Sex-determination gene and pathway evolution in nematodes

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Summary

The pathway that controls sexual fate in the nematode *Caenorhabditis elegans* has been well characterized at the molecular level. By identifying differences between the sex-determination mechanisms in *C. elegans* and other nematode species, it should be possible to understand how complex sex-determining pathways evolve. Towards this goal, orthologues of many of the *C. elegans* sex regulators have been isolated from other members of the genus *Caenorhabditis*. Rapid sequence evolution is observed in every case, but several of the orthologues appear to have conserved sex-determining roles. Thus extensive sequence divergence does not necessarily coincide with changes in pathway structure, although the same forces may contribute to both. This review summarizes recent findings and, with reference to results from other animals, offers explanations for why sex-determining genes and pathways appear to be evolving rapidly. Experimental strategies that hold promise for illuminating pathway differences between nematodes are also discussed. *BioEssays* 25:221–231, 2003.

Introduction

The adoption of one of the two sexual fates is an event that has been studied in great detail, particularly in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*.[1–4] In these species, numerous genes and their corresponding proteins have been characterized and assembled into pathway models that attempt to explain how an initial chromosomal signal is read and transmitted to downstream targets that cause sex-specific differentiation. An important issue that remains largely unresolved is: how do complex sex-determining pathways such as these arise during evolution? Whether sex-determining pathway evolution can be considered representative of pathway evolution in general is the subject of debate. Studies thus far suggest that those controlling sexual fate are much more evolutionarily labile. Indeed, for many, it is this feature of sex determination that makes its evolution intriguing. Nonetheless, some general themes may emerge from comparative studies of sex determination regarding which parts of pathways are most evolutionarily stable and the order in which pathway segments are assembled.

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C. elegans and *Drosophila* rely on distinct sets of proteins and interactions to make the sexual fate decision. In the somatic cells of the worm, there is a series of inhibitory interactions involving, among other proteins, an extracellular ligand, a transmembrane receptor, a protein phosphatase, and a zinc finger protein. In the fly somatic pathway, the sex-determining signal is transduced largely by RNA splicing proteins, which activate their targets and ultimately control splicing of a DM-domain transcription factor. The only known similarity involves *C. elegans mab-3* and the *Drosophila* gene *doublesex*. The proteins encoded by these genes belong to the same sequence family, control some related aspects of sexual differentiation, and occupy downstream positions in their respective pathways.[5] This pattern of relatedness is consistent with the retrograde model of evolution, in which pathway growth occurs through the addition of new upstream elements.[6] However, the worm and fly pathways alone are not enough comparative material. Information from multiple closely related species will be necessary for small steps in pathway evolution to be observed, and hence for models of pathway evolution to be developed and evaluated. Analyses of several different fly species have generated some interesting results.[4] Here we focus on studies of sex determination in *C. elegans* and other nematodes.

C. elegans as the paradigm

Our understanding of how the sexual fate decision is made in *C. elegans* should serve as an excellent starting point in the exploration of sex-determining pathway evolution. Characterization of the worm pathway began with the identification of the primary signal—the ratio of X chromosomes to sets of autosomes (the X:A ratio).[7] Animals with two X chromosomes develop as hermaphrodites, which can be identified by their large body size, two-armed gonad, vulva and tapered tail (Fig. 1A). The presence of hermaphrodites does not prevent *C. elegans* from serving as a model for conventional male/female species, since *C. elegans* hermaphrodites resemble females of other species, apart from their ability to produce a

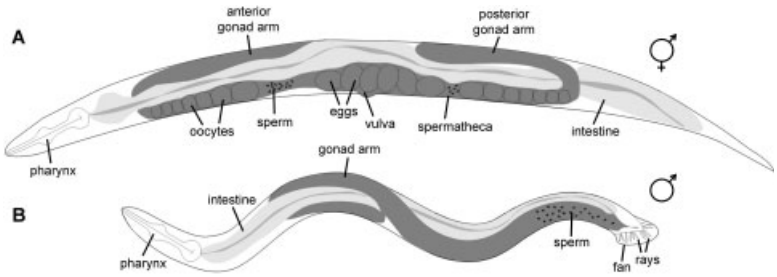

Figure 1. Overt sexual differences between *Caenorhabditis* **A:** hermaphrodites and **B:** males. The two gonad arms in the hermaphrodite generate about 150 sperm each and then switch to oocyte production. The initial sperm that they produce and any sperm obtained through mating are stored in the spermathecae. Oocytes are fertilized when they are forced through the spermathecae by muscular contractions. After fertilization, the eggs remain in the adjoining uterus for a short time before being deposited through the vulva. The single-armed gonad in the male produces sperm continuously, which can be delivered to the hermaphrodite vulva via the specialized male tail.

small number of sperm in their germline prior to oogenesis. Animals with a single X chromosome develop as males, which are smaller than hermaphrodites, have a single-armed gonad, lack a vulva, and have a fan-like tail used for mating (Fig. 1B). The X:A ratio does not have the final say in whether a male or hermaphrodite is formed. A large number of genes necessary for interpreting the ratio has been identified through the isolation of mutations that cause worms to develop as members of the incorrect sex (Table 1). The epistatic interactions between several of these suggest that they constitute a regulatory cascade.[8,9] Subsequent characterization of their protein products has in many cases helped explain the observed genetic interactions, and a pathway model combining the molecular and genetic information has been constructed (Fig. 2). In somatic cells, the most downstream global regulator of sex is *tra-1*; if *tra-1* is active the soma is female, and if *tra-1* is inactive the soma is male. In the germline, a similar pathway operates, but there are additional genes involved, as mutations exist that cause inappropriate feminization or masculinization of the germline but not the soma (Fig. 3). The regulatory mechanisms used to modulate gene activity also differ, in part because germ cells in the hermaphrodite adopt different sexual fates at different times during development.

Isolation of sex-determination gene orthologues from other nematodes

Many studies exploring the evolution of sex-determining mechanisms in nematodes begin with the isolation of sequence orthologues from *C. briggsae* and *C. remanei* (Table 2). These species are closely related to each other and to *C. elegans*.[10,11] The most obvious difference among them is that *C. remanei* reproduces using conventional male and female sexes, while *C. briggsae* and *C. elegans* exist as males and hermaphrodites. Most initial attempts to obtain sex-determining gene orthologues by hybridization failed, suggesting either that the genes were not present, or that they had diverged too much to be recognized. Alternative approaches eventually proved to be more successful. Five of the 14 putative orthologues described to date were isolated by taking advantage of conserved gene order between the species. In these cases, a *C. elegans* gene located near the sex-determining gene was used as a probe in screens of *C. briggsae* or *C. remanei* genomic libraries. Several of the other sequences were obtained using PCR and degenerate primers designed to anneal to small motifs broadly conserved among members of a particular gene family. For each sequence, attempts have been made to substantiate the claim of orthology. Conserved gene order, protein segments, and gene structure have all been used as supporting evidence. The most challenging comparisons remain to be made, and will involve genes that are members of large and closely related sequence families. For example, *fog-2* has many paralogues in *C. elegans*, none of which are known to be necessary for hermaphrodite spermatogenesis.[12] Some of these are located in a cluster that includes *fog-2*, suggesting that the family has expanded in part by local duplications. In *C. briggsae* and *C. remanei*, the *fog-2* family structure may turn out to be conserved and discernable. However, in more distant nematodes, the accumulated results of gene loss and duplication events in conjunction with sequence divergence may make it more difficult or impossible to distinguish between orthologous and paralogous relationships.

Rapid sequence divergence of sex-determination proteins

The orthologues of a gene of interest are often isolated from other species as a means of identifying protein segments that are functionally important. Although several conserved regions were identified through the sex-determining gene comparisons, the most intriguing observation, made not only in nematodes but also in flies[13,14] and mammals,[15,16] was that sex-determining proteins evolve at an accelerated pace.

Table 1. Genes and proteins that regulate sexual fate in *C. elegans*

Gene[a]	Loss-of-function phenotype[a]	Protein domains, motifs, or signals[a]	Protein functional information[a]
fbf-1	XX excess sperm	Related to RNA-binding proteins	Binds NOS-3; binds *fem-3* 3′UTR
fbf-2	XX excess sperm	Related to RNA-binding proteins	Binds NOS-3; binds *fem-3* 3′UTR
fem-1	XX fertile females; XO fertile females	Ankyrin repeats	Binds FEM-2
fem-2	XX fertile females; XO fertile females	Protein phosphatase type 2C	Has phosphatase activity; binds FEM-3; binds FEM-1
fem-3	XX fertile females; XO fertile females	Novel	Binds FEM-2; binds TRA-2
fog-1	XX oocytes only; XO oocytes only	Related to CPEB proteins	—
fog-2	XX oocytes only; XO normal	F-box	Binds GLD-1/*tra-2* mRNA complex
fog-3	XX oocytes only; XO oocytes only	Similar to vertebrate Tob, BTG1, and BTG2	—
fox-1	XX normal; suppresses XO-specific lethality and feminization caused by duplications of left end of X	RRM-type RNA-binding	Binds RNA
gld-1	XX oocytes only; XO normal	STAR RNA-binding	Binds many RNA targets, including *tra-2* mRNA
her-1	XX normal; XO form hermaphrodites	Secretory signal	Acts cell non-autonomously
laf-1	XX lethality and feminization; XO lethality and feminization in soma and germline	—	—
mab-3	XX normal; XO abnormal tail and synthesizes yolk proteins	DM DNA-binding motif	Binds site in *vit-2* promoter
mog-1	XX sperm only; XO normal	DEAH-box RNA helicase	—
mog-2	XX sperm only; XO normal	—	—
mog-3	XX sperm only; XO normal	—	—
mog-4	XX sperm only; XO normal	DEAH-box RNA helicase	—
mog-5	XX sperm only; XO normal	DEAH-box RNA helicase	—
mog-6	XX sperm only; XO normal	—	—
nos-1	XX excess sperm	Related to *Drosophila* Nanos	—
nos-2	XX excess sperm	Related to *Drosophila* Nanos	—
nos-3	XX excess sperm	Related to *Drosophila* Nanos	Binds FBF-1; binds FBF-2
sdc-1	XX weak masculinization; XO normal	Zinc fingers	—
sdc-2	XX masculinization and lethality; XO normal	Novel	Binds *her-1* promoter and localizes to X chromosomes
sdc-3	XX masculinization and lethality; XO normal	Zinc fingers	Localizes to X chromosomes
sex-1	XX show dosage compensation defects and masculinization; XO normal	Nuclear hormone receptor	Binds *xol-1* promoter
tra-1	XX low fertility males; XO low fertility males	Zinc fingers	Binds to DNA near *egl-1* and *mab-3*; binds TRA-2
tra-2	XX non-mating pseudomales; XO normal	Transmembrane domain	Binds FEM-3; binds TRA-1
tra-3	XX sterile pseudomales; XO normal	Calpain protease	Cleaves TRA-2
xol-1	XX normal; XO die as embryos or small crumpled feminized L1 larvae	Novel	Binds *sdc-2* promoter

[a]For additional information and references see reviews by Goodwin and Ellis,[2] and Cline and Meyer.[3]

Sequence comparisons are not the only indicator, as interspecies hybrids often develop normally except that they show signs of sexual transformation, likely because the sex-determining proteins from the two parents do not interact properly.[17] What is the reason for this rapid divergence? Two broad explanations are usually cited: the proteins may be "allowed" to change more quickly than other proteins (rapid neutral evolution), or positive selection may be promoting change. Attempts have been made to distinguish between these possibilities using nonsynonymous to synonymous substitution ratios and patterns of intraspecies sequence variation (polymorphisms).[14–16,18,19] The conclusions vary depending on the method used and on the species and genes examined. In nematodes these analyses have been hampered by the high level of synonymous divergence between species and by the low frequency of sequence polymorphisms among *C. elegans* populations.[19–22]

Although the rapid neutral evolution/positive selection debate continues, it is interesting to speculate about what might lead to either or both in the case of nematode sex-determining proteins. First we will consider why the proteins might be allowed to diverge. A study comparing orthologous sequences between *Saccharomyces cerevisiae* and *C. elegans* found that proteins with less interactors evolve more quickly, because a smaller proportion of their residues are involved in function.[23] The high-throughput methods used to quantify the yeast protein interactions have not been applied to nematodes. If the *C. elegans* sex-determining

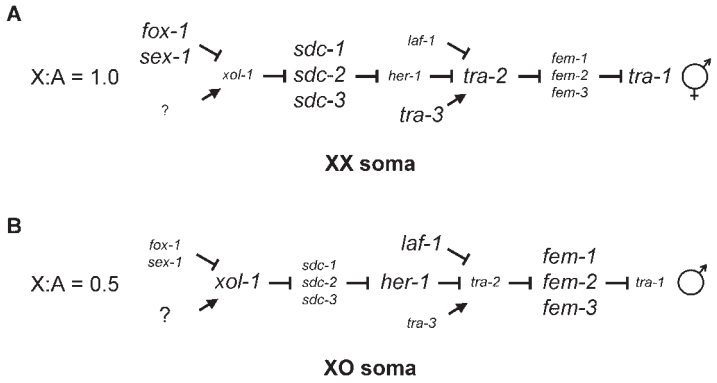

Figure 2. A model of the pathway that controls somatic sex in *C. elegans*. Gene activities that are dispensable for the given sexual fate are shown in small font. Arrows represent positive interactions and bars represent negative interactions. The ratio of X chromosomes to autosome sets (X:A ratio) is the initial signal that determines the state of the pathway.[7] Two genes, *fox-1* and *sex-1*, serve as X signal elements (they contribute to "X" in the X:A ratio) and act to reduce *xol-1* expression.[58,59] **A:** When *xol-1* is sufficiently inhibited (X:A = 1.0), the *sdc* genes are able to promote hermaphrodite development by repressing *her-1* transcription.[60] In the absence of HER-1 protein, the transmembrane protein encoded by the *tra-2* gene negatively regulates the FEM proteins,[40,61] allowing the transcription factor encoded by *tra-1* to promote hermaphrodite development.[62] The role of TRA-3 in XX animals is to activate TRA-2 by proteolysis.[63] In addition to inhibiting the FEMs, TRA-2 may increase the activity of TRA-1 by a direct interaction.[35,36] **B:** In animals with one copy of the X chromosome (X:A = 0.5), male development ensues because the *sdcs* are inhibited by active *xol-1*.[60] The extracellular protein encoded by *her-1* is expressed and inhibits *tra-2*,[64–66] allowing the *fem* genes to negatively regulate *tra-1*.[67] In the absence of *tra-1* activity, the male fate is established. The *laf-1* gene may function in parallel to *her-1* to reduce *tra-2* activity.[68] The common position of the *fems* in the genetic pathway, and demonstrated interactions between FEM-3 and FEM-2,[69] and FEM-2 and FEM-1,[70] have led to the proposal that the three proteins function as a complex. However, to date it is not known how the FEMs promote the male fate, either in terms of the way that they interact in vivo, or the targets that they act on.

proteins are found to have fewer interactors than most other proteins, this might account to some extent for their faster evolution.[6] In some cases of rapid evolution, protein dispensability is thought to be an important factor. More dispensable proteins should experience weaker purifying selection (selection against deleterious alleles), and thus should accumulate slightly deleterious substitutions more rapidly.[24,25] However, given that reproductive capacity and sexual development are tightly linked, it is difficult to view sex-determining proteins as more dispensable than proteins that regulate other aspects of development.

Why might positive selection promote changes in sex-determining proteins? One explanation is that changes in sex-determining proteins facilitate shifts in the ratio of self to outcross progeny. Self-fertilization allows for rapid population growth but can bypass the proposed advantages of sexual reproduction. Perhaps the optimal ratio of self to outcross progeny depends on environmental conditions. Males arise spontaneously in *C. elegans* populations, and half the outcross progeny from a male/hermaphrodite cross are male. Thus there is already the capacity for extensive outcrossing. However, under conditions of outcrossing, the time hermaphrodites spend making sperm would be better spent making oocytes, as sperm production delays oogenesis and increases generation time.[26] Consequently, mutations in sex-determining proteins that adjust the timing of the sperm-to-oocyte switch in hermaphrodites might sometimes be advantageous. Another force that might drive change in sex-determining proteins is genomic conflict. It can be initiated by cytoplasmically inherited organelles or parasites. They are transmitted uniparentally through females, and thus may attempt to interfere with sex determination so that the sex ratio is skewed towards the female fate.[27,28] In hermaphroditic nematodes like *C. elegans*, these cytoplasmic elements might also seek to hasten the switch from spermatogenesis to oogenesis in the hermaphrodite germline, so that more oocytes are produced (provided males are available in the population to supply sperm). If these adjustments reduce the fitness of the host, there will be selection for changes in sex-determining proteins that allow the pathway to escape or offset the foreign modifier. Genomic conflict can also arise if the optimal sex ratio differs for maternal-effect genes and zygotically expressed genes.[29] Again the consequence could be selection for modifications in sex-determining proteins, with the long-term result being interspecies sequence divergence as each species follows its own evolutionary trajectory. Having a large pathway may sometimes prove to be advantageous for the worm, as it could allow refinements to be made more rapidly, through mutations in any of the components, or through new allele combinations.

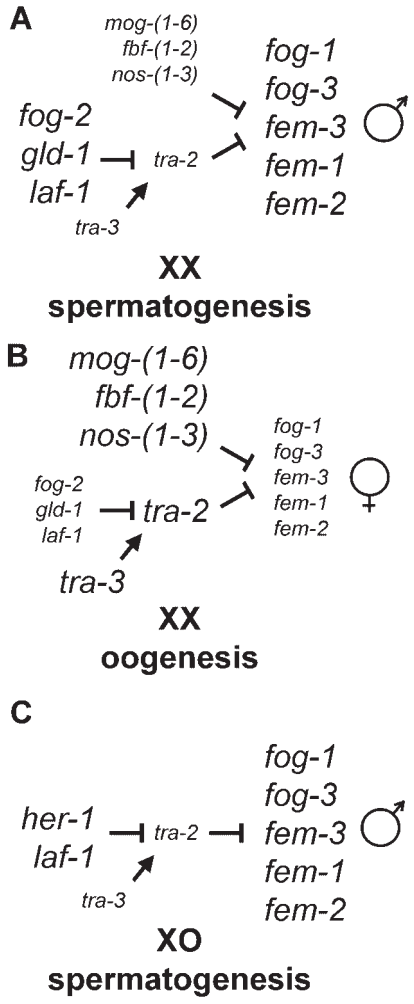

Figure 3. Models of the pathways that control germline sex in *C. elegans*. Arrows represent positive interactions and bars represent negative interactions. Gene activities that are dispensable for the given sexual fate are shown in small font. In the XO soma, specification of the male fate involves the inhibition of TRA-2 by HER-1, likely by a direct interaction.[66] **A:** During the male fate phase in the XX germline, *her-1* transcripts are not detected.[71] Instead, *tra-2* translation is repressed by *fog-2*, *gld-1* and *laf-1*.[12,68] The reduced level of TRA-2 allows the *fems* to function, along with two germline-specific genes, *fog-1* and *fog-3*.[72,73] How the FEM, FOG-1, and FOG-3 proteins promote spermatogenesis is not known. An interaction between TRA-2 and TRA-1 is also required but is not shown.[35,36] **B:** As in the XX soma, adoption of the female fate in the XX germline occurs when *tra-2* is active and able to inhibit its downstream targets. Several genes that inhibit *fem-3* translation are also required. These are the *mog, fbf*, and *nos* genes, and they are thought to act by binding to *fem-3* mRNA, or by altering the splicing of other genes involved in *fem-3* repression.[74–79] **C:** In the XO germline, TRA-2 is inactivated by *her-1* and *laf-1*,[61,68] allowing the downstream *fem* and *fog* genes to function. *tra-1* has a complex role in sperm production in both XX and XO animals, and is not shown in this figure.[80,81]

Sequence change and pathway change

The relationship between sex-determining protein divergence and pathway dissimilarity is not clear. Some proteins, HER-1 and TRA-2 for example, have diverged extensively in sequence between *C. elegans* and *C. briggsae* yet are members of the sex-determination pathway in both species.[30,31] In contrast, the SXL protein is well conserved between *D. melanogaster* and the housefly *Musca domestica*, yet appears to lack a sex-determining role in the latter.[32] Thus rapid protein divergence and changes in pathway structure are not necessarily coincident. Perhaps the proposed causes of protein sequence divergence discussed in the previous section can contribute to pathway changes. For example, if a cytoplasmic parasite caused a skewed sex ratio in a host population, then selection would favor changes in the sequence of host sex-determining proteins that offset this effect. If these became fixed in the species, they could be observed as interspecies sequence divergence. Alternatively, sequence changes that lead to the incorporation of a new regulator into the pathway could become fixed, because they have a similar beneficial effect. This second type of alteration could be observed as pathway divergence—new components and new forms of regulation being incorporated in one species but not in another. The two types of changes might occur in succession when one type of modification, although having a selective advantage, does not yield an optimized pathway.

Testing orthologues for function in *C. elegans*

Foreign genes are often introduced into mutant *C. elegans* worms to test whether they can restore a wild-type phenotype. Briefly, DNA is injected into the germline of adult hermaphrodites, where it can associate with developing oocytes.[33] Worms arising from these oocytes can express the DNA, which is usually maintained as an extrachromosomal array. Several sex-determination gene orthologues have been tested in this manner (Table 2). The results of these interspecies complementation studies need to be interpreted carefully. If rescue is observed, it is sometimes said to indicate that the orthologue has a conserved sex-determining role. However, the foreign protein may resemble the *C. elegans* protein enough to supply activity even though it is not actually part of the sex-determining cascade in the foreign species. It could, for example, have an altered expression pattern in the other species that prevents it from fulfilling the same role, or the target that is sex determining in *C. elegans* could have diverged in the other species such that it is no longer a target. Conversely, if rescue is not observed, it is sometimes interpreted as indicating that the orthologue does not regulate sex determination. However, this conclusion may be incorrect because the foreign gene has evolved in its own molecular world, which includes its evolving targets. The *fog-3* gene from *C. remanei* cannot replace *C. elegans fog-3*, even when

Table 2. Orthologues of *C. elegans* sex-determination genes isolated from other nematode species

Gene	Other species	Isolation method	Protein sequence identity[a]	*C. elegans* rescue?	Similar RNAi phenotype?[c]	References
fem-1	*C. briggsae*	Degenerate oligo PCR	71%	Poorly	In soma but not in germline	A. Spence Lab, pers. comm.
fem-1	C. sp. (CB5161)	Degenerate oligo PCR	69%	—	—	A. Spence Lab, pers. comm.
fem-2	*C. briggsae*	Low stringency hybridization	63%	In soma but not in germline	Not in germline	20,82
fem-2	C. sp. (CB5161)	Degenerate oligo PCR	59%	In soma but not in germline	Not in germline	20
fem-3	*C. briggsae*	Conserved gene order	38%	—	In soma but not in germline	37
fem-3	*C. remanei*	Conserved gene order	31%	—	In soma but not in germline	37
fog-3	*C. briggsae*	Degenerate oligo PCR	56%	Yes	Yes	34
fog-3	*C. remanei*	Degenerate oligo PCR	57%	Poorly or not at all	Yes	34
her-1	*Brugia malayi*	EST project	35%	Poorly or not at all[b]	—	30
her-1	*C. briggsae*	Conserved gene order	57%	Yes[b]	Yes	30
tra-1	*C. briggsae*	Low stringency hybridization	44%	In soma except somatic gonad, not in germline	—	83
tra-2	*C. briggsae*	Conserved gene order	43%	—	Yes	31,84
tra-2	*C. remanei*	Conserved gene order	43%	—	Yes	39

[a]Compared with the *C. elegans* orthologue. These values are taken from the referenced sources and thus alignment and calculation methods may differ.
[b]Wild-type XX animals carrying *her-1* transgenes were examined for signs of masculinization.
[c]Compared with the *C. elegans* RNAi phenotype when known, otherwise compared with the phenotype observed in *C. elegans* null mutants.

regulated by the *C. elegans* regulatory sequences. However, RNA interference indicates that *fog-3* is required for establishing the male fate in the germline of both species.[34] In the case of TRA-1 and TRA-2, there is more direct evidence of coevolution.[35] These proteins interact in *C. elegans* and in *C. briggsae* but not between species.[35,36] Cross-species binding of FEM-3 to TRA-2 does not occur either, despite maintenance of the interaction in *C. elegans*, *C. briggsae*, and *C. remanei*.[37] Thus the inability of many of the sex-determination orthologues to fully replace their *C. elegans* counterparts is symptomatic of their sequence dissimilarity and says nothing about potential differences in their biological roles. Similarly, successful complementation might occur for reasons other than conserved biological function.

Using RNAi to explore gene function in other nematodes

To understand the extent to which the roles of sex-determining genes are conserved, it is necessary to look at the functions of their orthologues in the species from which the orthologues are isolated. One technique that has been widely used to examine gene function in *C. elegans* and other nematodes is RNA interference (RNAi).[38] Worms can be injected, fed, or soaked in a solution containing dsRNA corresponding to a particular gene. The expression of the gene is then reduced in the progeny of the treated animal because the dsRNA, which is transferred to oocytes, continually targets its related mRNA product for degradation. RNAi has been performed against several sex-determining orthologues from *C. briggsae* and *C. remanei*, and, with the exception of the *fem* genes, the phenotypes that are observed are identical or similar to those seen in *C. elegans* (Table 2). In the case of the *fems*, the non-*elegans* species fail to show the germline abnormalities expected based on the *C. elegans* results. RNAi against any of the *fems* causes highly penetrant germline feminization in *C. elegans* hermaphrodites.[20,37,38] In contrast, *C. briggsae* hermaphrodites show no signs of germline feminization when their *fem* genes are targeted (A. Spence Lab, pers. communication, Refs. 20,37). *C. remanei* males exposed to *fem-3* dsRNA are also unaffected in their germline.[37] These species are not completely resistant to RNAi, as the predicted germline phenotypes are obtained for the *C. briggsae* and *C. remanei* orthologues of *glp-1*,*tra-2*, and *fog-3*.[11,31,34,39] The results suggest that, in *C. briggsae* and *C. remanei*, the *fems* might not regulate germline sex to the extent that they do in *C. elegans*. However, RNAi is not a rigorous test of gene function, as genes can be resistant to its effects for unknown reasons. Null mutations in the *fem* orthologues, and any other sex-determination gene orthologues for which RNAi does not produce a phenotype, will have to be isolated before more definitive statements about pathway evolution can be made.

Resolving pathway details

RNAi might eventually reveal that many of the orthologues of the *C. elegans* sex-determining genes regulate sexual fate in other species. However, the details of the regulatory connec-

tions between these components in the other species will need to be resolved before precise pathway comparisons can be made. Let us consider the role of the TRA-2 protein in *C. elegans*. It is thought to promote the female fate by binding and inhibiting FEM-3, such that FEM-3 can no longer inhibit TRA-1.[40] Several lines of evidence suggest that TRA-2 also regulates sex independently of FEM-3, by binding directly to TRA-1.[35,36] These two TRA-2 mechanisms could have different levels of importance in other species. In some, for example, TRA-2, FEM-3 and TRA-1 might regulate sexual fate, but exclusively through the TRA-2–FEM-3 interaction. In *C. briggsae* and *C. remanei*, TRA-2 and FEM-3 physically interact.[37] Furthermore, double RNAi experiments suggest that *tra-2* promotes the female fate by inhibiting *fem-3*, as *fem-3* (*RNAi*) suppresses the somatic masculinization produced by *tra-2* (*RNAi*).[37] Thus the association between these proteins seems to serve as a regulatory mechanism in the three species that have been examined. The germline importance of TRA-2/FEM-3 in *C. briggsae* and *C. remanei* is not clear, as *fem-3* (*RNAi*) yields no germline phenotype. In *C. briggsae*, there is evidence that the TRA-2/TRA-1 mechanism has also been maintained, as the two proteins interact in yeast two-hybrid assays.[35] The *C. remanei* orthologue of TRA-1 has not been isolated. For the other sex-determining gene orthologues that have been identified, the various genetic and protein interactions observed in *C. elegans* remain to be verified.

Sex-determining pathway divergence in other taxa

Sex determination in a variety of insect species has been studied with reference to the *Drosophila melanogaster* pathway. One of the most upstream components in *D. melanogaster* is an RNA-binding protein encoded by *Sex-lethal* (*Sxl*).[41–43] Sex-specific splicing of *Sxl* mRNA yields active protein in females that ultimately leads to the production of a female isoform of the *doublesex* (*dsx*) transcription factor.[44,45] In the absence of active SXL, the default male splice form of *dsx* is produced. In *Drosophila virilis*, the orthologue of *Sxl* is sex-specifically spliced, consistent with it having the same role in sex determination.[46] In four dipteran species from outside the *Drosophila* genus, *Sxl* does not show sex-specific splicing and therefore is not thought to be part of the sex-determining cascade.[32,47–49] In contrast, *dsx* is sex-specifically spliced in non-*Drosophila* flies.[50,51] Comparisons have also been made among the pathways in mammals and non-mammalian vertebrates. The *Sry* gene, which is an upstream regulator in some mammals,[52,53] is completely missing from others,[54] and has not been identified outside mammals. In contrast, analyses of the expression of some downstream regulators suggest that they have conserved sex-determining roles in mammals, birds and alligators.[55,56] These results resemble those arising from comparisons of sex determination between distant phylogenetic groups. Worms and flies for example, use a similar downstream element (*mab-3/dsx*),[5] while the upstream regions are unrelated.

Whether the upstream differences revealed by these studies represent independent addition of new upstream components to a shorter ancestral pathway, greater evolutionary flexibility in the upstream regions, or both is not clear (Fig. 4). Perhaps the pathway in the common ancestor of worms and flies was very simple, and after the lineages split each pathway incorporated several new elements. Another possibility is that the ancestral pathway extended several

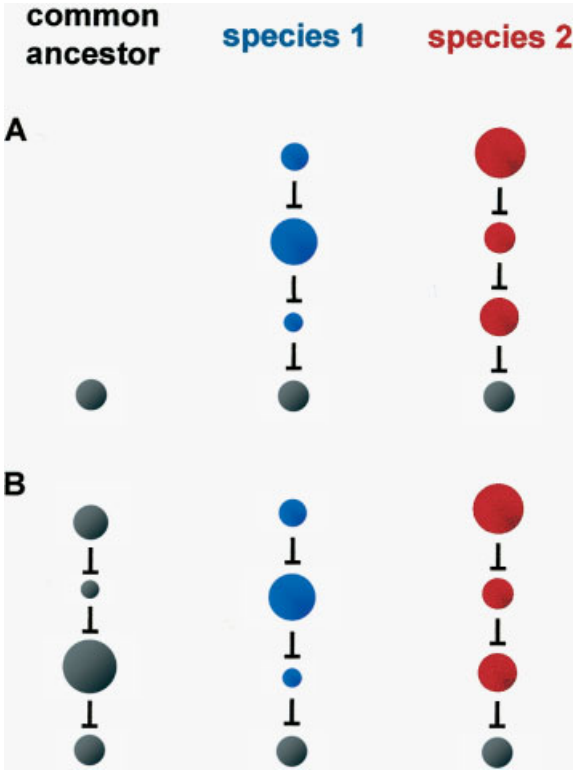

Figure 4. Pathway divergence occurring because of retrograde growth of a simple ancestral pathway, or because of evolutionary plasticity in the upstream regions. Circles represent proteins, and bars represent negative interactions. Proteins present in the pathway in the common ancestor are coloured grey, while those specific to the pathways in the species arising from the ancestor are shown in blue for species 1 and red for species 2. **A:** A simple pathway in the common ancestor expands through retrograde growth after separation of the two species.[6] Different-sized circles are used to indicate that a different set of proteins is added in each species. **B:** A complex pathway in the common ancestor changes through the removal and addition of new upstream components, while the downstream regulator is conserved because its activity is more difficult to replace. Again, different-sized circles are used to indicate that a different set of proteins is added in each species. The types of evolution shown in A and B can produce the same pattern of interspecies differences.

components upstream of *mab-3/dsx* and, through the removal and addition of components, the pathways became dissimilar. *mab-3* and *dsx* may have been maintained because they regulate multiple targets to produce coordinated sexual differentiation. Replacement of their activity might require more specific evolutionary changes than those needed for replacement of sex-determining proteins that only regulate another sex-determining protein. One possible mechanism for replacing a large portion of an existing pathway with new components is outlined in Figure 5. It involves shrinkage of an existing pathway, through modifications that allow the sexes to be specified independently of upstream components. An implication of this type of growth is that the pathway components shared between two species will not necessarily represent the complete pathway in their common ancestor. Another is that several upstream elements found in one species may sometimes be missing from the pathway in another closely related species. However, the pathways will still possess the hallmark of retrograde growth—the more downstream that the component is the longer it has been part of the pathway. The pathway differences observed among insects and among vertebrates may be best explained strictly by independent upstream extension of an ancestral pathway. It will be interesting to see whether nematode species differ from each other only with respect to the upper few components, or if large upstream segments are sometimes lost or replaced. The emerging results concerning the *fem* genes in *Caenorhabditis* hint at the possibility that pathways may also change in the middle.

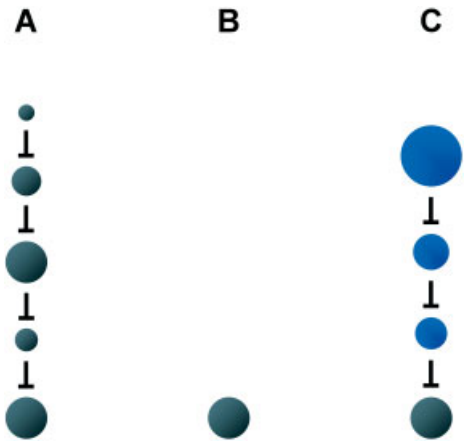

Figure 5. Shrinkage and then growth of a sex-determining pathway. **A:** A pathway consisting of several proteins (circles) is used to determine sex. **B:** The pathway is then reduced in size when changes in a downstream component make it the primary sex-determining signal. Hodgkin[85] showed that such changes can occur through simple mutations in one gene. He constructed a *C. elegans* strain containing two different versions of *tra-1*. One is a gain-of-function (gf) feminizing allele and the other is a loss-of-function (lf) masculinizing allele. The strain consists of *tra-1(lf)/tra-1(lf)* males and *tra-1(gf)/tra-1(lf)* females. The sex of individuals in this strain is determined independently of the X chromosome and the other components upstream of *tra-1*. Mutations in genes above *tra-1* could also be imagined to cause pathway shrinkage, as could the incorporation of a new regulator that acts on one of the downstream components rather than on the uppermost one. **C:** After pathway shrinkage, new components (blue circles) are added through retrograde growth.[6] Circles of different sizes are used to indicate that the new pathway uses proteins that are different from those used in the ancestral pathway.

Orthologues with different biological roles

The functional comparisons of the *fems* in *Caenorhabditis* and *Sxl* in flies could illustrate an important point: even when an orthologous gene is readily identified in another species, it may perform different biological functions. In this regard, genes and proteins may sometimes be like actors in movies. Two different movies (species) can contain the same actor (protein), and this actor might not change dramatically in appearance (sequence), but may play a much different role (biological function). In terms of applying gene-function information from model organisms to other organisms, changes in the biological function of genes conserved at the sequence level could be problematic. For example, one might want to control parasitic nematode reproduction by inhibiting one or more of the *fem* genes, based on what is known about the effects of *fem* inhibition in *C. elegans* (sperm are not produced). If the *fems* does not regulate germline sex, or regulate it to a much lesser degree, this inhibition may not have the desired effect on the other species. Large-scale interspecies comparisons of gene function, between *C. elegans* and *C. briggsae* for example, should reveal how often the biological roles of conserved genes change between closely related species.

Conclusions and future studies

The proteins and interactions that regulate sexual fate in *C. elegans* have been described in detail, and should serve as a useful starting point for exploring sex-determining pathway evolution in nematodes. Studies thus far have focused on isolating the orthologues of the *C. elegans* sex-determining genes, primarily from the closely related species *C. briggsae* and *C. remanei*. For reasons that are not clear, the sequences of these genes are changing rapidly. However, the orthologues (perhaps with the exception of those of the *fem* genes) appear to regulate sexual fate in the other species. Thus rapid sequence divergence does not necessarily reflect changes in pathway structure, although the same forces might cause sequence and pathway changes. Based on models of how pathways arise[6] and on differences observed in other taxa, the sex-determining pathways among nematodes may be expected to differ in terms of which proteins are found near the top. However, some nematodes may show more drastic

differences because of changes that bypass much of an ancestral pathway.

Further comparisons of the pathways in *C. elegans* and *C. briggsae* will be greatly facilitated by the isolation of genetic nulls in the latter. A method for introducing mutations into specific genes in nematodes has not been developed. However, PCR-based screens for worms carrying deletions in genes of interest have been productive. Using the recently completed *C. briggsae* genomic sequence, a PCR screen for deletions in genes of interest can be performed. By comparing null mutants of one species to those of another, it should be possible to make more rigorous conclusions regarding pathway changes. A complementary approach will be to perform genetic screens in *C. briggsae*, modeled after the ones used to study the sex-determining pathway in *C. elegans*. It is reasonable to assume that many of the mutations will be in orthologues of known components of the *C. elegans* pathway. The more intriguing mutations, if they are obtained, will be those that occur in genes with *C. elegans* orthologues that have no known sex-determining role. These genes might be specific to the *C. briggsae* pathway, or the mutant phenotype in *C. elegans* might be more difficult to observe. In either case, the results will be of interest. If *C. elegans* and *C. briggsae* turn out to use very similar sex-determining mechanisms, orthologue deletions and genetic screens in more distant nematodes may be necessary. Genetic and physical maps are being constructed for *Pristionchus pacificus*; hence it should serve as a suitable distant relative for incorporating into studies of pathway evolution.[57] Even if substantial pathway differences are observed between *C. elegans* and *C. briggsae*, knowledge of the pathway in *P. pacificus* could provide clues as to which of the differing components were present in their common ancestor. Determining the extent to which proteins move in and out of developmental pathways as well as which parts of pathways are most evolutionarily labile are important endeavors, and will require continued comparisons involving several species.

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