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Rapid Coevolution of the Nematode Sex-Determining Genes fem-3 and tra-2

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Summary

Unlike many features of metazoan development, sex determination is not widely conserved among phyla [1–5]. However, the recent demonstration [4] that one gene family controls sexual development in Drosophila, C. elegans, and vertebrates suggests that sex determination mechanisms may have evolved from a common pathway that has diverged radically since the Cambrian. Sex determination gene sequences often evolve quickly (e.g., [5, 6, 7]), but it is not known how this relates to higher-order pathways or what selective or neutral forces are driving it. In such a rapidly evolving developmental pathway, the fate of functionally linked genes is of particular interest. To investigate a pair of such genes, we cloned orthologs of the key C. elegans male-promoting gene fem-3 from two sister species, C. briggsae and C. remanei. We employed RNA interference to show that in all three species, the male-promoting function of fem-3 and its epistatic relationship with its female-promoting upstream repressor, tra-2, are conserved. Consistent with this, the FEM-3 protein interacts with TRA-2 in each species, but in a strictly species-specific manner. Because FEM-3 is the most divergent protein yet described in Caenorhabditis and the FEM-3 binding domain of TRA-2 is itself hypervariable [8, 9], a key protein-protein interaction is rapidly evolving in concert. Extrapolation of this result to larger phylogenetic scales helps explain the dissimilarity of the sex determination systems across phyla.

Results and Discussion

The System

The sexual dimorphisms of C. remanei (dioecious) and C. briggsae (hermaphroditic) are similar to those of C. elegans (Figure 1). As in C. elegans, the haploid chromosome number in C. remanei (Figure 1C) and C. briggsae [10] is six. A quantitative PCR assay (Figures 3E and 3F) indicates that the X chromosome dose of C. remanei females is twice that of males, as it is in C. briggsae [11].

Syntenic Cloning of fem-3 Homologs

The physical interaction of the FEM-3 and TRA-2 proteins [12] is probably the major way in which TRA-2 promotes female mates, and TRA-2 homologs [8, 9] are notable for the hypervariability of their FEM-3 binding domains. Similar sequence divergence likely thwarted previous efforts to identify fem-3 homologs, leading us to use a genomics-based synteny approach [13]. Attempts to identify fem-3 homologs by using the C. elegans ion channel gene twk-19 were thwarted by a breakage in syneny relative to C. elegans in both C. briggsae and C. remanei (Figure 2A). We next used the predicted C. elegans gene C01F6.1, which lies 7 kb from Ce-fem-3 and encodes a copine family protein [14]. Low-stringency hybridization with a C01F6.1 fragment produced single bands on C. briggsae and C. remanei genomic Southern blots (our unpublished data). This probe was then used to identify C. remanei and C. briggsae genomic clones that contained homologs of both C01F6.1 and fem-3. The gene structures (see the Supplementary Material available with this article online) and amino acid sequences (Figures 2B and 2C) were determined by the cloning of cDNAs from both high-stringency library screens and RT-PCR.

The FEM-3 proteins are remarkably divergent and range from 31.2% to 37.5% pairwise amino acid identity (Figures 2C and 2D). This makes fem-3 the most rapidly evolving worm sex determination gene [7–9, 15–18] and thus puts it among the most rapidly evolving loci in the genome [19]. In addition to many amino acid substitutions, there is significant length variation due to start codon location and internal indels unique to each species. Patches of conservation in FEM-3 do exist, but sensitive Hidden Markov Modeling searches [20] using the alignment failed to reveal any other homologs. The residues affected in both null and temperature-sensitive missense mutations [21] are in three cases identical in two of three species; in one case, they are conserved in all three. However, none are located in the few blocks of strongest conservation, defined as four or more consecutive, universally conserved amino acids.

The Ce-fem-3 3’ UTR contains the point mutation element (PME), whose interaction with the FBF RNA binding proteins [22] is required for cessation of hermaphrodite spermatogenesis [23]. The longest sequence common to the fem-3 3’ UTR of all three species was a 9 nt stretch (Figure 2E) that partially overlaps the canonical PME and includes another residue affected in an unpublished fem-3(3f) allele (A. Spence, personal communication). Because the exact nucleotides of the 3’ UTR contacted by FBF are not known and the conserved region has been present in all experiments demonstrating a PME/TFB interaction, this conserved motif may be as important for FBF binding as the PME itself. The Ce-fem-3 5’ UTR is notable for the presence of a 17 nt perfect inverted repeat [21], but no such element exists in Cr-fem-3 or Cb-fem-3.

Cb-fem-3 and Cr-fem-3 Are Required for Male Somatic Fates

The extreme divergence of FEM-3 called into question the conservation of its male-promoting role. To assess this, we used RNA interference [24, 25] to reduce the activity of Cb-fem-3 and Cr-fem-3 (Figure 3). Half of the
Cr-fem-3(RNAi) worms were normal, fertile females, and half were apparently male worms with varying degrees of somatic feminization. That they represented XX and XO animals, respectively, was verified through a novel quantitative PCR assay that directly measures the relative dosage of X chromosomes to autosomes (Figure 3E). Somatic feminization of XO animals could be rendered complete by high concentrations of injected RNA (Figures 3C and 3D), was insensitive to growth at elevated temperature (Table 1), and was never accompanied by the production of oocytes. A small but consistent fraction of female Cr-fem-3(RNAi) animals had one-armed gonads (the Oag phenotype), multiple vulvae (Muv), or undifferentiated germ cells well into adulthood (our unpublished data). To be certain they were not extremely feminized XO animals, we employed the quantitative single-worm PCR assay, and all abnormal females were XX (Figure 3F). Such low penetrance effects of lowered fem-3 activity on normal female development have not been reported in *C. elegans*.

We also examined Cb-fem-3(RNAi) animals (Figure 3G, Table 1). The progeny of unmated mothers were completely unaffected by injection with double-stranded Cb-fem-3 dsRNA, as judged by somatic anatomy and the ability to produce abundant self progeny. This differs dramatically from XX Ce-fem-3(RNAi) animals, which like Ce-fem-3 mutants [26] are 100% Fog (Figure 4H; Table 1). No Fogs were seen after coinjection with Cb-fem-2 dsRNA, which on its own also fails to feminize hermaphrodite germlines [18]. To be certain that our Cb-fem-3 dsRNA preparation was effective and to assess whether male somatic tissues were sensitive to it, we injected mated hermaphrodites. The XO progeny produced were roughly half wild-type and half variably feminized. This penetrance was not sensitive to dose or temperature (our unpublished data). As with Cr-fem-3(RNAi), XO Cb-fem-3(RNAi) animals never produced oocytes.

Assessing the Conservation of the tra-2/fem-3 Interaction

Rapid evolution of FEM-3 and TRA-2 could reflect a gain or loss of physical interactions in evolution, compensatory coevolution of binding partners, selection for different affinities or other variables, or simply relaxed constraint. To distinguish among these possibilities, we employed the yeast two-hybrid system to perform directed tests for interaction with all possible combinations of FEM-3 and TRA-2 homologs. All three conspecific TRA-2/FEM-3 pairs interacted strongly in yeast, as judged by β-galactosidase expression (Figure 4A). None of the cross-species combinations produced any activity above that seen in the negative control strains (our unpublished data), and the positive interactions were within a 4-fold range of each other (Figure 4B).

As a more genetic test of the tra-2/fem-3 functional relationship, simultaneous RNAi against both genes was employed in a pseudo-epistasis experiment. If the primary function of tra-2 activity in females is to repress fem-3 activity, then the absence of fem-3 should suppress the masculinization of tra-2(RNAi) XX animals [8, 9]. *Cb-tra-2/Cb-fem-3(RNAi) and Cr-tra-2/Cr-fem-3(RNAi) animals were generated, and in both cases the somatic phenotypes of tra-2(RNAi) alone were completely rescued (Table 1; Figures 4C and 4D). Interestingly, the germline masculinization of *Cr-tra-2(RNAi)* [9] and *Cb-tra-2(RNAi)* (Table 1) animals was not suppressed. The overwhelming phenotype of XX *Cr-tra-2/Cr-fem-3(RNAi)* animals was therefore Mog, a perfect male soma with a completely masculinized germline (Figure 4D; Table 1). The low penetrance of *Cb-tra-2(RNAi)* XX masculinization predicts that most of the XX double RNAi progeny would be self-fertile hermaphrodites, which they were. However, a Mog population also appeared at a frequency of 20% (Table 1). To be certain that the two dsRNAs were not somehow interfering with each other, we repeated the double RNAi experiment with mated mothers and found the expected phenotype of *Cb-fem-3(RNAi)* alone in the XO progeny (Table 1).

These data show that fem-3 is required for proper male somatic fates in both *C. briggsae* and *C. remanei* and that fem-3 function is regulated by its interaction with tra-2, as in *C. elegans*. Conservation of the tra-2/fem-3 interaction in all three species makes it unlikely that the sequence change seen in these genes is driven by selection to add or remove the interaction between them. The lack of any cross-species two-hybrid interac-

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**Figure 1. Features of Sexual Dimorphism in *C. remanei***

(A) Inseminated female, with medial vulva bearing copulatory plug (arrowhead) and elongated tail (to left, bracket). Male-derived sperm (s) are visible in the two spermathecae flanking the vulva. The posterior gonad arm is filled proximally with oocytes (o); the anterior arm lies beneath the gut (g). This animal bears an embryo (e) in the posterior half of the uterus (u).

(B) Adult male, showing modified tail (bracket) and single-armed testis with sperm (s), g. gut.

(C) *C. remanei* has six pairs of chromosomes. Diakinesis oocyte nuclei were stained with DAPI, and in all cases six bivalents were visualized. One of these is the X as judged by chromosome-specific quantitative PCR (see Figures 3E and 3H). The chromosomes of one nucleus are circled in yellow. Scale bars = 50 μM.
Figure 2. Evolution of fem-3 and Surrounding Region

(A) Synteny is largely intact in all three species, but the twk-19 ion channel gene is greatly separated from the microfilarial antigen in C. briggsae and C. remanei relative to C. elegans. Hybridization experiments indicate that it remains linked in C. briggsae (data not shown), consistent with an inversion or insertion/deletion event. Although Cb/Cr-twk-19 is depicted as being in the conserved orientation relative to C. elegans, it may be inverted. Also note the absence in C. briggsae and C. remanei of the cytochrome p450 homolog nearest fem-3 in C. elegans. "Nov con" means the gene in question is novel, but conserved beyond nematodes.

(B) Alignment of FEM-3 homologs. Residues conserved in two or more species are boxed in black, and conservative substitutions are boxed in gray. Alignment was initially generated by the Pileup program of the GCG Wisconsin Package, then manually edited to force amino acids 324–333 of Cb-FEM-3 and amino acids 355–364 of Cr-FEM-3 into alignment. Red octagons indicate residues mutated to stop codons in the null Ce-fem-3 alleles e1996, e2037, and e2068 [21, 26]. Asterisks mark residues affected in the null missense Ce-fem-3 allele e2063 (Thr142) and the temperature-sensitive Ce-fem-3 alleles e2006, e2143, and q77 [21, 26]. Both Cb-fem-3 and Cr-fem-3 sequences have been deposited with GenBank under accession numbers AY143174 and AY142113, respectively.

(C) Pairwise sequence identities of the homologs as aligned above. The amino acid alignment was used as a guide for aligning coding sequences, and similarities were calculated with the Distances program of the GCG Wisconsin Package.

(D) The longest exact sequence found in all three 3' H11032 UTRs is a 9 nt stretch that overlaps the PME on its 3' end (large red box). Another potentially conserved nucleotide, the 5' most PME uridine, is also boxed. The three residues that are mutated in the Ce-fem-3(gf) alleles that initially defined the PME [23] are marked with blue asterisks. The red asterisk marks a residue mutated in an additional Ce-fem-3(gf) allele (A. Spence, personal communication).

tion also eliminates a model in which the binding partner sequences are largely unconstrained. The inability of conserved FEM-3 domains to mediate an interaction with TRA-2 indicates that independent lineages evolve internally compatible but externally incompatible solutions. Such concerted evolution could enable the sex determination pathway to be an early arising, if inadvertent, source of reproductive isolation. Consistent with this, XO C. briggsae-C. remanei hybrid animals are feminized [11], which may be due to an inability of heterologous forms of sex determination factors to interact in vivo.

Our results cannot address the possibility that selection for differences in details of the tra-2/fem-3 interaction, such as binding constants or stoichiometric composition, may be optimized to suit fleeting or lineage-specific
conditions. The TRA-2/FEM-3 complex may involve other proteins as well; FEM-2/FEM-3 [27] and TRA-2/TRA-1 [28, 29] interactions have been reported. The latter interaction is also conserved in *C. briggsae* and, like TRA-2/FEM-3, is species-specific [29], even though the domains that mediate the TRA-2/TRA-1 interaction are well conserved relative to the case in question here. This suggests that only a few residues need be changed to produce a species-specific interaction.

**Do the fem Genes Have Variable Germline Roles?**

RNAi-based studies of *fem-1* (A. Spence, personal communication), *fem-2* [18], and *fem-3* (this work) in non-*Caenorhabditis* species have also failed to produce the germline sexual transformations expected from the mutant phenotypes of their *C. elegans* homologs. However, *Ce-fem-2*(RNAi) [18] and *Ce-fem-3*(RNAi) (Figure 3H) produce highly penetrant germline phenotypes, so *fem* germline function can be affected by RNAi. In addition, *Cr-tra-2*(RNAi) ([9]; Figure 4C), *Cb-tra-2*(RNAi) ([8], Table 1), *Cb-glp-1*(RNAi), and *Cr-glp-1*(RNAi) animals all manifest germline phenotypes [30], so *C. briggsae* and *C. remanei* germlines are not generally refractory to RNAi. Underscoring the distinct nature of this process, numerous *C. elegans* mutations affect only germline sex determination [31–38]. Taken to-

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**Figure 3. fem-3*(RNAi)* Phenotypes in Three Species**

At a concentration of 1.5 μg/μL (A, B), *Cr-fem-3* dsRNA produced intersexual XO animals with nearly normal male (not shown) or largely feminized (B) tails. Gonad morphologies included both two reflexed arms and asymmetric gonads with one large arm (la) and one small arm (sa, [A]). Protruding vulvae (pv) were common (A). At a concentration of 4 μg/μL, *Cr-fem-3* dsRNA completely feminized the soma of XO animals, whose tails were elongated (C) and whose complete vulvas (D) were capable of supporting insemination by males, as judged by the presence of a copulatory plug (arrowhead). In no case were oocytes produced, and abundant sperm were made even in animals with completely feminized somas (D), s, sperm. The scale bar represents 50 μm in panels (A) and (D) and 31.25 μm in panels (B) and (C). (E) Quantitative PCR assay to demonstrate that intersexual *Cr-fem-3*(RNAi) animals are XO. Each data point represents the ratio of signal from an autosome-derived PCR product to that of a simultaneously amplified X-derived product, with a single worm used as a template. Control animals were adult progeny from uninjected mothers; wild-type females and intersexual worms were siblings from the same *Cr-fem-3* dsRNA-injected mothers. See the Experimental Procedures for assay details. (F) Use of the quantitative PCR assay to demonstrate that abnormal *Cr-fem-3*(RNAi) females are XX. Both Oag and Muv females were tested (see text). (G) Similar intersexual phenotypes, including a protruding vulva and a partially feminized tail, were observed in XO *Cb-fem-3*(RNAi) animals. (H) *Ce-fem-3*(RNAi) XX animals have feminized germlines.
Table 1. Summary of RNA Interference Experiments

<table>
<thead>
<tr>
<th>Target gene(s) (N)</th>
<th>XO Progeny Phenotypes</th>
<th>XX Progeny Phenotypes</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Intersex¹</td>
</tr>
<tr>
<td>Cr-fem-3</td>
<td>3.6%</td>
<td>44.5%</td>
</tr>
<tr>
<td>(1017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr-fem-3</td>
<td>51.9% (combined³)</td>
<td>0%</td>
</tr>
<tr>
<td>(257) (208)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr-tra-2²</td>
<td>63.1%</td>
<td>0%</td>
</tr>
<tr>
<td>(236)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr-fem-3 &amp; Cr-tra-2 (209)</td>
<td>56.0% (combined³)</td>
<td>0%</td>
</tr>
<tr>
<td>Ce-fem-3 unmated (≥300)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cr-fem-3 unmated (≥300)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cb-fem-3 unmated (273)</td>
<td>19.0%</td>
<td>17.9%</td>
</tr>
<tr>
<td>Cb-tra-2 unmated (244)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cb-fem-3 &amp; Cb-tra-2 unmated (140)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cb-fem-3 &amp; Cb-tra-2 mated (207)</td>
<td>15.5%</td>
<td>22.2%</td>
</tr>
<tr>
<td>Cb-fem-3 &amp; Cb-fem-2 unmated (≥200)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

¹Intersex animals had a range of somatic feminization, including vulvas (often protruding) and two-armed gonads. None produced oocytes.
²Both germline and soma were feminized.
³Includes only animals with a clear spermatogenic testis, no vulva, and at least a partially masculinized tail.
²Individual classes were not counted; the two progeny classes were grossly similar to those in the above experiment.
⁴Data recompiled from experiments in [9].
⁵Includes animals that had a delayed sperm-oocyte switch but were eventually self-fertile.
⁶Includes animals with female or disorganized somatic gonads. These animals produced large numbers of sperm, had vulval defects, or had partially masculinized tails. Some of these animals eventually produced oocytes as well.

Together, these results suggest that the unexpected RNAi phenotypes noted above are biologically meaningful and that sex determination is even more rapidly reconfigured in the germline than in the soma. In particular, the Cr-tra-2(RNAi)/Cr-fem-3(RNAi) results imply that Cr-tra-2 may act independently of Cr-fem-3 in the germline. Such a fem-3-independent mechanism, mediated by a direct TRA-2/TRA-1 interaction, was recently discovered in C. elegans [28, 29]. However, we note that RNAi is an incomplete test of gene function. Definitive statements will therefore require the isolation of null mutations in non-elegans species.

What Forces Drive Compensatory Coevolution in Sex Determination?

A few cases of rapid concerted evolution of interacting proteins have been documented—for example, those mediating fertilization specificity in marine invertebrates (reviewed in [39]). Here, prevention of gamete loss via nonproductive hybridization provides a plausible source of diversifying selection. However, blocking hybridization post-zygotically yields no increase in fitness to the parents because the gametes are already wasted and there is no further parental investment. What, then, drives concerted evolution of the tra-2/fem-3 interaction? Viable hypotheses must provide a continual change-promoting force independent of the sexual phenotype of a species. Genomic conflict is one such force. This can arise when the optimal sex ratio is different from the perspectives of maternal-effect (i.e., parental) and zygotic (i.e., offspring) genes or when sex determination is manipulated by cytoplasmic organelles or parasites whose transmission is enhanced by female-biased sex ratios (reviewed in [40]). If these conflicts create suboptimal host sex ratios, suppressing variants of sex determination genes would be favored by selection. This could initiate a “Red Queen” process in which a constant sexual phenotype masks rapid change in the components that produce it. Maternal-zygotic conflict requires the existence of inbreeding [41] or divergent costs of producing progeny of each sex ([42], pp. 142–143). As inbreeding is the norm in hermaphroditic nematodes, this may be relevant. Cytoplasmic sex-manipulating factors have not been described in Caenorhabditis, but the intracellular bacterium Wolbachia does infect filarial nematodes [43].

Whatever the forces driving the evolution of sex determination systems, data now exist to show how they go from initial identity to nearly complete disparity. In early stages, demonstrated by intra-genus comparisons, sex determination is accomplished by homologous but rapidly diverging genes (e.g., [7, 44, 45]; this work). In more distantly related taxa, diversity is seen in the primary sex-determining signal [1, 46] and in the role of specific sex determination genes [47], whereas other essential features are conserved. In distinct phyla, only a few similarities remain. Although the evolution of sex determination is especially rapid, many developmental processes show similar divergence in their underlying mechanisms [48, 49]. An understanding of such change is crucial to complement examples of deep conservation and to complete our view of how development evolves.
Figure 4. Conservation and Species Specificity of the tra-2/fem-3 Interaction

(A) Yeast two-hybrid interaction assay. Yeast bearing a LexA binding site upstream of a lacZ reporter were transformed with various pairs of plasmids, one encoding a LexA DNA binding domain (DBD) fusion with FEM-3 or lamin control, and another a GAL4 activation domain (AD) fusion with TRA-2c or the plain activation domain vector pACT2. Interaction between the hybrid proteins is indicated by activation of the lacZ reporter.

(B) Quantitation of the three species-specific interactions. Liquid assays were performed on four independent transformants of each plasmid pair. E indicates C. elegans constructs; B indicates C. briggsae constructs; and R indicates C. remanei constructs. The stronger activity of the C. briggsae strains was consistent over several assays, but the small difference between C. elegans and C. remanei was not.

(C) Cr-tra-2(RNAi) XX animal, showing masculinization of both somatic gonad and germ cells. The gonad is a single, reflexed arm, and all differentiated germ cells are sperm (s).

(D) Suppression of somatic, but not germline, masculinization of Cr-tra-2(RNAi) XX animals by Cr-fem-3(RNAi). Note the two proximal gonad arms full of sperm (s) and the normal vulva with copulatory plug (arrowhead), indicating a male has mated with this animal.

Supplementary Material

Supplementary material, including primer sequences, Experimental Procedures, and a supplementary figure, are available with this article online at http://images.cellpress.com/supmat/supmatin.htm.

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