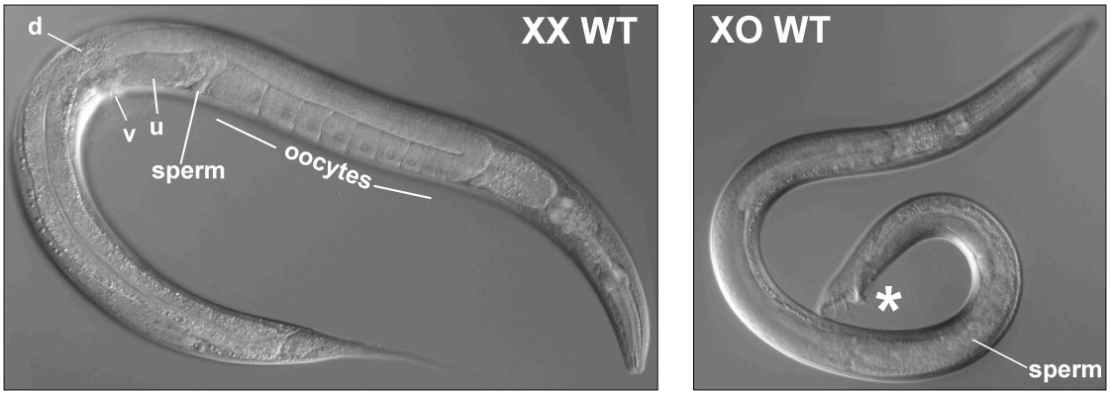

Figure 3. DIC images of a *C. briggsae* wild type XX hermaphrodite (left) and a wild type XO male (right). Hermaphrodites possess a two-armed gonad (anterior arm shown above) extending distally from a common uterus (u) and vulva (v). Each arm contains a single distal tip cell (d), where a syncitial stem-cell population of mitotically dividing germ cells exists. As the germ cells move toward the vulva, they enter meiosis and undergo gametogenesis to produce sperm and then oocytes. Sperm is housed in the spermatheca and oocytes are fertilized after passage through this organ. Embryos are held in the uterus prior to expulsion from the vulva. Males (right panel) possess a sperm-producing, one-armed gonad consisting of a testis, seminal vesicle, vas deferens, and cloaca. The male tail (*) is made up of specialized mating organs such as spicules and sensory rays (RIDDLE *et al.* 1997). Note: DIC images taken by E. Haag.

nematode *tra* genes tends to yield a good number of Tra mutants following mutagenesis. F_2 screening of approximately 10,000 haploid genomes has yielded

fourteen recessive *Cb-tra* mutants (Table 1). Of the mutants vigorously studied thus far, three linkage groups have been identified through genetic mapping (Fig. 4).

3.2 Linkage Group III Tra Alleles

C. briggsae tra mutants in linkage group III possess similar phenotypic characteristics as *C. elegans tra-1* mutants, such as sperm-filled, one-armed gonads, masculinized tails, and male mating behavior (HODGKIN 1987; HODGKIN and BRENNER 1977) (Table 1 and Fig. 5). Like *C. elegans tra-1* mutants, the masculinization of the tail in this class of *Cb-tra* mutants is variable and ooids/oocytes can be seen following a limited period of sperm production in several strains. However, small, misshapen gonads, which are commonly seen in *Ce-tra-1* null mutants (HODGKIN 1987; MATHIES *et al.* 2004), have not been observed in linkage group III *Cb-tra* mutants.

Based on synteny with *Ce-tra-1*, *Cb-tra-1* should be positioned on the right end of chromosome III. If *Cb-tra-1* has remained in this location, *Cb-cby-1* should be a suitable genetic marker for *Cb-tra-1* linkage mapping (Fig. 4). Based on this assumption, *Cb-cby-1* was crossed into *Cb-tra(nm2)*, because *Cb-tra(nm2)* animals strongly resemble *Ce-tra-1* mutants phenotypically. As expected, *Cb-tra(nm2)*was observed to be loosely linked to *Cb-cby-1*. Therefore, based on its near complete male phenotype and chromosome III linkage results, *Cb-tra(nm2)* was classified as a likely *Cb-tra-1* allele.

To strengthen the link between *Cb-tra(nm2)* and the *Cb-tra-1* locus, a *Cb-tra-1*-specific DNA polymorphism test was developed (E. Haag, pers. comm.) (Fig. 6).

Table 1. Cb-tra Allele Summary

Allele	***tra* phenotype**	**Linkage Group**	**DNA Indel Linkage**	**Fails To Complement**	**Sequence Lesion**	**Candidate Gene**
nm2	Complete, moderate mating behavior, produces "ooids", infertile	III [*Cb-cby-1*] & [*Cb-let(nm28)]*	*Cb-tra-1*	?	Q512stop	*Cb-tra-1*
nm10	Incomplete, stubby tail, some mating behavior, infertile	III [*Cb-let(nm28)*]	*Cb-tra-1*	?	?	*Cb-tra-1*
nm30	Incomplete, good oocytes/occasional embryos	?	*Cb-tra-1*	?	?	*Cb-tra-1*
ed23ts	Incomplete, occasional pv, complete rescue at 15C	II [*Cb-cby-15)*]	*Cb-tra-2*	*nm1*	D587A	*Cb-tra-2*
nm1	Incomplete	II [*Cb-dpy(nm4)*]	*Cb-tra-2*	*ed23ts*	R1197stop	*Cb-tra-2*
nm9ts	Incomplete, frequent pv, complete rescue at 15C	II [*Cb-dpy(nm4)*]	?	*ed23ts*	P1214L	*Cb-tra-2*
nm21	Incomplete, dominant at 25C	II [*Cb-dpy(nm4)*]	?	?	?	*Cb-tra-2*
nm29	Incomplete	II [*Cb-dpy(nm4)*]	*Cb-tra-2*	?	?	*Cb-tra-2*
nm36ts	Incomplete, frequent pv, complete rescue at 15C	?	*Cb-tra-2*	?	?	*Cb-tra-2*
ed24ts	Incomplete, frequent pv, partial rescue at 15C	IV [*Cb-cby-7*]	*Cb-tra-3*	?	?	*Cb-tra-3*
nm22	Incomplete, small size	not X	?	?	?	?
nm23	Sdc	not X	?	?	?	?
nm24	Sdc	?	?	?	?	?
nm26	Incomplete	not II	?	?	?	*Cb-tra-1* or *Cb-tra-3*
nm34	Complete, moderate mating behavior, possible ts, fertility unknown	?	?	?	?	*Cb-tra-1*
nm37	Incomplete, frequent pv, slight ts (not rescued at 15C)	?	?	?	?	*Cb-tra-1* or *Cb-tra-2* or *Cb-tra-3*

pv = protruding vulva, ts = temperature sensitive (25ºC restrictive temperature)

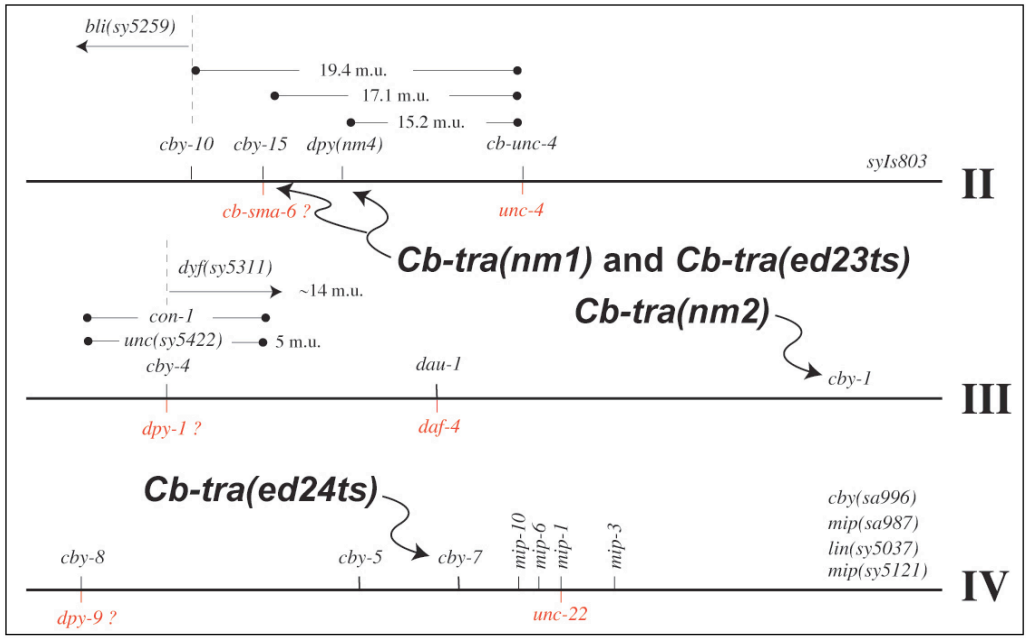

Figure 4. Linkage of *tra* mutants to the condensed *C. briggsae* genetic map. *Cb-tra(ed23ts)* and *Cb-tra(nm1)* mapped to chromosome II using *Cb-cby-15* and *Cb-dpy(nm4)* as markers. *Cb-tra(nm2)* mapped to chromosome III using *Cb-cby-1* as a marker. *Cb-tra(ed24ts)* mapped to chromosome IV using *Cb-cby-7* as a marker. Based on synteny with *C. elegans*, *Cb-tra-2*, *Cb-tra-1*, and *Cb-tra-3* should be located on chromosome 2, 3, and 4 respectively. Note: Red markers indicate *C. elegans* homologs.

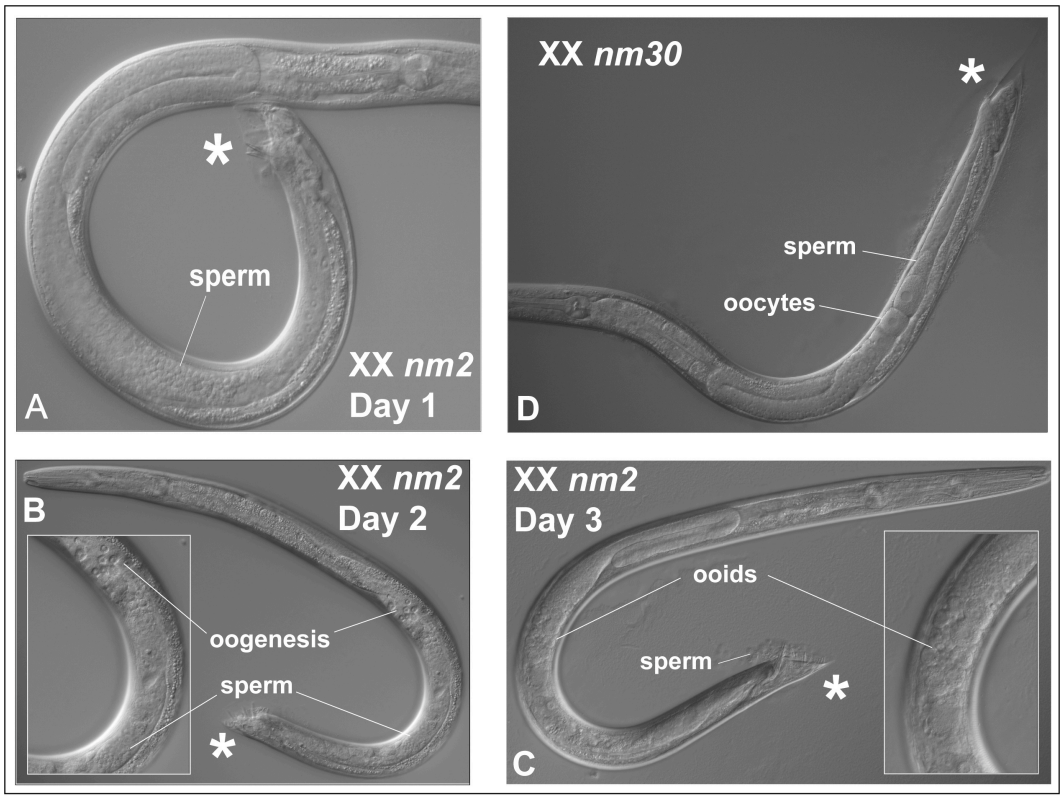

Figure 5. DIC images of several linkage group III *Cb-tra* mutants. All linkage group III homozygotes viewed under DIC possess a sperm-producing, one-armed gonad, and male tail (*). (A) *Cb-tra(nm2)* possesses a complete male tail and produces only sperm during the first day of adulthood. (B-C) Later in adulthood, *Cb-tra(nm2)* homozygotes switch to oogenesis and produce ooids (resemble oocytes, but lack a clearly distinguishable nucleolus). The expulsion of sperm from the tail in panel C was likely due to microscopy-induced pressure, since *Cb-tra(nm2)* mutants are infertile. Sperm usually accumulates in the proximal gonad of *Cb-tra(nm2)* mutants. (D) *Cb-tra(nm30)* possesses an incomplete male tail with reduced sensory rays and spicules. Oocytes and the occasional embryo have been observed in *Cb-tra(nm30)* homozygotes.

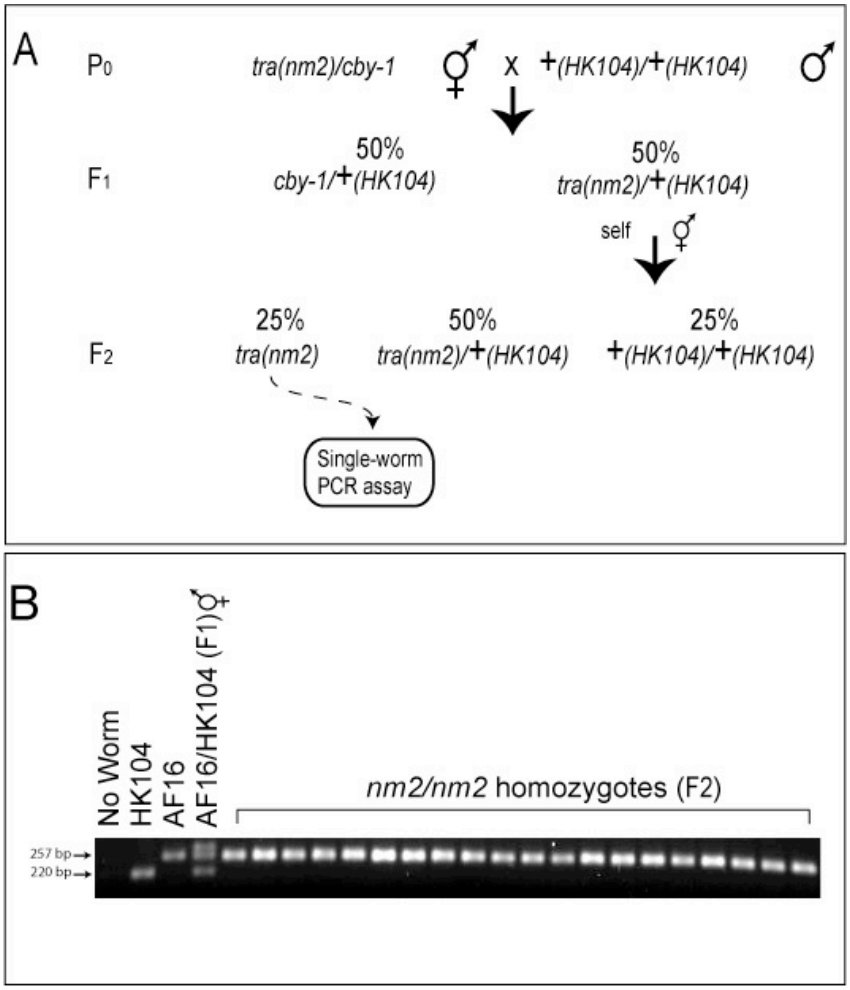

Figure 6. *Cb-tra(nm2)* is linked to a *Cb-tra-1* DNA indel. (A) *Cb-tra(nm2)* was crossed with HK104 to obtain F_2 *tra* homozygotes whose genomes contain both AF16 and HK104 sequences. (B) The results of *Cb-tra-1* single worm-derived PCR shows that the F_1 hermaphrodite carried both types of sequences (the upper band represents the AF16/HK104 sequence hybridization, which forms a loop structure that causes the movement of this band to be retarded). All of the F_2 *tra* progeny assayed were homozygous for the AF16 *Cb-tra-1* sequence.

This assay exploits a natural DNA polymorphism within the *Cb-tra-1* locus of two *C. briggsae* populations, AF16 and HK104, in order to determine the segregation pattern between the *Cb-tra-1* locus and an allele-specific Tra phenotype. The crossing scheme outlined in figure 6A enables the production of wild type F_1 progeny whose genomes contain both AF16-specific and HK104-specific sequences. Following

selfing, *Cb-tra* mutants produced in the F_2 are assayed using *Cb-tra-1*-specific primers flanking the region containing the AF16/HK104 DNA polymorphism. Since AF16 is the genetic background of all *Cb-tra* mutants, the overrepresentation of the AF16 *Cb-tra-1* product would indicate that the Tra phenotype and the *Cb-tra-1* locus were linked. In the case of non-linkage, only 1/4 of the selected Tra mutants would be expected to be homozygous for the AF16 sequence by chance. When *Cb-tra(nm2)* was tested using the assay described above, all of the F_2 *tra* mutants selected were homozygous for the AF16 *Cb-tra-1* sequence, indicating that the mutation responsible for the Tra phenotype in these mutants is tightly linked to *Cb-tra-1* at the molecular level (Fig. 6B). In addition to *Cb-tra(nm2)*, several other mutants in linkage group III have also shown positive linkage to *Cb-tra-1* using the assay outlined above (Table 1). However, DNA polymorphism testing and positive linkage of a mutant to a specific DNA locus does not definitively prove that the mutant causing lesion and the locus being tested are and the same.

In order to more directly correlate the Tra phenotype of *Cb-tra(nm2)* mutants with a deficiency in their *Cb-tra-1* gene, overlapping *Cb-tra-1* PCR fragments were prepared from AF16 and *Cb-tra(nm2)* homozygotes for use in the Surveyor Mutation Detection Kit (Transgenomic). A single DNA lesion was observed and subsequent sequencing of the mismatched region revealed a nonsense mutation located in the TRA-1 C-terminus of *Cb-tra(nm2)* mutants (Fig. 7A). Based on the nature of the mutation, two plausible explanations are sufficient to correlate the null-like Tra-1 phenotype of *Cb-tra(nm2)* mutants with their *Cb-tra-1* lesion: 1) Lack of TRA-1 protein function, due to the loss of over half the TRA-1 protein (including the TRA-2

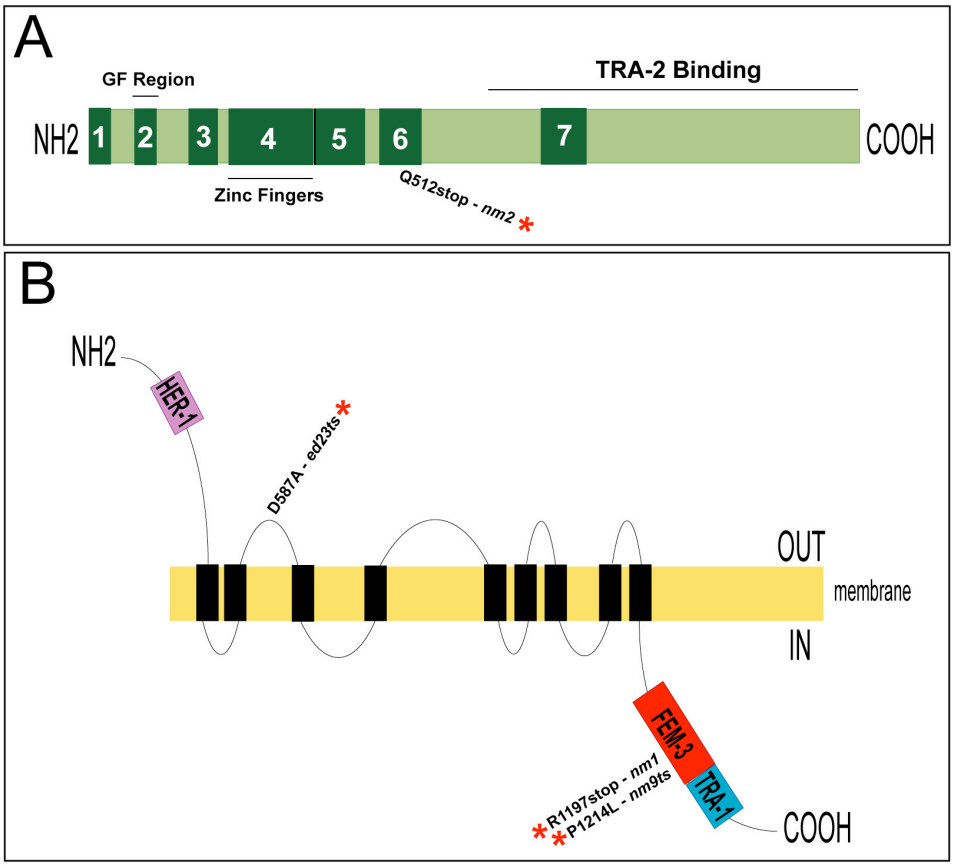

Figure 7. Locations of *Cb-tra* lesions within the *C. briggsae* TRA-1 and TRA-2 proteins. (A) Depiction of the *C. briggsae* TRA-1 protein and the relative location of one *Cb-tra* mutant's lesion within the protein (DE BONO and HODGKIN 1996; WANG and KIMBLE 2001). Dark green boxes indicate the seven regions of high conservation between the *C. elegans* and *C. briggsae* TRA-1 proteins, while light green boxes indicate less conserved regions between the two proteins (DE BONO and HODGKIN 1996). (B) Depiction of the *C. briggsae* TRA-2 transmembrane protein and the relative locations of three *Cb-tra* mutant lesions within the protein. The HER-1 (purple), FEM-3 (red), and TRA-1 (blue) binding sites, as well as the nine transmembrane domains (black) of TRA-2 are shown above (KUWABARA 1996a; WANG and KIMBLE 2001).

binding site) or 2) Loss of the entire TRA-1 protein, due to the degradation of the *Cb-tra-1* transcript through nonsense-mediated decay (NMD).

In order to investigate the possible role that NMD might play in producing the strong Tra phenotype of *Cb-tra(nm2)* homozygotes, reverse transcriptase polymerase

chain reaction (RT-PCR), using *Cb-tra-1*-specific primers, was performed on these mutants (Fig. 8). Based on the results from this experiment, the *Cb-tra-1* transcript

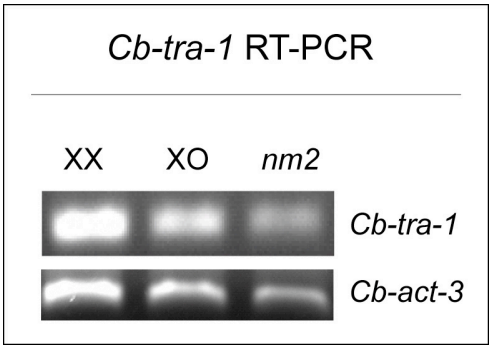

Figure 8. *Cb-tra-1* RT-PCR. The total RNA levels of *Cb-tra-1* and *Cb-act-3* in AF16 hermaphrodites (XX), AF16 males (XO), and *Cb-tra(nm1)* animals was assessed through gene-specific RT-PCR. Based on the *Cb-act-3* loading control, the relative total RNA levels in the samples were: XX>XO>*nm2*. The band intensity ratios for both RT-PCR experiments was 1.73:1.46:1 (XX:XO:*nm2*). Based on this data, the level of *Cb-tra-1* total RNA in *Cb-tra(nm2)* has not been reduced by NMD in these mutants.

appears to be present in *Cb-tra(nm2)* mutants, which rules out the possibility that the Tra phenotype in these animals is a consequence of *Cb-tra-1* NMD. However, the possibility that *Cb-tra(nm2)* mutants are nullomorphs of TRA-1 at the level of protein function is still an option for several reasons. First, the severity of the Tra phenotype in these mutants, due to the loss of over half of the TRA-1 protein, suggests that *Cb-tra(nm2)* animals possess a severely defective TRA-1 protein. Second, *Cb-tra(nm2)* mutants treated with *Cb-tra-1* RNAi continued to produce ooids following spermatogenesis. Therefore, attempts to further reduce the TRA-1 protein in *Cb-tra(nm2)* mutants did not result in a stronger Tra phenotype, suggesting that these animals are likely already null for TRA-1. In the future, chromosomal deficiency studies should be performed to confirm that *Cb-tra(nm2)* mutants are TRA-1 nulls.

Based on the evidence presented thus far, *Cb-tra(nm2)* is clearly at least an allele of *Cb-tra-1*, if not a TRA-1 null. However, in order to further prove that the Tra phenotype of these mutants is directly related to their mutated *Cb-tra-1* gene, transgenic rescue of *Cb-tra(nm2)* with the wild-type copy of *Cb-tra-1* was attempted. Unfortunately, all transgenic rescue attempts using *Cb-tra-1* containing BAC constructs have been unsuccessful (see methods). Perhaps transgenic studies utilizing different methods, such as ballistic bombardment, will be more successful in the future.

Because *Cb-tra(nm2)* is the strongest *Cb-tra-1* allele isolated to date, an XO assay utilizing *Cb-tra(nm2)* mutants has been developed in order to determine the *Cb-tra-1* loss of function XO phenotype (Fig. 9). Although this assay has not been successfully completed, all of the necessary strains have been constructed and the allele-specific PCR, which is critical for identifying *Cb-tra(nm2)* homozygotes, has been perfected (Fig. 9A-B). In the meantime, *Cb-tra-1* RNAi results have indicated that *Cb-tra-1* loss of function XO animals produce a limited amount of sperm during L4 and switch into the production of oocytes during adulthood (Fig. 9C and R. Ellis, pers. comm.). Should *Cb-tra(nm2)* XO mutants possess a similar phenotype (limited number of sperm followed by oocytes), this would be a more severe phenotype than their XX counterparts (sperm for many days followed by ooids). Such a finding would indicate that the role of TRA-1 in sex determination is not identical between *C. briggsae* and *C. elegans*, due to the fact that *Ce-tra-1* XO males tend to mimic the phenotype of their XX counterparts (HODGKIN 1987).

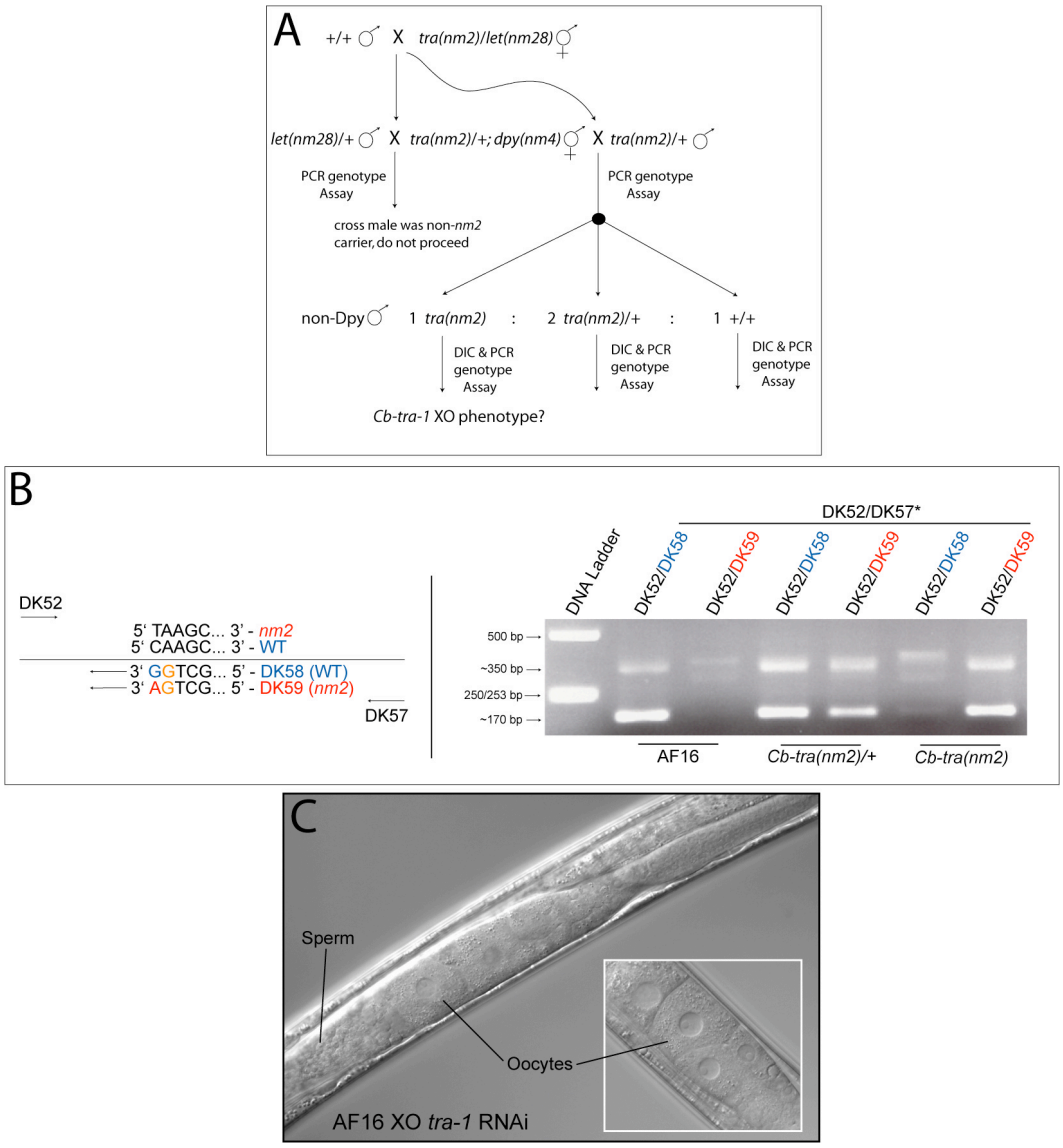

Figure 9. *Cb-tra-1* RNAi and *Cb-tra(nm2)* XO assay. (A) Diagram of the genetic scheme necessary to produce *Cb-tra(nm2)* XO homozygotes. (B) Depicts the allele-specific PCR used to genotype worms for the presence or absence of the *nm2*-specific *Cb-tra-1* sequence lesion. Red indicates wild type sequence/primers, while blue indicates *nm2* sequence/primers. The orange bases in the *Cb-tra-1* allele specific primers are intentional mismatches used to improve genotyping capabilities. Three sets of PCR are performed on each worm: flanking primers (DK52/DK27) produce an ~350 bp band off of which *Cb-tra-1* allele-specific primers (DK52/DK58 = WT and DK52/DK59 = *nm2*) produce the ~170 bp genotyping band. (C) A limited amount of sperm followed by the production of oocytes, which improve over time (inset), are produced in AF16 XO males following *Cb-tra-1* RNAi.

3.3 Linkage Group II Tra Alleles

C. briggsae tra mutants in linkage group II were genetically mapped using both *Cb-dpy(nm4)* (M. Layton, pers. comm.) and *Cb-cby-15* (Fig. 4). These mutants possess the same phenotypic characteristics as *C. elegans tra-2* mutants, such as a one-armed gonad with sperm, an abnormal male tail with reduced sensory rays, lack of male mating behavior, and infertility (HODGKIN and BRENNER 1977) (Table 1 and Fig. 10). Based on synteny with *C. elegans*, the association of *tra-2*-like mutants with

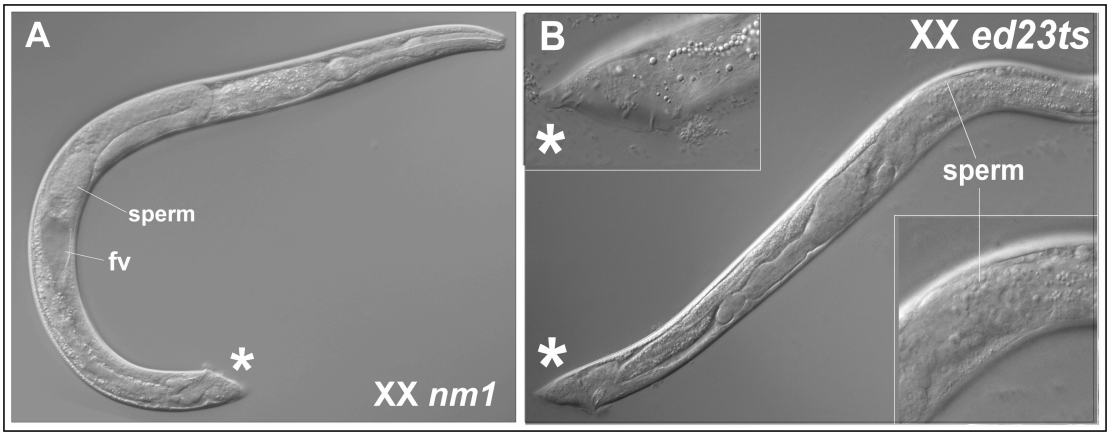

Figure 10. DIC images of linkage group II *Cb-tra* mutants. All linkage group II mutants possess a sperm-producing, one-armed gonad and an abnormal male tail (*) with reduced sensory rays and spicules (A-B). Fluid vacuoles (fv), noted in panel A, have been observed in many linkage group II *Cb-tra* strains. In addition, protruding vulvas (not shown) are often observed at the restrictive temperature in ts *tra* strains, regardless of *tra* genotype.

linkage group II is not surprising. However, like the case with linkage group III mutants, the correlation of phenotype and chromosomal location only helped to identify *Cb-tra-2* as the candidate gene for this group of mutants.

To further tie linkage group II mutants with *Cb-tra-2*, a DNA polymorphism assay (E. Haag, pers. comm.) was developed for the *Cb-tra-2* locus (Fig. 11). A representative *Cb-tra* mutant in linkage group II, *Cb-tra(nm1)*, was crossed with

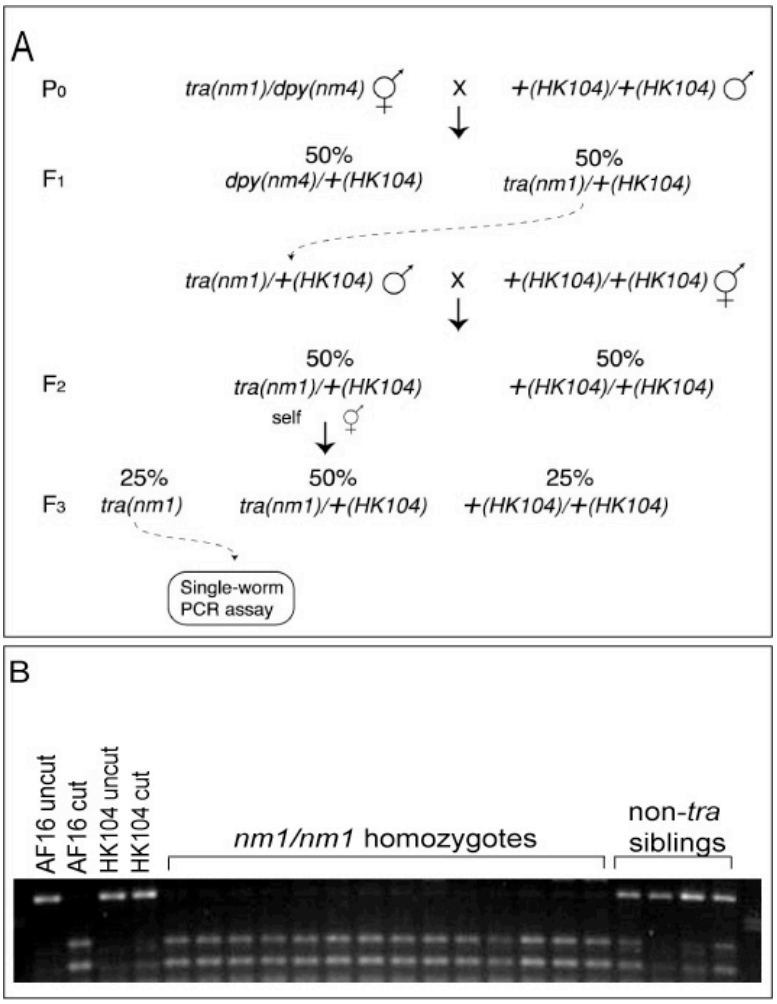

Figure 11. *Cb-tra(nm1)* is linked to a *Cb-tra-2* DNA polymorphism. (A) *Cb-tra(nm1)* was crossed with HK104 to obtain F_3 *tra* homozygotes whose genomes contain both AF16 and HK104 sequences. (B) The results of *Cb-tra-2* single worm-derived PCR shows that the non-*tra* F_3 hermaphrodites carried both types of sequences. All of the *tra* progeny assayed were homozygous for the AF16 *Cb-tra-2* sequence.

HK104 two times in order to produce wild type F_2 progeny whose genomes contain mostly HK104 sequence. *Cb-tra* mutants produced in the F_3 were assayed using a *Cb-tra-2*-specific PCR reaction and subsequent restriction digestion. Only one in sixteen F_3 *tra* animals would be expected to be homozygous for the AF16 sequence by chance if the *tra* mutation and the *Cb-tra-2* region being tested were unlinked. All

sixteen of the *Cb-tra(nm1)* mutants assayed were homozygous for the AF16 sequence. This result confirms that the *Cb-tra(nm1)* mutation and the *Cb-tra-2* locus are linked at the molecular level. In addition, this result confirms that *Cb-tra-2* is still located on chromosome II in *C. briggsae*. The *Cb-tra-2* SNP mapping assay has successfully been used to positively link several other alleles in linkage group II to *Cb-tra-2* (Table 1). Clearly, however, linkage of *Cb-tra* alleles to *Cb-tra-2* using the polymorphism assay above does not definitively prove that these animals are *Cb-tra-2* mutants.

In order to determine whether some of the *Cb-tra* alleles in linkage group II possessed a *Cb-tra-2* mutation, sequencing of three reference alleles was performed. The results identified missense and nonsense mutations in the *Cb-tra-2* genes from these alleles (Fig. 7B & Table 1). The TRA-2 lesion of *Cb-tra(ed23ts)* changes conserved amino acid 587 (HAAG and KIMBLE 2000; KUWABARA *et al.* 1992), which is located in the second loop of the transmembrane protein, from aspartic acid (D) to alanine (A) (C. Carvalho, pers. comm.). In the case of *Cb-tra(nm9ts)*, non-conserved amino acid 1214, which is located in the FEM-3 binding domain of TRA-2, was changed from a proline (P) to a leucine (L) (J. Salogiannis, pers. comm.). The above mutations have no effect on the animals at 15°C, but cause a Tra phenotype at 25°C. The TRA-2 lesion in *Cb-tra(nm1)* is a premature stop approximately a third of the way into the protein's intracellular FEM-3 binding domain near the C-terminus. Therefore, based on the presence and location of their respective *Cb-tra-2* lesions, *Cb-tra(nm1)*, *Cb-tra(nm9ts)*, and *Cb-tra(ed23ts)* have been classified as *Cb-tra-2* mutants.

To fully explore what effect the *Cb-tra-2* mutation of *Cb-tra(nm1)* homozygotes may have on their TRA-2 protein, a *Cb-tra-2*-specific RT-PCR experiment was performed on these mutants to determine the stability of their *Cb-tra-2* transcript. Despite several attempts, *Cb-tra-2* message could not even be detected in wild type XX and XO *C. briggsae* animals (data not shown) using a protocol similar to the *Cb-tra-1* RT-PCR experiment presented above. Therefore, at this point, it is unclear whether the *Cb-tra(nm1)* mutation causes reduction in TRA-2 function through the action of NMD or due to the loss of the TRA-2/FEM-3 interaction. However, either scenario supports the notion that *Cb-tra(nm1)* is likely a *Cb-tra-2* null allele.

Although use of a *Cb-tra-2* null allele may have been ideal, the *Cb-tra-2* XO phenotype was observed in a *Cb-tra(ed23ts)* background, due to the fact that this allele possesses a *Cb-tra-2* mutation and because it is genetically facile (Fig. 12).

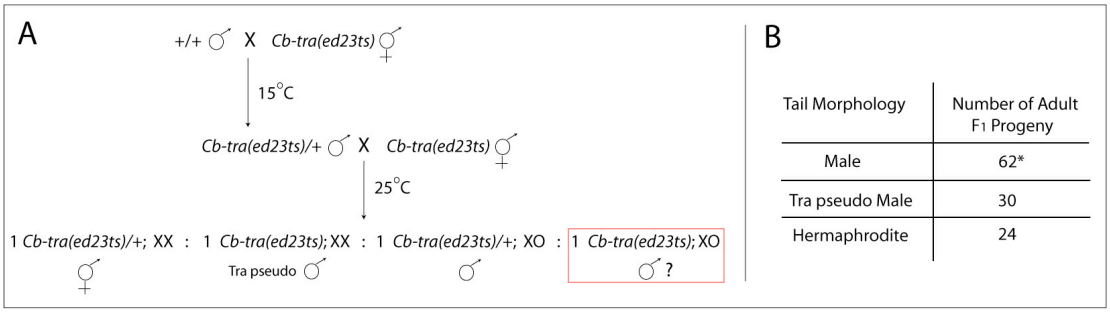

Figure 12. *Cb-tra-2* XO phenotype. (A) Depiction of the genetic crossing scheme necessary to produce *Cb-tra(ed23ts)* XO homozygotes in the F_1. (B) Scoring of the earliest adult F_1 progeny for tail morphology, using two independent crosses, produced three phenotypic classes: male, Tra pseudo male (reduced rays/spicules), and hermaphrodite. The observation that *Cb-tra(ed23ts)* XO homozygotes are somatically male, instead of non-male, is statistically significant (**P* Value < 0.001). To assess possible germline differences between *Cb-tra(ed23ts)* homozygous males and carrier males, 34 somatically male animals were viewed under DIC. All 34 were indistinguishable from AF16 males.

Based on the crossing scheme outlined in Figure 12, four genotypic classes of F_1 progeny will be produced in equal frequencies when this assay is performed. However, only three phenotypic classes (1 hermaphrodite : 1 Tra : 2 male) will be represented if the XO homozygous *Cb-tra(ed23ts)* animals behave like their *C. elegans* counterparts (HODGKIN and BRENNER 1977). Based on the data presented in Figure 12, the XO *Cb-tra-2* phenotype is indistinguishable from that of wild type *C. briggsae* males, which is consistent with the XO phenotype of *Ce-tra-2* homozygotes.

3.4 Linkage Group IV Tra Alleles

Cb-tra(ed24ts) homozygotes phenotypically resemble *Cb-tra-2* mutants (Table 1 and Fig. 13). However, *Cb-tra(ed24ts)* was genetically mapped to linkage group IV

Figure 13. DIC image of a linkage group IV *Cb-tra* mutant. At 25°C, *Cb-tra(ed24ts)*, possesses a sperm-producing, one-armed gonad, an abnormal male tail (*) with reduced sensory rays and spicules, and often displays a protruding vulva phenotype (not shown).

using *Cb-cby-7* (Fig. 4) (C. Carvalho, pers. comm.). Although this strain maps to the *Ce-tra-3* syntenic region of *C. briggsae* chromosome IV, the phenotype of *Cb-tra(ed24ts)* mutants at 25°C is slightly more masculinized than *Ce-tra-3* mutants (HODGKIN and BRENNER 1977). In addition, *Cb-tra(ed24ts)* behaves like a typical zygotic recessive allele, whereas *Ce-tra-3* mutants are only observed in the F_3 due to

maternal rescue of the F_2 *tra* homozygotes. Despite the phenotypic and genetic differences, a *Cb-tra-3*-specific DNA polymorphism assay performed on *Cb-tra(ed24ts)* mutants indicates that *Cb-tra(ed24ts)* is a likely allele of *Cb-tra-3* (Fig. 14). Surprisingly, during the AF16/HK104 polymorphism assay, only approximately 3% of the F_2 progeny were observed to be Tra, even though 25% of the animals should be *Cb-tra(ed24ts)* homozygotes following a single HK104 cross and subsequent F_1 hermaphrodite selfing. Also, many obvious intersexual animals were observed. Therefore, it appears that the majority of *Cb-tra(ed24ts)* homozygotes are naturally suppressed in the HK104 genetic background.

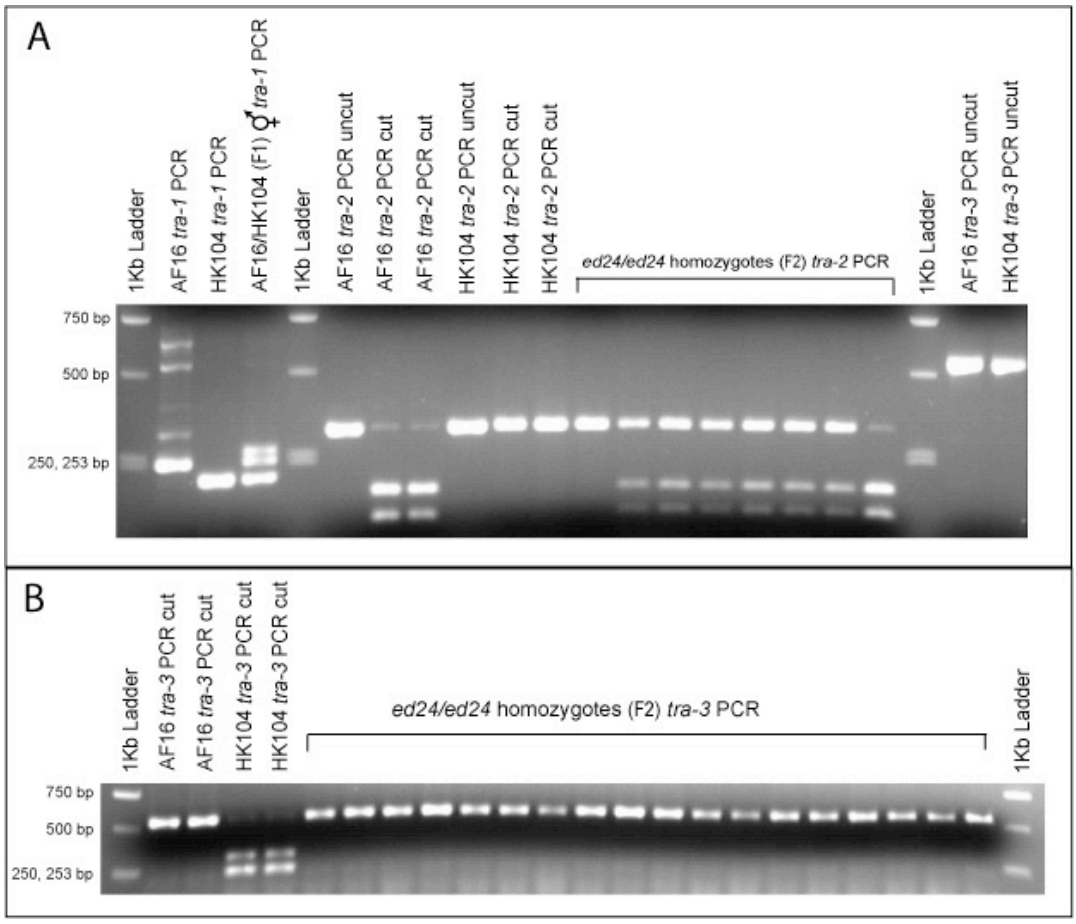

Figure 14. *Cb-tra(ed24ts)* is linked to a *Cb-tra-3* DNA polymorphism. (A) The results of *Cb-tra-1* single worm-derived PCR shows that the F_1 hermaphrodite carried both AF16 and HK104 sequences following a single round of crossing with HK104. PCR of the F_2 *tra* homozygotes with *Cb-tra-2* specific primers and subsequent restriction digestion shows non-linkage of *Cb-tra(ed24ts)* with the *Cb-tra-2 locus*. (B) When the *Cb-tra-3* assay was performed on several F_2 *tra*, all of the animals were homozygous for the *Cb-tra-3* AF16 sequence.

CHAPTER 4: CONCLUSIONS/DISCUSSION

4.1 Isolation, Characterization, and Role of *Cb-tra* Mutants.

Reconstruction of the *C. briggsae* sex determination pathway is necessary to test the hypothesis that the evolution of hermaphroditism in nematodes was convergent and it will serve as the foundation to study mating system change between *C. briggsae* and *C. remanei*. Therefore, in order to aid in the reconstruction of the *C. briggsae* sex determination pathway, forward F_2 screens were performed to identify *Cb-tra* mutants. Previous RNA interference (RNAi) studies have suggested that F_2 EMS mutagenesis in *C. briggsae* would yield *Cb-tra-2* and *Cb-tra-1* mutants (E. Haag pers. comm., HAAG and KIMBLE 2000). In addition, novel, zygotically acting Tra alleles could potentially be recovered, due to the unbiased nature of forward screening. Through the screening of approximately 10,000 haploid genomes, three distinct *C. briggsae* genetic linkage groups, which map to the syntenic regions of the three known *C. elegans tra* homologs, have been identified.

Based on morphology, pseudomales of linkage group III and linkage group II resemble *Ce-tra-1* and *Ce-tra-2* mutants, respectively. In addition, *Cb-tra-1* and *Cb-tra-2* DNA polymorphism assays have linked several mutants to their candidate gene. Finally, sequence analysis from several alleles in linkage group II and one allele in linkage group III has identified a variety of DNA lesions in these alleles that could affect the function of their candidate TRA protein. Therefore, based on morphology, genetic linkage (synteny with *C. elegans tra* homologs), molecular linkage, and sequence analysis, alleles of linkage group III and linkage group II appear to be mutants of *Cb-tra-1* and *Cb-tra-2*, respectively. At this point, definitive nulls have

not been confirmed in either *Cb-tra-1* or *Cb-tra-2*, due to the lack of known chromosomal deficiencies in *C. brigssae*. However, *Cb-tra(nm2)* and *Cb-tra(nm1)* are potential null alleles of *Cb-tra-1* and *Cb-tra-2*, respectively, based on the nature of their Tra phenotypes, their *tra* molecular lesions, and RNAi studies (*Cb-tra-1* only).

Although mutants that map to chromosome IV (syntenic region of *Ce-tra-3*) were not expected to be isolated through F_2 screens, because *Ce-tra-3* is maternally rescued, *Cb-tra(ed24ts)* was found to be a member of linkage group IV (C. Carvalho, pers. comm.). Phenotypically, homozygous *Cb-tra(ed24ts)* animals bear a strong resemblance to *Cb-tra-2* animals. However, this allele has been linked to *Cb-tra-3* through a DNA polymorphism assay. Therefore, based solely on its genetic and molecular linkage results, *Cb-tra(ed24ts)* is potentially a zygotically acting allele of *Cb-tra-3*.

As far as function is concerned, the role of *Cb-tra-1*, *Cb-tra-2*, and potentially *Cb-tra-3* in promoting female fates is largely conserved between *C. elegans* and *C. briggsae*. In addition, *C. elegans* (HODGKIN 1987), *P. pacificus* (PIRES-DASILVA and SOMMER 2004), and *C. briggsae tra-1* mutants share the inability to maintain spermatogenesis. However, in spite of these similarities, the role of *tra-1* during male spermatogenesis and during gonadogenesis appears to have changed between *C. briggsae* and *C. elegans*.

First, with respect to spermatogenesis, *tra-1* may play a greater role in the maintenance of sperm production in *C. briggsae* XO animals than it does in *C. elegans* XO animals, based on the RNAi results presented above. Since a minor

sperm activation role for *tra-1* has previously been suggested in *C. elegans* (CHEN and ELLIS 2000), it is plausible that *Cb-tra-1* may utilize these proposed sperm activation sites to a greater extent in order to significantly promote spermatogenesis in *C. briggsae* males (R. Ellis pers. comm., CHEN *et al.* 2001). Second, with respect to gonadogenesis, *C. briggsae tra-1* does not appear to play a role during gonadogenesis, since *Cb-tra(nm2)* animals (putative TRA-1 nulls) do not possess defective gonads (this study and L. Mathies, pers. comm.). Therefore, although Mathies *et al.* (2004) have recently proven that *Ce-tra-1* plays an active role in gonadogenesis, this *tra-1* function does not appear to be conserved between *C. elegans* and *C. briggsae*. This finding casts doubt on the hypothesis that the original role of *tra-1* was related to gonadogenesis.

In addition to the *Cb-tra-1* discrepancies described above, should *Cb-tra(ed24ts)* be found to possess a *Cb-tra-3* mutation, the Tra phenotype associated with this allele would be more severe than *Ce-tra-3* mutants. Also, the observation that *Cb-tra(ed24ts)* acts zygotically would indicate that all *C. briggsae* hermaphrodites are required to produce their own *tra-3* transcripts. Theoretically, production of the cytoplasmic, C-terminal portion of the TRA-2 protein may only be possible through TRA-3 cleavage in *C. briggsae*, due to the fact that *C. briggsae* does not possess an equivalent transcript of the 1.8 kb *Ce-tra-2* message (KUWABARA 1996a). In *C. elegans*, the 1.8 kb transcript encodes the cytoplasmic, C-terminal region of the TRA-2 protein; therefore, *C. elegans* could produce this portion of the protein without TRA-3 cleavage. To this end, *Cb-tra-3* may act zygotically to compensate for the lack of the 1.8 kb *tra-2* transcript and may possess a more severe Tra phenotype than

Ce-tra-3 alleles because the presence the 1.8 kb *tra-2* transcript in *C. elegans* may lessen its dependence on the TRA-3 protein during sex determination.

In summary, the *C. briggsae* homologs of *tra-1*, *tra-2*, and possibly *tra-3* have been isolated through forward F_2 screens. Upon further characterization, the function of the *tra* genes between *C. elegans* and *C. briggsae* appears to be largely conserved. However, notable differences between these two species, with respect to the role of *tra-1* and potentially the role of *tra-3,* have been observed. Perhaps most importantly, reconstruction of the *C. briggsae* sex determination pathway, for the purposes of studying the convergent evolution of hermaphroditism and mating system change in nematodes, not only requires a solid understanding of the role *Cb-tra* mutants play in sex determination, but it also requires the mutants themselves.

For example, *Cb-tra* suppressor screens will be necessary to isolate downstream components of the *C. briggsae* sex determination pathway, while epistasis analysis between *Cb-tra* mutants and sex mutants of an opposing phenotype will be critical for determining the order in which these components act within the pathway. Recently, both of these techniques have been performed using some of the available *Cb-tra* alleles. *Cb-tra-2* suppressor screens, using both *Cb-tra(ed23ts)* and *Cb-tra(nm9ts)*, have resulted in the isolation of many self-fertile *Cb-tra-2* suppressors (C. Carvalho and J. Salogiannis, pers. comm.). In addition, epistasis analysis between *Cb-tra(ed23ts)* and *Cb-fem-2(nm27)* revealed that *Cb-fem-2* acts downstream of *Cb-tra-2* (C. Carvalho, pers. comm.). Therefore, the use of *Cb-tra* mutants in the reconstruction of the *C. briggsae* sex determination pathway through epistasis analysis and suppressor screen analysis has clearly shown that the order of *Cb-tra-2*

and *Cb-fem-2* is conserved between *C. elegans* and *C. briggsae* and that the components immediately downstream of TRA-2 are not necessary to perform the *C. briggsae* sperm/oocyte switch.

4.2 Natural Suppression of *Cb-tra(ed24ts)* in the HK104 Background.

Several sex determination protein interactions, such as FEM-3/TRA-2 and TRA-1/TRA-2, have been shown to be conserved, but species-specific in *C. elegans*, *C. briggsae*, and *C. remanei* (HAAG *et al.* 2002; WANG and KIMBLE 2001). In addition, S. E. Baird (2002) has shown that *C. remanei* interspecies hybridizations with AF16 or HK104 produce varied XO sex reversal phenotypes depending on which *C. briggsae* strain is used. According to S. E. Baird (pers. comm.) a population-specific *Cb-tra-2* polymorphism has been linked to this intra-species variation. Interestingly, the ray pattern of XO males in the two *C. briggsae* populations is also variable (BAIRD *et al.* 2005). In addition, the results shown in this thesis indicate that the Tra phenotype of *Cb-tra(ed24ts)* homozygotes is suppressed by natural variants in the HK104 background. Taken together, the evidence presented thus far suggests that the interactions of sex determination pathway components in the two geographically isolated populations of *C. briggsae*, AF16 and HK104, may not be identical and that these two populations may be cryptic species within *C. briggsae*.

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