Current Biology, Vol. 12, 2035–2041, December 10, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)01333-7

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

Eric S. Haag,[1,2,3,4] Shanping Wang,[2] and Judith Kimble,[1,2]
[1]Department of Biochemistry and
[2]Howard Hughes Medical Institute
University of Wisconsin
433 Babcock Drive
Madison, Wisconsin 53706

Summary

Unlike many features of metazoan development, sex determination is not widely conserved among phyla [1–3]. 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 fates, 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 synteny 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(gf)* 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/FBF 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 *Cb-fem-3* or *Cr-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

[3]Correspondence: ehaag@wam.umd.edu
[4]Present address: Department of Biology, University of Maryland, College Park, Maryland 20742.

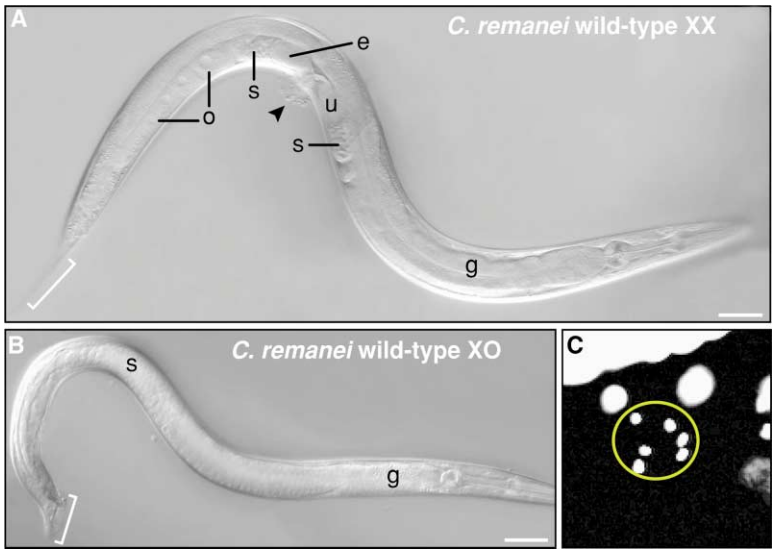

Figure 1. Features of Sexual Dimorphism in *C. remanei*

(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.

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* RNA, 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)* ([8]; Table 1) animals was not suppressed. The overwhelming phenotype of XX *Cr-tra-2/Cr-fem-3(RNAi)* animals was therefore Mog, a perfect female 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-

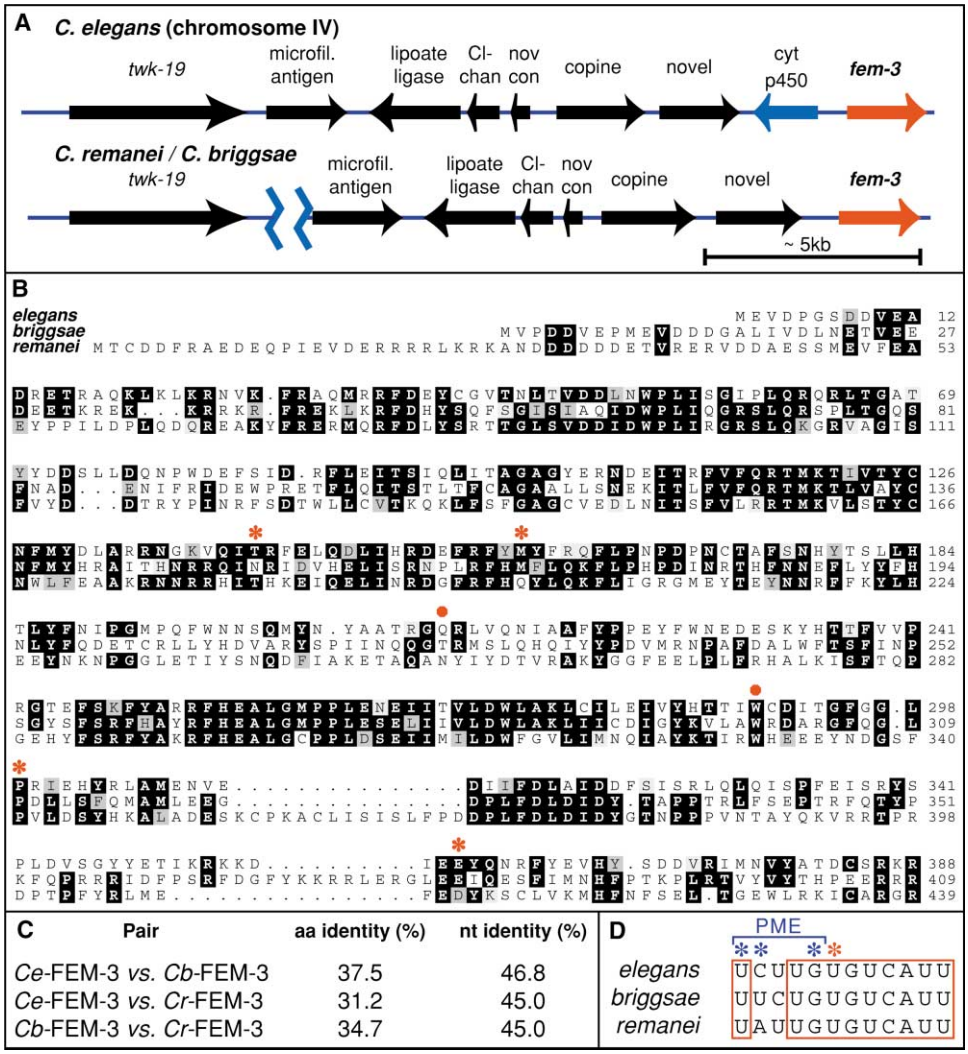

Figure 2. Evolution of *fem-3* and Surrounding Region

(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′ 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 uradine, 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

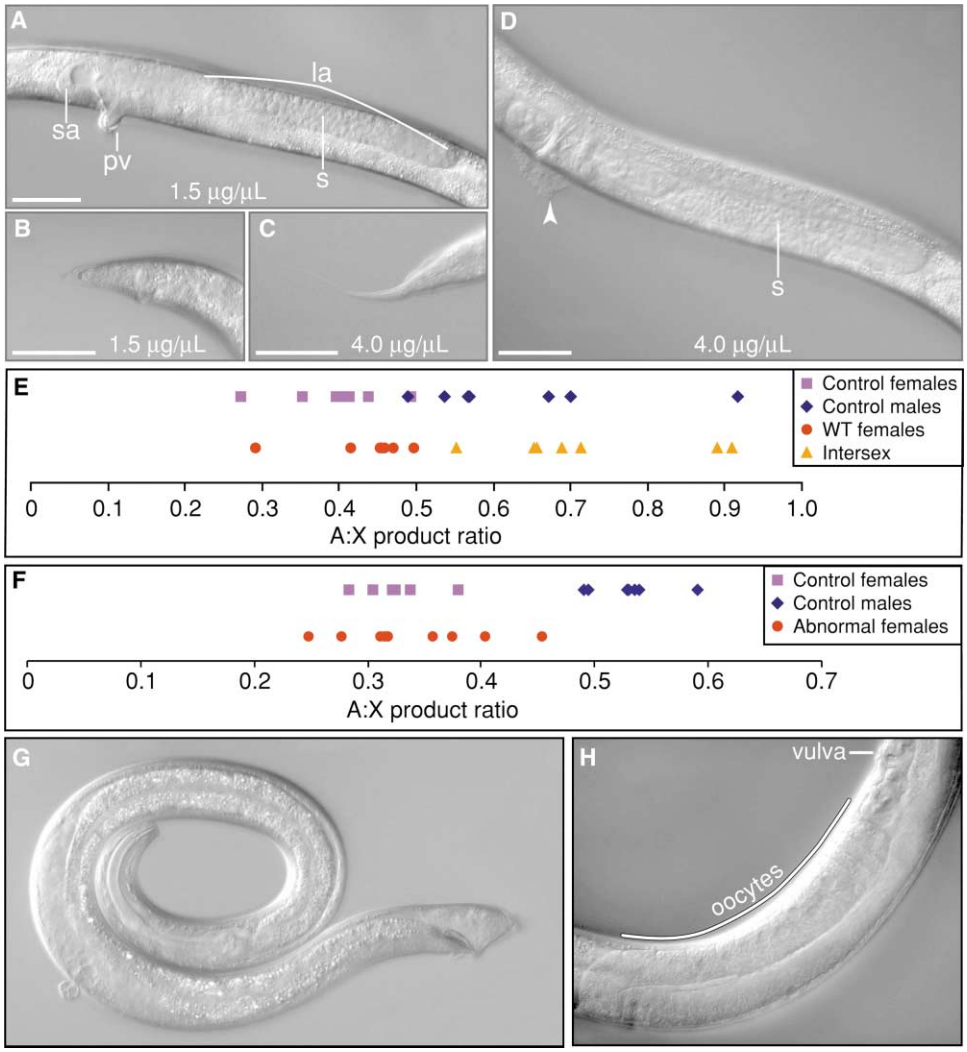

Figure 3. *fem-3(RNAi)* Phenotypes in Three Species

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.

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-*elegans 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-

Table 1. Summary of RNA Interference Experiments

Target gene(s) (N)	XO Progeny Phenotypes			XX Progeny Phenotypes					
	WT	Intersex[a]	Fem[b]	WT	Oag, Muv	Fog	Intersex	Mog	Tra[c]
Cr-fem-3 (1017)	3.6%	44.5%	0%	49.7%	2.2%	0%	0%	0%	0%
Cr-fem-3 (25°) (208)	51.9% (combined[d])		0%	48.1% (combined[d])		0%	0%	0%	0%
Cr-tra-2[e] (236)	63.1%	0%	0%	6.4%	0%	0%	24.6%	0%	5.9%
Cr-fem-3 & Cr-tra-2 (209)	56.0% (combined[d])		0%	0%	0%	0%	0%	44.0%	0%
Ce-fem-3 unmated (>300)	–	–	–	0%	0%	100%	0%	0%	0%
Cb-fem-3 unmated (>300)	–	–	–	100%	0%	0%	0%	0%	0%
Cb-fem-3 mated (273)	19.0%	17.9%	0%	63.0%	0%	0%	0%	0%	0%
Cb-tra-2 unmated (244)	–	–	–	90.2%[f]	0%	0%	5.3%[g]	0%	4.5%
Cb-fem-3 & Cb-tra-2 unmated (140)	–	–	–	80.0%	0%	0%	0%	20.0%	0%
Cb-fem-3 & *Cb-tra-2* mated (207)	15.5%	22.2%	0%	51.2%	0%	0%	0%	11.1%	0%
Cb-fem-3 & *Cb-fem-2* unmated (>200)	–	–	–	100%	0%	0%	0%	0%	0%

[a] Intersex animals had a range of somatic feminization, including vulvas (often protruding) and two-armed gonads. None produced oocytes.
[b] Both germline and soma were feminized.
[c] Includes only animals with a clear spermatogenic testis, no vulva, and at least a partially masculinized tail.
[d] Individual classes were not counted; the two progeny classes were grossly similar to those in the above experiment.
[e] Data recompiled from experiments in [9].
[f] Includes animals that had a delayed sperm-oocyte switch but were eventually self-fertile.
[g] Includes 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.

gether, 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.

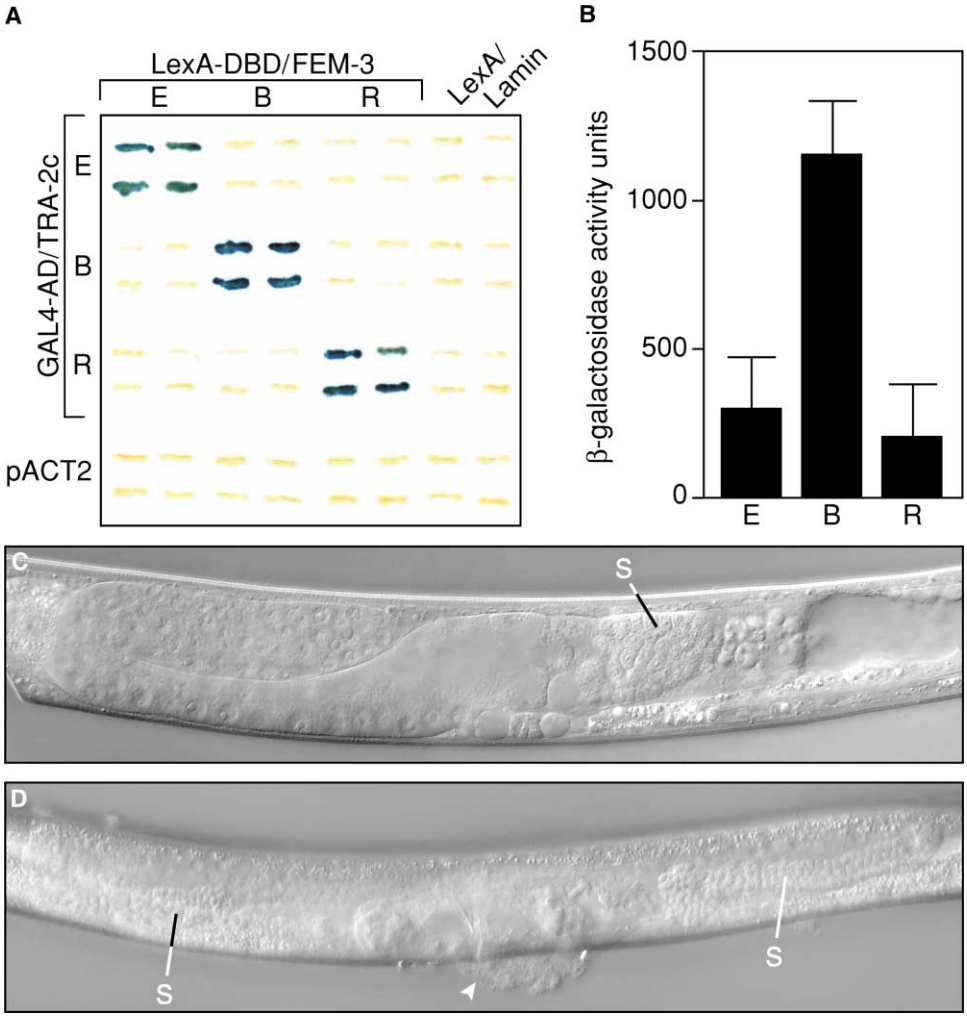

Figure 4. Conservation and Species Specificity of the *tra-2/fem-3* Interaction

(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.

Received: August 1, 2002
Revised: August 27, 2002
Accepted: September 23, 2002
Published: December 10, 2002

Acknowledgments

We thank S. LaMartina, V. Prahlad, C. Eckmann, and N. Buter for technical assistance and advice. We also thank D. Pilgrim (University of Alberta), U. Vivegananthan, and A. Spence (both from the University of Toronto) for sharing results prior to publication and all members of the Kimble Lab for their support. E.S.H. was supported by the Jane Coffin Childs Memorial Fund for Medical Research. J.K. is an Investigator with the Howard Hughes Medical Institute.

References

1. Schütt, C., and Nothinger, R. (2000). Structure, function, and evolution of sex-determining systems in Dipteran insects. Development *127*, 667–677.
2. Goodwin, E., and Ellis, R. (2002). Turning clustering loops: sex determination in *Caenorhabditis elegans*. Curr. Biol. *12*, R111–R120.
3. Graves, J.A.M., and Shetty, S. (2001). From W to Z: evolution of vertebrate sex chromosomes and sex determining genes. J. Exp. Zool. *290*, 449–462.

4. Zarkower, D. (2001). Establishing sexual dimorphism: conservation amidst diversity? Nature Rev. Genet. *2*, 175–185.
5. Whitfield, L., Lovell-Badge, R., and Goodfellow, P. (1993). Rapid sequence evolution of the mammalian sex-determining gene *SRY*. Nature *364*, 713–715.
6. O'Neil, M., and Belote, J. (1992). Interspecific comparison of the *transformer* gene of Drosophila reveals an unusually high degree of evolutionary divergence. Genetics *131*, 113–128.
7. de Bono, M., and Hodgkin, J. (1996). Evolution of sex determination in *Caenorhabditis*: unusually high divergence of *tra-1* and its functional consequences. Genetics *144*, 587–595.
8. Kuwabara, P.E. (1996). Interspecies comparison reveals evolution of control regions in the nematode sex-determining gene *tra-2*. Genetics *144*, 597–607.
9. Haag, E., and Kimble, J. (2000). Regulatory elements required for development of *C. elegans* hermaphrodites are conserved in the *tra-2* homologue of *C. remanei*, a male/female sister species. Genetics *155*, 105–116.
10. Nigon, V., and Dougherty, E. (1949). Reproductive patterns and attempts at reciprocal crossing of *Rhabditis elegans* Maupas, 1900, and *Rhabditis briggsae* (Nematoda: Rhabditidae). J. Exp. Zool. *112*, 485–503.
11. Baird, S. (2002). Haldane's Rule by sexual transformation in *Caenorhabditis*. Genetics *161*, 1349–1353.
12. Mehra, A., Gaudet, J., Heck, L., Kuwabara, P.E., and Spence, A.M. (1999). Negative regulation of male development in *Caenorhabditis elegans* by a protein-protein interaction between TRA-2A and FEM-3. Genes Dev. *13*, 1453–1463.
13. Kuwabara, P.E., and Shah, S. (1994). Cloning by synteny: identifying *C. briggsae* homologues of *C. elegans* genes. Nucleic Acids Res. *22*, 4414–4418.
14. Creutz, C., Tomsig, J., Snyder, S., Gautier, M., Skouri, F., Beisson, J., and Cohen, J. (1998). The copines, and novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from *Paramecium* to humans. J. Biol. Chem. *273*, 1393–1402.
15. Hansen, D., and Pilgrim, D. (1998). Molecular evolution of a sex determination protein. FEM-2 (pp2c) in *Caenorhabditis*. Genetics *149*, 1353–1362.
16. Streit, A., Li, W., Robertson, B., Schein, J., Kamal, I., Marra, M., and Wood, W. (1999). Homologs of the *Caenorhabditis elegans* masculinizing gene *her-1* in *C. briggsae* and the filarial parasite *Brugia malayi*. Genetics *152*, 1573–1584.
17. Chen, P., Cho, S., Jin, S., and Ellis, R. (2001). Specification of germ cell fates by FOG-3 has been conserved during nematode evolution. Genetics *158*, 1513–1525.
18. Stothard, P., Hansen, D., and Pilgrim, D. (2002). Evolution of the PP2C family in *Caenorhabditis*: rapid divergence of the sex-determining protein FEM-2. J. Mol. Evol. *54*, 267–282.
19. Kent, W.J., and Zahler, A.M. (2000). Conservation, regulation, synteny, and introns in a large-scale *C. briggsae-C. elegans* genomic alignment. Genome Res. *10*, 1115–1125.
20. Eddy, S. (2001). HMMER: profile hidden Markov models for biological sequence analysis. http://hmmer.wustl.edu/. (St. Louis, MO: Washington University).
21. Ahringer, J., Rosenquist, T., Lawson, D., and Kimble, J. (1992). The *Caenorhabditis elegans* sex determining gene *fem-3* is regulated post-transcriptionally. EMBO J. *11*, 2303–2310.
22. Zhang, B., Gallegos, M., Puoti, A., Durkin, A., Fields, S., Kimble, J., and Wickens, M.P. (1997). A conserved RNA binding protein that regulates sexual fates in the C. elegans hermaphrodite germ line. Nature *390*, 477–484.
23. Ahringer, J., and Kimble, J. (1991). Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3′ untranslated region. Nature *349*, 346–348.
24. Guo, S., and Kemphues, K.J. (1995). *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell *81*, 611–620.
25. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.
26. Hodgkin, J. (1986). Sex determination in the nematode *Caenorhabditis elegans*: analysis of *tra-3* suppressors and characterization of *fem* genes. Genetics *114*, 15–52.
27. Chin-Sang, I.D., and Spence, A.M. (1996). *Caenorhabditis elegans* sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3. Genes Dev. *10*, 2314–2325.
28. Lum, D., Kuwabara, P., Zarkower, D., and Spence, A. (2000). Direct protein-protein interaction between the intracellular domain of TRA-2 and the transcription factor TRA-1A modulates feminizing activity in *C. elegans*. Genes Dev. *14*, 3153–3165.
29. Wang, S., and Kimble, J. (2001). The TRA-1 transcription factor binds TRA-2 to regulate sexual fates in *Caenorhabditis elegans*. EMBO J. *20*, 1363–1372.
30. Rudel, D., and Kimble, J. (2001). Conservation of *glp-1* regulation and function in nematodes. Genetics *157*, 639–654.
31. Schedl, T., and Kimble, J. (1988). *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics *119*, 43–61.
32. Barton, M., and Kimble, J. (1990). *fog-1*, a regulatory gene required for specification of spermatogenesis in the germ line of *Caenorhabditis elegans*. Genetics *125*, 29–39.
33. Ellis, R., and Kimble, J. (1995). The *fog-3* gene and regulation of cell fate in the germ line of *Caenorhabditis elegans*. Genetics *139*, 561–577.
34. Graham, P., and Kimble, J. (1993). The *mog-1* gene is required for the switch from spermatogenesis to oogenesis in *Caenorhabditis elegans*. Genetics *133*, 919–931.
35. Graham, P.L., Schedl, T., and Kimble, J. (1993). More *mog* genes that influence the switch from spermatogenesis to oogenesis in the hermaphrodite germ line of *Caenorhabditis elegans*. Dev. Genet. *14*, 471–484.
36. Doniach, T. (1986). Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. Genetics *114*, 53–76.
37. Barton, M.K., Schedl, T.B., and Kimble, J. (1987). Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. Genetics *115*, 107–119.
38. Clifford, R., Lee, M., Nayak, S., Ohmachi, M., Giorgini, F., and Schedl, T. (2000). FOG-2, a novel F-box-containing protein, associates with the GLD-1 RNA-binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. Development *127*, 5265–5276.
39. Swanson, W., and Vacquier, V. (2002). The rapid evolution of reproductive proteins. Nature Rev. Genet. *3*, 137–144.
40. Werren, J., and Beukeboom, L. (1996). Sex determination, sex ratios, and genetic conflict. Annu. Rev. Ecol. Syst. *29*, 233–261.
41. Werren, J.H., and Hatcher, M.J. (2000). Maternal-zygotic gene conflict over sex determination: effects of inbreeding. Genetics *155*, 1469–1479.
42. Fisher, R. (1930). The genetical theory of natural selection (London, UK: Oxford University Press).
43. Bandi, C., Trees, A., and Brattig, N. (2001). *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet. Parasitol. *98*, 215–238.
44. Erickson, J., and Cline, T. (1998). Key aspects of the primary sex determination mechanism are conserved across the genus *Drosophila*. Development *125*, 3259–3268.
45. Tucker, P., and Lundrigan, B. (1993). Rapid evolution of the sex determining locus in Old World mice and rats. Nature *364*, 715–717.
46. Western, P., and Sinclair, A. (2001). Sex, genes, and heat: triggers of diversity. J. Exp. Zool. *290*, 624–631.
47. Meise, M., Hilfiker-Kleiner, D., Dübendorfer, A., Brunner, C., Nothinger, R., and Bopp, D. (1998). *Sex-lethal*, the master sex-determining gene in *Drosophila*, is not sex-specifically regulated in *Musca domestica*. Development *125*, 1487–1494.
48. True, J., and Haag, E. (2001). Developmental system drift and flexibility in evolutionary trajectories. Evol. Dev. *3*, 109–119.
49. Weiss, K., and Fullerton, S. (2000). Phenogenetic drift and the evolution of genotype-phenotype relationships. Theor. Popul. Biol. *57*, 187–195.