Sex and the single worm: sex determination in the nematode C. elegans

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Accepted 27 January 1999

Abstract

The study of sex determination in model organisms has been especially fruitful in increasing our understanding of developmental biology, gene regulation and evolutionary mechanisms. The free living nematode, Caenorhabditis elegans, can develop as one of two sexes; male or self-fertilizing hermaphrodite. Here we discuss the progress toward a genetic and molecular understanding of that decision. Numerous genetic loci have been identified that affect sexual fate, and epistasis analysis of these genes has led to a model of a regulatory hierarchy with stepwise negative interactions. It is becoming evident that many of the genes have numerous levels of regulation. We also discuss the apparent rapid rate of evolution that many of the sex determination proteins have undergone. Protein sequences of homologues from closely related species are more divergent than homologues of proteins involved in other developmental processes. Rapid evolution of sex determination genes may be a common theme throughout the animal kingdom. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Sex determination; Caenorhabditis elegans; Signal transduction; Gene regulation; Evolution

1. Introduction

Over 2000 years ago, Aristotle suggested that men mate during the hot summer months if they wished to have sons, based on his belief that sex was determined by temperature. The rediscovery of Mendel’s work at the turn of this century allowed for the subsequent observation that in most animals, the ratio of males to females was consistent with sex being determined by a nuclear component. We now know that in most animal species, the primary signal for determining sex is chromosomal, and we are beginning to make progress in understanding the mechanisms involved in reading and implementing this signal. The mechanisms are varied between species, and numerous levels of gene regulation are involved. Besides mammals, we know most about how sex is determined in model organisms, particularly Drosophila melanogaster and the free living soil nematode, Caenorhabditis elegans (Fig. 1). Classical genetic screens have identified numerous loci that affect the determination of sex in C. elegans. Most of the associated genes have now been cloned allowing predictions of their biochemical functions due to their similarity to other known proteins or motifs. Many of these predictions have been confirmed, and interactions between the molecules have been directly demonstrated in several cases. Our current model of sex determination in C. elegans initiates with a signal that is based on the number of X chromosomes in a diploid animal. This code is used to co-ordinate sex determination and dosage compensation. In sex determination, the signal is passed through a novel transduction cascade, with an overall theme of stepwise negative interactions, culminating in the expression of sex specific genes (Fig. 2). Herein we describe our current understanding of how the initial signal is interpreted and the mechanisms involved in transducing the signal, as well as providing a discussion as to how these molecules may have evolved.

2. Sexual dimorphism

’Hermaphrodites. That’s what this whole thing’s about, you know. Or haven’t you heard?’ – John Berendt, ‘Midnight in the Garden of Good and Evil’.

C. elegans exists in two sexes, male and self-fertilizing hermaphrodite (Fig. 1). Cell lineage determination has
shown that virtually every tissue contains sexually specialized cells (Sulston and Horvitz, 1977), with 30% of the somatic nuclei in hermaphrodites, and 40% in males, specific to their respective sex. Hermaphrodites are anatomically similar to females of closely related nematode species (e.g. Caenorhabditis remanei), therefore they are most conveniently thought of as somatic females that are transiently able to produce sperm. The gonad of the hermaphrodite consists of two arms that join at a common uterus and open to the environment through a vulva (Fig. 1). The regions of the gonad arms most distal from the vulva consist of a syncytium that contain mitotic nuclei, which serve as stem cells for sperm and oocytes. With time, the nuclei migrate down the gonad arms towards the uterus. Once they reach a certain distance from the distal end of their respective gonad arm, they begin meiosis and differentiate as sperm or oocytes. The first germ nuclei to differentiate do so as sperm, which are produced during the ultimate (fourth) larval stage, and are stored in the spermatheca at the proximal end of each gonad arm. Approximately 150 sperm are made per arm, and since over a thousand oocytes can be produced (Hodgkin, 1986), sperm are limiting as to the number of offspring a hermaphrodite can produce through self-fertilization. After the brief period of sperm production, the hermaphrodite produces oocytes for the rest of her life. These are fertilized as they pass through the spermatheca to the uterus. The eggs are then pushed out into the environment through the vulva.

The male is smaller than the hermaphrodite, although it has 1031 somatic nuclei and the hermaphrodite has only 959. Unlike the hermaphrodite, the male gonad has only one arm and produces exclusively sperm. The male tail is a very elaborate specialized structure that is able to clamp onto the hermaphrodite vulva during mating. Pseudopodal sperm cells are transferred into the hermaphrodite through the vulva and crawl through the uterus to the spermatheca.

Fig. 1. Basic anatomy of adult C. elegans hermaphrodite (top) and male (bottom). The gray portions of the animals depict their respective gonads. The hermaphrodite gonad consists of two reflexed arms, opening at the vulva in the middle of the ventral side, while the male only has one, opening at the cloaca in the tail.

![Genetic interactions involved in somatic sex determination. Arrowheads represent positive interactions while vertical bars represent negative or inhibitory interactions. Genes with blue letters are primarily involved in promoting the male fate, while those with red letters promote the female fate. The two pathways represent the activities of the genes in promoting the male (top) and female (bottom) fates. Gene activities that have a role in promoting that sexual fate are shown in large font, while gene activities that are dispensable are shown in small font. The initial signal is the ratio of the number of X chromosomes to the number of sets of autosomes. In XO animals (X/A ratio of 0.5), the xol-1 gene is active, which passes the signal in a series of negative interactions, culminating in the inactivity of tra-1. In hermaphrodites, the X/A ratio (1.0) results in active tra-1, which in turn promotes the female somatic fate. It is still unclear how the tra-3 Iaf-1 pathway receives its initial signal. The dosage compensation pathway is downstream of the sdc genes, separate from her-1. The male specific gene mab-3 functions downstream of tra-1.](image-url)
where they are stored. If both male and hermaphrodite sperm are present in the spermatheca, the male sperm is used preferentially (Ward and Carrel, 1979). Through cross-fertilization, a single hermaphrodite is able to produce upwards of 1400 offspring (Hodgkin, 1986). Equal numbers of males and hermaphrodites are produced from cross-fertilization, however self-fertilization results in virtually all hermaphrodite progeny, with the rare male arising via nullo-X gametes produced through random non-disjunction of the X chromosome.

3. Primary signal for sex determination and dosage compensation

The primary signals involved in making the decision as to what sex a given organism will display are quite varied between genera. Mammals use the presence or absence of the dominant testis determining factor (SRY) present on the Y chromosome, while those possessing it develop as males (reviewed in Graves, 1998). In defense of Aristotle, many reptiles use temperature as the primary signal for determining sex. For example, *Emys orbicularis* (European pond turtle) eggs incubated above 30°C develop as females while eggs incubated below 25°C develop as males (Pieau et al., 1994). The primary signal for sex determination in *C. elegans* is the ratio of X chromosomes (X/A), to sets of autosomes (A) (Nigon, 1949; Hodgkin et al., 1979; Madl and Herman, 1979). Both sexes are diploid, however hermaphrodites have two X chromosomes (XX) and males only have one (XO). Therefore, males have an X/A ratio of 0.5 and hermaphrodites a ratio of 1.0. This ratio is also the signal for the sex-specific trait of dosage compensation. Since males and hermaphrodites differ in their number of X chromosomes, hermaphrodites potentially can produce twice as much X-linked gene product as males. In order to equalize the amount of gene products between the sexes, the hermaphrodite transcript levels of X-linked genes are reduced by half (Meyer and Casson, 1986; Donahue et al., 1987). This difference from how dosage compensation is achieved in *Drosophila melanogaster*, where the single X chromosome in the female (Mukherjee and Beermann, 1965; reviewed in Lucchesi, 1998).

The worm's ability to interpret the X/A ratio is very sensitive, as demonstrated by triploid and tetraploid worms. Animals with two X chromosomes and three sets of autosomes (2X:3A, X/A ratio of 0.67) develop as males whereas tetraploid animals with the slightly higher X/A ratio of 0.75 (3X:4A) develop as hermaphrodites (Nigon, 1949; Hodgkin et al., 1979; Madl and Herman, 1979; Hodgkin, 1987a). Although no denominator elements (i.e. on the autosomes) have been characterized, a number of numerator elements on the X chromosome have been proposed. These were identified using the basic premise that raising the X/A ratio by introducing numerator elements (through duplica-

![Fig. 3. X/A numerator elements on the X chromosome. The regions of the X chromosome labelled one through three represent the three regions identified through duplication and deletion analysis that contain X/A ratio numerator elements (Akerib and Meyer, 1994).](image-url)

*Sex-1*, *Fox-1*, and *Fox-2* are examples of gene loci on the X chromosome that influence the establishment of the X/A signal. The molecular lesions are in a gene that encodes a putative RNA-binding protein, which is now referred to as FOX-1 (Nicoll et al., 1997). By convention, *C. elegans* genes are referred to in lowercase (fox-1), while their protein products are shown in uppercase (FOX-1).

XX animals hemizygous for all three regions on the left end of one of the X chromosomes are viable and only partially masculinized. If all numerator elements are located in these three regions, then deleting all three from one of the X chromosomes in an XX animal should result in a lethal dose of X-linked transcripts and complete masculinization. Since this is not the case, numerator elements must also be located in other regions of the X chromosome. *Sex-1* (signal element on X) is a numerator element near the centre of the chromo-
some, and encodes a member of a nuclear hormone receptor family (Carmi et al., 1998) (Fig. 3). Loss-of-function mutations in sex-1 cause partial XX lethality and masculinization, while extra copies of sex-1 in XO animals result in partial lethality (Carmi et al., 1998), consistent with its classification as a numerator element. When animals are homozygous for both sex-1 and fox-1, XX lethality is 100%, while no lethality is seen with fox-1 homozygotes and only partial lethality in animals homozygous for sex-1. Therefore sex-1 and fox-1 have additive effects in establishing the X/A ratio. Genes other than fox-1 and sex-1 must be involved in numerator activity because, as mentioned, there are two other regions on the left end of the X chromosome that each affect the X/A ratio (Akerib and Meyer, 1994; Nicoll et al., 1997) (Fig. 3). No specific autosomal denominator elements have yet been identified, however the simplest model would lead us to expect that a loss of some of these elements would cause a raising of the X/A ratio and feminization. Thus, somatic sex, germ-line sex and X-chromosome gene expression are controlled by the dosage of several genes on the X chromosome, which seem to act additively by a mechanism that is still unclear.

4. Signalling elements that are involved in both sex determination and dosage compensation

All numerator elements described above appear to control the expression of a single master switch gene, xol-1 (XO lethal) (Nicoll et al., 1997). Loss-of-function mutations in xol-1 cause lethality in XO animals and a switch to the hermaphrodite sexual fate (Miller et al., 1988), therefore xol-1 functions to promote male development and allow for male X-transcript levels. xol-1 expression seems to be regulated in a number of ways. The transcript level of xol-1 is ten times higher in males than hermaphrodites as measured on Northern blots, and expression of a xol-1::lacZ reporter construct is only detectable in males (Rhind et al., 1995), implicating transcriptional regulation in the control of xol-1. sex-1 is involved in this regulation because XO animals hemizygous for loss-of-function mutations of sex-1 inappropriately express the xol-1::lacZ reporter (Carmi et al., 1998). In fact, the screen that was originally used to identify sex-1 relied on inappropriate expression of the xol-1::lacZ reporter in XX animals (Carmi et al., 1998). An elegant experiment using transgenic arrays carrying the xol-1 promoter, the lac operator (lacO), and the lac repressor fused with the green fluorescent protein (lacI::GFP), demonstrated that SEX-1 (identified with a SEX-1 antibody), colocalizes with the array carrying the xol-1 promoter. The array fluoresces green due to lacI::GFP binding to lacO. This supports the idea that SEX-1 acts as a transcriptional repressor of xol-1 by directly binding to its promoter (Carmi et al., 1998). Elements in region one on the left end of the X chromosome are also involved in the control of xol-1 transcription since XX animals hemizygous for xol-1 can be thought of as a master switch gene that is expressed in XO animals to promote the male fate, and inactive in XX animals to allow for the hermaphrodite fate as well as a lowering of X-linked transcript levels. In XO animals, xol-1 appears to act by repressing the activity of the three sdc (sex and dosage compensation) genes. Loss-of-function mutations in sdc-1 and sdc-2 and certain mutations in sdc-3 cause XX animals to become masculinized, as well as having increased X-linked transcript levels (Nusbaum and Meyer, 1989; Villeneuve and Meyer, 1990; DeLong et al., 1993). One clue as to how xol-1 may turn off the sdc activity comes from an experiment where XOL-1 is ectopically expressed in XX animals (Rhind et al., 1995). In XX animals this would normally cause lethality, however the lethality is suppressed when multiple copies of sdc-2(lf) are present in the animal as a transgenic array. The suppression is not due to the SDC-2 protein per se, since expression of SDC-2 from a heterologous promoter is insufficient to suppress the lethality, while plasmid constructs that contain only the 5' upstream region and two-thirds of the coding region of sdc-2 are able to suppress the lethality. This has been explained by a model in which XOL-1, acting as a repressor, is titrated by the extra copies of the control region, thereby allowing the endogenous sdc-2 gene to be expressed. Arrays carrying sdc-1 or sdc-3 are unable to suppress the lethality, suggesting XOL-1 acts primarily on sdc-2. In fact, overexpression of SDC-2 is able to fully compensate for loss of sdc-3 activity in sex determination (Davis and Meyer, 1997). This leaves sdc-3 with an unclear role in sex determination, because null alleles do not have obvious sex determination defects (DeLong et al., 1993). Only certain non-null alleles (sdc-3(Tra)) cause masculinization, but have no effect on dosage compensation (DeLong et al., 1993; Klein and Meyer, 1993). Conversely, other alleles do not affect sex determination, but disrupt dosage compensation (sdc-3(Dpy)). It has been suggested that the sex determination defect of the null alleles is suppressed by the dosage compensation defect, through a feedback mechanism (DeLong et al., 1993). The Dpy and Tra mutations of sdc-3 disrupt different portions of the protein, leading to the interpretation that separate domains may be involved in independent functions. The Dpy mutations eliminate two zinc fingers at the carboxy terminus of SDC-3, while the Tra mutations affect an apparent ATP-binding domain. Obviously, the null alleles eliminate both...
of these domains (Klein and Meyer, 1993). The zinc finger motifs are necessary for association of SDC-3 with the X-chromosome in hermaphrodites, presumably for its dosage compensation function (Davis and Meyer, 1997). SDC-1 also has zinc fingers predicting that it too acts by binding to nucleic acids (Nonet and Meyer, 1991).

5. Genes involved in sex determination, but not dosage compensation

All genetic elements discussed thus far play a role in both sex determination and dosage compensation. In contrast, all factors acting epistatically downstream of the sdc genes are involved in either dosage compensation or sex determination, but not both (Fig. 2). We will discuss only those involved in sex determination (for a review on dosage compensation, see Lucchesi, 1998). First, we will introduce factors involved in the male versus female somatic sex decision, along with recent advances in our understanding of how these factors are regulated. Next, we will discuss the factors involved in germ-line sex determination. Here, there is a male/female decision, but also a level of temporal control. Although there are some genes whose activity is restricted to germ-line sex determination (fog genes, for example), most of the factors involved in somatic sex determination are also involved in the germ line, however their functions and regulation can be slightly different in the two types of tissue.

5.1. Somatic sex determination

The sdc genes promote the hermaphrodite fate in XX animals, at least in part, by lowering the transcript level of the male specific gene, her-1 (hermaphroditization) (Schauer and Wood, 1990; Trent et al., 1991). Loss-of-function mutations in her-1 transform XO animals into hermaphrodites (Hodgkin, 1980), but have no effect on XX animals, implicating her-1 specifically in the promotion of the male somatic fate. An absence of sdc-1 or sdc-2 activity causes a dramatic increase in the transcript level of her-1 in XX animals, thus their normal role is likely to repress her-1 transcription in XX worms, while the inactivity of the sdc genes in XO animals allows for the expression of her-1 (Trent et al., 1991). Mosaic analysis has shown that HER-1 can act cell non-autonomously to promote cells to take on the male fate (Hunter and Wood, 1992). The HER-1 protein has a potential signal sequence, and may act as a secreted ligand that binds a cell surface receptor (Perry et al., 1993). Cell non-autonomy would help to ensure that adjacent cells adopt the same sexual fate. If an XO cell were to misread the X/A ratio, and start a signalling cascade promoting the female cell fate, HER-1 from neighbouring cells could bypass previous signals and cause the cell to adopt the proper male fate.

The tra-2 locus makes three transcripts (Okkema and Kimble, 1991), with the largest being both necessary and sufficient for tra-2’s role in somatic sex determination (Kuwabara and Kimble, 1995). TRA-2A, the product of the largest transcript, is predicted to have membrane spanning domains with an intracellular carboxy terminus and is the most likely candidate for the HER-1 receptor (Kuwabara et al., 1992) (Fig. 4). In XO animals, HER-1 is thought to inactivate TRA-2A by binding, thereby allowing for the male fate (Kuwabara et al., 1992). The absence of HER-1 in XX animals allows TRA-2A to promote the female fate. The putative HER-1 binding site is thought to be in a region of TRA-2A characterized by strong gain-of-function missense mutations called tra-2(eg), for enhanced gain-of-function. An amino acid substitution in this extra-cellular region presumably renders TRA-2A insensitive to negative regulation by HER-1 causing XO animals to be transformed from males into hermaphrodites (Kuwabara, 1996a). However, regulation of tra-2 cannot fully be explained by protein-protein interaction. For example, the transcript encoding TRA-2A is 15 times more abundant in hermaphrodites than males (Okkema and Kimble, 1991), suggesting transcriptional regulation as well. The difference in transcript levels may be due to feedback from genes that are epistatically downstream of tra-2 (namely tra-1 and fem-3), and is dependent upon the phenotypic sex of the animal rather than the X/A ratio (Okkema and Kimble, 1991). TRA-2 is also regulated at the translational level. Gain-of-function (gf) alleles of tra-2 are due to molecular lesions in the 3′ untranslated regions of the transcript.
lateral region (3’UTR) (Goodwin et al., 1993), and prevent hermaphrodites from making sperm (Doniach, 1986; Schedl and Kimble, 1988). The strongest tra-2(ge) allele also results in some somatic feminization of XO animals (Doniach, 1986). The tra-2 3’UTR contains two 28 nt direct repeats (DREs) that are thought to bind a factor (DRF) that inhibits translation (Goodwin et al., 1993). One protein that may be a component of DRF is encoded by the 
\( laf-1 \) (lethal and feminized) gene. Animals homozygous for 
\( laf-1 \) loss-of-function alleles arrest as embryos or early larvae, while animals (both XX and XO) heterozygous for 
\( laf-1(ge) \) are feminized (Goodwin et al., 1997). Part of 
\( laf-1 \)’s wild-type function seems to be to promote male development. The removal of the DRE’s from the 3’UTR allows for an increase in expression from tra-2 reporter constructs. A lacZ reporter construct with a heat shock promoter and the wild-type tra-2 3’UTR resulted in lower \( \beta \)-Gal expression than when a similar construct missing the DREs was used (Goodwin et al., 1997). The removal of the DRE elements could result in the removal of the DRF repressor-binding site, thereby releasing it from translational repression. When the reporter construct with the complete 3’UTR is put in a 
\( laf-1(ge) \) heterozygous background, \( \beta \)-Gal expression is increased. The increase in \( \beta \)-Gal expression in 
\( laf-1(ge) \) heterozygous animals could be due to a release of translational repression in the 3’UTR (Goodwin et al., 1997). Therefore LAF-1 could be a component of DRF that binds to the tra-2 3’UTR, or it could be involved in regulating DRF activity. By genetic epistasis, tra-3 is either upstream of 
\( laf-1 \) or in a parallel pathway, therefore it should also be upstream of tra-2 (Goodwin et al., 1997). Loss-of-function mutations in 
\( tra-3 \) cause masculinization in XX animals. 

\( tra-3 \) is similar to the calpain family of calcium-regulated cystolic proteases (Barnes and Hodgkin, 1996), although there is no evidence that calcium is involved in \( tra-3 \) regulation. It may regulate tra-2 by destroying LAF-1, or another component of the DRF by proteolytic cleavage (Goodwin et al., 1997).

The current model of somatic sex determination involves active \( tra-2A \) preventing the FEM proteins from promoting the male fate (Fig. 4) (Kuwabara and Kimble, 1992). Loss-of-function mutations in any of the three \( fem \) genes result in feminization of XX and XO animals (Doniach and Hodgkin, 1984; Kimble et al., 1984; Hodgkin, 1986). \( fem-2 \) encodes a protein with ankyrin repeats, which are thought to be involved in protein-protein interaction (Spence et al., 1990). It is expressed in virtually all cell types in both sexes suggesting that any regulation must be post-translational (Gaudet et al., 1996). \( fem-2 \) encodes a serine/threonine protein phosphatase (PP2C) (Pilgrim et al., 1995; Chin-Sang and Spence, 1996), with an unusually long amino terminus similar only to its \( Caenorhabditis \) 
\( briggsae \) homologue (Hansen and Pilgrim, 1998). \( fem-3 \) encodes a novel protein, therefore its sequence has so far shed no light on how it may function to promote the male fate (Ahringer et al., 1992). It is possible that the three FEM proteins work together as a complex. Indeed, \( fem-2 \) and \( fem-3 \) do interact, as shown through the yeast two-hybrid system and co-immunoprecipitation of the proteins expressed in rabbit reticulocyte lysates (Chin-Sang and Spence, 1996). Since \( fem-2 \) is a promiscuous phosphatase in vivo and in vitro (Hansen and Pilgrim, 1998), it is possible that \( fem-3 \) serves to regulate the phosphatase activity of \( fem-2 \), either through conferring target specificity or subcellular localization. The phosphatase activity of \( fem-2 \) is necessary for its masculinizing function (Chin-Sang and Spence, 1996), although its target(s) has not yet been identified. The kinase that must act on the same target(s) has also not been identified. One would predict that loss-of-function mutations in such a kinase would result in a masculinized animal. There could be many reasons why the kinase has proved elusive, including the possibility that more than one kinase is involved (redundancy), reducing the possibility that either would be isolated in a standard genetic screen.

The kinase could also be involved in other developmental processes (pleiotropic), therefore its loss could result in a phenotype that could mask the sex determination component of its function. It is possible that \( tra-2 \) inactivates the FEM proteins by binding to one or more of them, and preventing them from interacting with their downstream target(s). \( fem-3 \) can interact with the carboxy terminus of \( tra-2A \), as shown by yeast-two-hybrid (A. Spence, personal communication). When the carboxy terminus of \( tra-2A \) is overexpressed in XO animals, partial somatic feminization is seen, possibly due to titration of one or more of the FEM proteins, thereby preventing them from promoting the male fate (Kuwabara and Kimble, 1995).

\( tra-1 \) is thought to be the terminal regulator of somatic sex determination by functioning as a transcription factor that regulates sex specific genes. The FEM proteins exert their masculinization function (directly or indirectly), by inactivating \( tra-1 \). Its function is to promote the hermaphrodite fate, as loss-of-function alleles of \( tra-1 \) result in somatic masculinization of XX animals (Hodgkin, 1987b). The \( tra-1 \) locus makes two mRNAs that encode two different proteins (Zarkower and Hodgkin, 1992). The smaller protein contains two zinc finger motifs while the larger contains five, and only the larger is capable of binding DNA in vitro (Zarkower and Hodgkin, 1993). The zinc fingers show striking similarity to the zinc finger domains of the \( cubitus interruptus Dominant (c;iD) \) from \( Drosophila \) (Orenic et al., 1990), and the products of GLI and GLI3 from humans (Kinzler et al., 1988; Ruppert et al., 1990). Although \( tra-1 \) does not seem to have the same general function as any of these three genes, the strong similarity could reflect the conservation of a specific activity. Gain-of-function alleles of \( tra-1 \) are caused by molecular lesions that substitute or delete amino acids in a region common to both the large and small forms of \( tra-1 \). Perhaps this region is involved in binding to another protein, although the identity of such a partner remains obscure (de Bono et al., 1995). Disruption of the protein-binding site is thought to result in
a lack of negative regulation of tra-1, allowing for gain-of-function female somatic development. One of the FEM proteins may bind to TRA-1, thereby inactivating it, and the other FEM proteins may be needed for this binding. The ankyrin repeats on FEM-1 make it the most likely of the three existing candidates to bind TRA-1.

As mentioned, tra-1 appears to be the terminal global regulator of somatic sex determination. Both the zinc finger domains on the TRA-1 proteins, and the ability of the larger protein to bind DNA in vitro (Zarkower and Hodgkin, 1993), support the argument that TRA-1 functions as a transcriptional regulator that controls the expression of sex specific genes. mab-3 (male abnormal) functions downstream of tra-l and promotes the development of V rays in the male tail, and represses the production of vitellogenin in the male intestine (Shen and Hodgkin, 1988).

tra-1 activity is necessary to prevent mab-3 mRNA from mis-accumulating in XX animals (Raymond et al., 1998), therefore TRA-1 may regulate the expression of mab-3, either directly or indirectly. Presumably, TRA-1 regulates the activity of many genes, in addition to mab-3, that are each involved in the differentiation of a subset of sex specific tissues.

Therefore, in order for a male soma to form, tra-2 is repressed by her-1, presumably through a ligand binding mechanism (Fig. 4). tra-2 is also repressed at the transcriptional and translational levels, which likely results in less TRA-2A protein made than in XX animals. With tra-2 repressed, the fem genes are able to inhibit the terminal regulator tra-1 (Fig. 2). A female soma forms when tra-1 is active. In order for this to occur, the fem gene products are inactivated by TRA-2A (Fig. 2).

5.2. Germ-line sex determination

Many nematode species do not have a hermaphrodite sex, but rather consist of males and ‘true’ females. The soma of the closely related species Caenorhabditis remanei looks very similar to C. elegans, however C. remanei XX animals do not make sperm. Since the C. elegans XX animal makes sperm and oocytes in each of its ovotestes, it is reasonable to assume that additional levels of genetic control are needed in the germ line, compared to the soma. One of the major obstacles that the hermaphrodite must address is how a differentiated male tissue (sperm), can be made in a female soma, while the environment remains competent for subsequent oocyte production. Spermatogenesis occurs for a short period of time, before switching to oogenesis, implying that the regulation of genetic factors involved in promoting sperm and oocyte production must also change at this point.

5.2.1. Hermaphrodite germ-line sex determination

Gamete production in the XX germ line is divided into two temporal phases: spermatogenesis and oogenesis. How is a male sexual fate accomplished in the germ line, even though the X/A ratio is that of the female (i.e. 1.0)? First, the terminal regulator of sex in the germ line differs from that in the soma. tra-1 is the terminal regulator in the soma and functions by promoting the female fate when active and allowing for a male soma when absent (Hodgkin, 1987b). In the germ line, epistatic analysis suggests that tra-1 is not the terminal regulator, although it is necessary for continued spermatogenesis (Hodgkin, 1987b; Schedl et al., 1989). The three fem genes are the apparent terminal regulators of sex determination in the germ line (Fig. 5). Two other genes (with no apparent somatic role), also work with the fems to promote spermatogenesis, namely fog-1 and fog-3 (feminization of the germ line) (Barton and Kimble, 1990; Ellis and Kimble, 1995). Loss-of-function mutations in either fog-1 or fog-3 result in the exclusive production of oocytes in somatically male or female animals (Barton and Kimble, 1990; Ellis and Kimble, 1995). For the purpose of this review, we will refer to fem-1, -2, -3, fog-1 and -3 collectively as the tfgs (terminal germ line fems and fogs) genes. In order for spermatogenesis to occur, the tfgs must be active. Their activity is dependent upon the inactivation of tra-2. Active tra-2 serves to repress at least one of the tfgs. A possible mechanism for tra-2 inactivation in the germ line involves a third fog gene (fog-2). Loss-of-function mutations in fog-2 feminize the germ line, however, unlike fog-
1 and fog-3, feminization occurs only in XX animals (Schedd and Kimble, 1988). Genetic epistasis places fog-2 upstream of the tgf5s (Schedd and Kimble, 1988), therefore it could function by turning off tra-2, as mentioned, or by turning on one or more of the tgf5s to promote spermatogenesis (Fig. 5). Even if fog-2 does function through the tgf5, down regulation of tra-2 is necessary for spermatogenesis to occur in the hermaphrodite. This is demonstrated by tra-2 gain-of-function alleles that result in feminization of the XX germ line (Hodgkin, 1980; Doniach, 1986; Schedd and Kimble, 1988). her-1 apparently is not involved in this down regulation since loss-of-function alleles of her-1 do not affect spermatogenesis in XX animals (Hodgkin, 1980), and XX animals lack detectable levels of her-1 transcript (Trent et al., 1991). The down regulation of tra-2 could be accomplished, at least in part, through translational repression by its 3’UTR. As discussed earlier, gain-of-function mutations of tra-2 are due to lesions of direct repeat elements (DRE) in the 3’UTR (Goodwin et al., 1993). This results in increased tra-2 activity that promotes XX animals from making sperm. It is thought that DRF binds to the DRE elements in the second or third larval stage to prevent translation of tra-2 and allow for spermatogenesis. laf-1 could be involved in the 3’UTR mediated regulation of tra-2 by promoting DRF binding, and tra-3 could restrict laf-1 to performing this function in the second and third larval stages of hermaphrodite development (Goodwin et al., 1997). GLD-1 binds the tra-2 3’UTR and is able to repress translation in a DRE dependent manner (Jan et al., 1999). Genetic analysis of glh-1 suggests that it has a necessary role in oogenesis, as well as in spermatogenesis in the hermaphrodite germ line (Ellis and Kimble, 1995; Francis et al., 1995a,b). Another mechanism regulating tra-2 in the hermaphrodite may involve a signal emanating from the somatic gonad. When certain cells from the hermaphrodite gonadal sheath or spermatheca are eliminated by laser ablation, the germ line is feminized (McCarter et al., 1997). These cells may secrete an inhibitory ligand for TRA-2, similar to the action of HER-1 in the male soma. It is possible that other domains exist in TRA-2 that serve as receptors for negative regulatory elements, such as the 22 amino acid MX region (Kuwabara et al., 1998). tra-2(mx) alleles cause a mixed phenotype; gain-of-function feminization in the germ line, resulting in a lack of spermatogenesis in XX animals, and partial loss-of-function masculinization in the soma (Doniach, 1986; Schedd and Kimble, 1988). The germ line gain-of-function phenotype could be due to a disruption of an inhibitory molecule-binding site (Kuwabara et al., 1998).

The transient down regulation of tra-2 thereby allows the tgf5s to promote a brief period of spermatogenesis, sufficient for approximately 150 sperm to be made per gonad arm, after which a switch is made to oocyte production. This requires that one or more of the sperm promoting tgf5 be turned off, and oocyte promoting factors be turned on. At least in part, this may be accomplished by repressing translation of fem-3 through its 3’UTR (Ahringer and Kimble, 1991). Gain-of-function mutations in fem-3 result in constitutive sperm production and are due to lesions in a region of the 3’UTR referred to as the point mutation element (PME) (Ahringer and Kimble, 1991). Zhang et al. (1997) were able to identify a factor that binds to the PME of the fem-3 3’UTR using a modified version of the yeast two-hybrid screen, where an RNA molecule (the 3’UTR) is used as bait. The protein is referred to as the fem-3 binding factor (FBF-1) and is encoded by the fbf-1 gene. The genome sequencing project identified an homologous, fbf-2, which encodes a protein with 91% identity to FBF-1. Both are able to bind PME and may have redundant functions. Together, they are referred to as FBF. It is thought that FBF down-regulates fem-3 expression by binding to its 3’UTR preventing translation. RNA interference (RNAi) (Fire et al., 1998), which knocks out the expression of FBF, causes a lack of oocyte production and most of the resulting animals make sperm (Zhang et al., 1997). This is consistent with FBF serving as a negative regulator of fem-3, since its phenotype is similar to that seen with fem-3(gf) mutations. Therefore, direct regulation of fem-3 appears to be involved in the switch from spermatogenesis to oogenesis.

Six other genes, namely mog-1, mog-2, mog-3, mog-4, mog-5, and mog-6 (masculinization of the germ line), are also involved in the switch from sperm to oocyte production (Graham and Kimble, 1993; Graham et al., 1993). In contrast to the fog genes, loss-of-function mutations in any of the mog genes, for the most part, result in masculinization that is restricted to the germ line. Genetic epistatic analysis places the mogs at the same location in the sex determination pathway as tra-2 (Graham and Kimble, 1993; Graham et al., 1993). Analysis of double mutants has shown that oocytes can still be made in the absence of the mogs, suggesting that they are not required for oocyte synthesis, but rather in the temporal switch from spermatogenesis to oogenesis (Graham and Kimble, 1993; Graham et al., 1993). Recently, a requirement for the mog genes has been demonstrated in the translational repression of transgenes by the fem-3 3’UTR (Gallegos et al., 1998). Surprisingly, however, this analysis has suggested that the fem-3 3’UTR-mediated repressor activity is found in somatic tissues, as well as in the germ line. Since fem-3 3’UTR gain-of-function mutant XX animals do not show somatic masculinization, Gallegos et al. (1998) suggest that although this repression machinery is ubiquitous, fem-3 mRNA is not normally found in XX somatic tissues, therefore translational repression of fem-3 may not occur in the soma. The fem-2 3’UTR is also able to inhibit expression of reporter transgenes in the soma, however, a dependency on the mog genes has not yet been shown for this repression to occur (Hansen and Pilgrim, unpublished results).

Hodgkin and Barnes (1991) demonstrated the importance of the timing of the switch from spermatogenesis to oogenesis. Since sperm are normally the limiting factor for the production of self-progeny by the hermaphrodite (a rare thing in the animal kingdom), the number of
sperm made reflects the number of progeny that can be produced. In one tra-3 allele, (e2333), sperm production is increased by about 200, resulting in a self-fertilized brood size of 500 (Hodgkin and Barnes, 1991). When the rate of population growth of tra-3(e2333) animals was compared to the wild-type, it was found that wild-type populations grew faster (Hodgkin and Barnes, 1991). At first glance, this may appear counterintuitive, since tra-3(e2333) animals have a much larger brood. However, since oocytes are not able to be produced until spermatogenesis is completed, the amount of time needed to produce the ‘extra’ sperm delays the onset of oogenesis and subsequent fertilization of the first progeny. Therefore, even though tra-3(e2333) animals produce more progeny, the increase in generation time decreases the rate of population growth. The regulation of tra-3 activity seems fine tuned by evolution to balance the number of progeny with generation time to allow for maximum population growth.

5.2.2. Germ-line sex determination in the male

In the male, spermatogenesis requires her-1 activity, which contrasts with spermatogenesis in the hermaphrodite, where her-1 activity is not required. Males homozygous for a temperature sensitive allele of her-1 grown at the permissive temperature, and then shifted to the restrictive temperature as adults, switch from spermatogenesis to oogenesis. As with male somatic tissue, a model has been proposed in which HER-1 binds and inactivates TRA-2 in the male, where inactivated TRA-2 would appear counterintuitive, since tra-2(e2333) animals have a much larger brood. However, since oocytes are not able to be produced until spermatogenesis is completed, the amount of time needed to produce the ‘extra’ sperm delays the onset of oogenesis and subsequent fertilization of the first progeny. Therefore, even though tra-3(e2333) animals produce more progeny, the increase in generation time decreases the rate of population growth. The regulation of tra-3 activity seems fine tuned by evolution to balance the number of progeny with generation time to allow for maximum population growth.

6. Evolution of sex determination proteins

As several of the genes in the sex determination pathway encode proteins that have only the most subtle of structural similarities with other proteins in the databases, developing structure-function relationships remains a problem. This could be due, in part, to the advanced state of the C. elegans genome project compared to other metazoans; and homologues may yet appear once other genomes are sequenced. In the meantime, one approach to elucidate structure-function relationships is to find a homologue from a closely related species, where the similarities are significant enough to allow isolation by low stringency hybridization, yet different enough that conserved regions are likely to be meaningful. For C. elegans genes, the related species Caenorhabditis briggsae has been used successfully (e.g. Jones and Candido, 1993), and homologues identified in this manner are often functionally interchangeable (Maduro and Pilgrim, 1996). Despite the superficial similarity of the secondary sexual development of the two species (both are male-hermaphrodite species), it has proven unexpectedly difficult to isolate C. briggsae homologues of C. elegans sex determining genes, and once isolated, they are often not fully interchangeable with their C. elegans counterparts. The Caenorhabditis sex-determining genes seem to be diverging much faster than most other protein coding genes examined to date. Below is a summary of the progress that has been made in identifying homologues of Caenorhabditis sex-determining genes.

6.1. tra-2

The tra-2 gene from C. briggsae does not cross-hybridize with C. elegans probes, even at low stringency (Kuwabara
and Shah, 1994). Instead, it was identified by synteny to a nearby gene in C. elegans that did cross hybridize (Kuwabara and Shah, 1994). The mRNA products from the tra-2 locus differ between the two species (Kuwabara, 1996b). Cb-tra-2 produces a single band on Northern blots, corresponding to the larger of the two C. elegans transcripts. Although a variety of alternatively spliced species can be produced, the largest proteins produced correspond to the TRA-2A protein of C. elegans. Cb-TRA-2A and Ce-TRA-2A are 64% similar and 43% identical over almost 1500 amino acids. Despite this low level of similarity, the EG site (potential site of interaction for HER-1), and several sites of the mx class of mutations are conserved. Both genes are trans-spliced to SL2, and contain a short (three codon) ORF in the 5'-UTR (Kuwabara, 1996b). The Cb-tra-2 3'-UTR shows some sequence similarity to the Ce-tra-2 3'-UTR and also seems to be involved in translational control (Jan et al., 1997). RNAi treatment of C. briggsae XX animals with the Cb-tra-2 gene did produce somatically masculinized progeny, suggesting that the C. briggsae homologue is involved in sex determination (Kuwabara, 1996b).

6.2. fem-1

Homologues of fem-1 have been cloned from C. briggsae, as well as from the male/female species Caenorhabditis remanei (J. Gaudet and A. Spence, personal communication). The C. briggsae homologue is able to partially rescue C. elegans animals mutant for fem-1 and RNAi inactivation of fem-1 in C. briggsae results in feminized animals suggesting that its masculinizing activity is conserved between the species. Other homologues have been cloned from mice (mFem-1a and mFem-1b) (Ventura-Holman, 1998). mFem-1a was isolated from a two-hybrid screen using a high mobility group protein (HMGI-C) as bait, while mFem-1b was identified by searching an EST database. mFem-1a and mFem-1b are 36% and 30% identical to Ce-FEM-1, respectively (Ventura-Holman, 1998), and mFem-1a has weak activity in worms (J. Gaudet and A. Spence, personal communication), however it is currently unknown if it has a role in sex determination in mammals.

6.3. fem-2

As with TRA-1, the predicted FEM-2 protein sequence immediately suggested a biochemical role, that of a protein phosphatase. However, the amino terminal portion of FEM-2 has no similarity to any protein in the database. A fem-2 homologue has been cloned from C. briggsae by low stringency hybridization (Hansen and Pilgrim, 1998). Since Caenorhabditis has several PP2C homologues that have been identified by the Genome Sequencing Consortium (Wilson et al., 1994), we have a reasonable internal control for any unusual degree of sequence divergence in FEM-2. The complete sequence of the FEM-2 homologue has been obtained, as well as a partial sequence of another PP2C homologue, from C. remanei (P. Stothard, D.H. and D.P., unpublished observations). A region common to all PP2C enzymes is 68% identical between the FEM-2 homologues while the non-sex-determining PP2C homologues are 95% identical in the same region. Thus, not only does the sequence divergence in the fem-2 gene exceed that expected for the ‘average’ gene, it is also much faster than that seen by its direct paralogues with no demonstrated role in sex determination.

6.4. tra-1

As a transcriptional regulator, tra-1 homologues were immediately apparent in the databases once the sequence was available, and a C. briggsae homologue was directly identified by low stringency hybridization (de Bono and Hodgkin, 1996). C. elegans tra-1 encodes two transcripts of 5 kb and 1.5 kb, which encode proteins differing greatly at their carboxy termini. As with tra-2, the transcript sizes differ between the two species. C. briggsae tra-1 also encodes two transcripts, but both are approximately 5 kb in size, and encode putative proteins that differ slightly at their amino termini rather than carboxyl. The only mRNA that is conserved between the two species corresponds to the TRA-1A protein of 1110 amino acids. The predicted Cb-TRA-1A protein is 55 amino acids longer, and shares about 45% similarity with the C. elegans homologue. As a transgene, the C. briggsae homologue shares some, but not all the properties of C. elegans tra-1. The somatic masculinization caused in XX animals by tra-1 null mutations can be partially rescued by expression of Cb-TRA-1, but the gonadal masculinization seemed completely unaffected (de Bono and Hodgkin, 1996), and no vulval development was seen. XO animals transgenic for the C. briggsae gene showed feminization of the tail and the intestine, however, there was no sign of vulval induction, and the gonad still had a male morphology and produced sperm (de Bono and Hodgkin, 1996). This latter observation (inappropriate feminization) suggests that the C. briggsae homologue is not properly regulated as a transgene in C. elegans, since Ce-tra-1 transgenic XO animals do not show such feminization.

7. Why are sex determination genes rapidly evolving?

All of the genes described above seem to be diverging much faster than genes involved in other developmental processes. This observation is not limited to Caenorhabditis. The mammalian sex-determining gene Sry has an unusually high rate of divergence over a wide range of species (Tucker and Lundrigan, 1993; Whitfield et al., 1993; Pamilo and O’Neill, 1997). The transformer (tra) sex-determining gene of Drosophila is extremely variable between closely related species (O’Neil and Belote, 1992; Civetta and Singh, 1998). Genes not necessarily involved in sex determination,
but in sex related behaviours, such as fertilization and mate recognition, also appear to be rapidly evolving (Ferris et al., 1997). Although there may be many genes of diverse functions rapidly evolving in a metazoan genome, a high rate of divergence is characteristic of genes involved in sex determination and sex-related behaviours. Many of the sex-related genes show a high degree of non-synonymous to synonymous nucleotide substitutions, suggesting that this rapid divergence has been driven by directional selection (Civetta and Singh, 1998). Alternative explanations have been proposed, such as that the sex-determination genes are tightly linked to unrelated genes that are selected for their influence on fitness levels. For *C. elegans*, one may argue that the recent evolution of a hermaphrodite sex from a female may also allow *C. elegans* genes to show more rapid evolution than expected, as the genes accommodate to spatial and temporal controls that are unnecessary in a male/female species. It has also been suggested that the rapid divergence of *Sry* is due, in part, to the lack of recombination on the non-pseudoautosomal region of the Y. This has led to theories that SRY divergence is a natural consequence of the evolutionary degradation of the Y (Graves, 1995). In *C. elegans*, there is no such restriction, and the effect is still seen. These arguments are increasingly harder to make, now that the number of species with sex-determining genes that show ‘rapid’ evolution continues to accumulate. A simpler explanation is that this is a general property of sex-determining genes in all systems.

Since there is extensive sequence divergence between the sex-determining loci of closely related species, as discussed above, and no similarity at all between the major sex-determining loci of any of the major ‘model systems’, it was somewhat surprising to find that one gene involved in sexual development does seem to be conserved throughout metazoan evolution. Raymond et al. (1998) cloned and characterized the *mab-3* gene from *C. elegans*, which acts downstream of *tra-1* to promote male-specific development of the intestine and the peripheral nervous system (tail). It contains a DNA-binding domain similar to that in the Drosophila doublesex (*dsx*) gene, and a human homologue with testes-specific expression was also identified (Raymond et al., 1998). The *Drosophila dsx* locus makes two proteins, one that promotes the male fate and the other the female fate (Burtis and Baker, 1989). The male-specific protein is able to rescue the *mab-3* tail phenotype in *C. elegans*, however the female-specific DSX protein cannot (Raymond et al., 1998). This raises the possibility that *mab-3* and *dsx* have similar functions in sex determination, which have been conserved throughout metazoan evolution. Both of these genes function ‘downstream’ of most of the other sex-determining genes in their respective organisms. Marin and Baker (1998) have suggested that changes in genes early in the sex-determining gene hierarchy are easily accommodated, because these genes are more likely to have a single or a small number of targets, than those further down the pathway (e.g. *mab-3, dsx*). Also, by mechanisms that are not fully understood, a new gene function that can ‘take control’ of the early stages in sex determination may start a new level of selection to begin. An example of this is in humans, where *Sry* may act in a dominant manner merely because it interferes with the action of another gene (*Sox-3*) which acts in a female determining manner (Graves, 1998).

8. Conclusions

Genetic epistatic analysis of many of the first isolated *C. elegans* sex-determination mutants suggested a series of negative interactions between genes in a signalling pathway (Hodgkin and Brenner, 1977; Nelson et al., 1978; Hodgkin, 1980, 1986; Doniach and Hodgkin, 1984). More genes have now been identified that have a role in sex determination, and numerous types of regulatory mechanisms have been identified that control the activities of these genes. Certainly, there are still many aspects of *C. elegans* sex determination that have not yet been elucidated. For example, many of the genes that are involved in the switch from spermatogenesis to oogenesis have been identified, however it is still unclear as to what initial signal initiates the switch. Also, the mechanism by which the *fen* genes negatively regulate *tra-1* in the soma is still unclear, as well as how they promote spermatogenesis. As these and many other questions relating *C. elegans* sex determination are being addressed, we cannot help but take note of how the work already accomplished has aided in our understanding of many different aspects of biology. Sex determination in a small worm with approximately 1000 somatic cells has proved to be a useful tool for studying such things as gene regulation, signal transduction, translational control, development, protein interactions and evolution.

Acknowledgements

We would like to thank Jeb Gaudet and Andrew Spence for sharing unpublished results, and Elizabeth Goodwin for keeping us apprised of work in progress. We would also like to thank Paul Stothard for unpublished results, and Shelagh Campbell and anonymous reviewers for comments on the manuscript.

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