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FEM-2 is a Rapidly Evolving Protein Phosphatase

by

David Donald Hansen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

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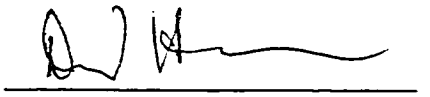

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Abstract

Cell fate determination is a fundamental aspect of development. For most multi-cellular organisms, this includes a decision as to what sex the organism will adopt. Arguably one of the most intensively studied systems for determining sex is that found in *Caenorhabditis elegans*. Based primarily on genetic and molecular analysis, numerous genes have been identified that work together in determining the sexual fate decision. One of these genes, *fem-2* (feminization), is involved in promoting the male fate in both the germ line and soma, therefore its loss-of-function phenotype is feminization of both males and hermaphrodites (Kimble *et al.*, 1984; Hodgkin, 1986). Alignment of the genetic and physical maps, and subsequent transgene rescue, revealed an Open Reading Frame (ORF), which encoded a putative protein phosphatase that was thought to encode FEM-2 (Pilgrim, 1993; Pilgrim *et al.*, 1995). Herein I describe experiments that confirm that this ORF does encode FEM-2, and that it does function as a protein phosphatase. The phosphatase domain of the protein is most similar to protein phosphatases of the type 2C (PP2C), however its long amino terminus only shows similarity to FEM-2 homologues from the closely related nematode species *Caenorhabditis briggsae* and *Caenorhabditis remanei*, the cloning of which are described herein. Sequence comparison of these proteins reveals an unusually high degree of divergence, which may be characteristic of proteins involved in sex determination. The two domains of the FEM-2 proteins seem to be evolving at different rates, raising the possibility that protein binding domains are less constrained in their abilities to evolve. Comparison of the sequences in the putative regulatory regions of the genes revealed possible conserved sequences, including some in the 3' untranslated regions (3'UTR). In this thesis, I demonstrate that the *fem-2* 3'UTR is able to negatively regulate expression of a heat inducible *lacZ* reporter construct. Deletion of part of the 3'UTR increases the level of expression, suggesting that these sequences are involved in repression. Therefore, 3'UTR

mediated repression may be a means of regulating expression of the protein, similar to that seen with other *C. elegans* sex determining genes.

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as well as my siblings and their spouses, have been a tremendous support during my schooling, and deserve special mention here.

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INTRODUCTION

General Introduction

Over 2000 years ago, Aristotle suggested that men mate during the hot summer months if they wished to have male offspring, based on his belief that sex was determined by temperature. Mendel's work in the middle of the 19th 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* (figure 1). Classical genetic screens have identified numerous loci that affect the determination of sex in *C. elegans*. One of the genes, *fem-2*, is involved in promoting spermatogenesis and male somatic development. Cloning and sequencing of the DNA corresponding to the minimal rescuing region identified an Open Reading Frame (ORF) that encodes a protein with similarity to protein phosphatases of the type 2C (Pilgrim *et al.*, 1995). The purpose of this thesis project was to; (a) confirm that the ORF encodes FEM-2 (b) determine if its similarity to protein phosphatases reflects actual phosphatase activity (c) identify possible regulatory regions in the gene. This was done in an effort to provide insight into how sexual fate is determined in *C. elegans*, as well as to increase our general understanding of how signal transduction mechanisms are used in development.

Before describing specifics about what is known about the *fem-2* gene, I will first describe other aspects of sex determination in *C. elegans*, in order to provide a context for a discussion of *fem-2*. This will first include a brief description of the behavior and

anatomy of the two *C. elegans* sexes. I will next describe what is known about the molecular mechanisms involved in the sexual fate decision. This includes the primary signal and interpretation of that signal. I will also point out the differences between the molecular mechanisms involved in somatic (figure 2) versus germ-line (figure 3) sex determination. Finally, I will describe what was known about *fem-2's* involvement in these processes prior to the initiation of this thesis.

Sexual Dimorphism

C. elegans exists in two sexes, male and self fertilizing hermaphrodite (figure 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 non-self fertile females of closely related species (e.g. *Caenorhabditis remanei*), therefore hermaphrodites are most conveniently thought of as somatic females that are transiently able to produce sperm. The gonad of the female or hermaphrodite consists of two arms that join at a common uterus and open to the environment through a vulva (figure 1). The regions of the gonad arms most distal from the vulva consist of a syncytium that contain mitotic nuclei that serve as stem cells for sperm and oocytes. With time these 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 a single 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 where they are stored. If both male and self (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 occasional random nondisjunction of the X chromosome.

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, where 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 obicularis* (European pond turtle) eggs incubated above 30° develop as females while eggs incubated below 25° develop as males (Pieau *et al.*, 1994). The primary signal for sex determination in *C. elegans* is the ratio of X chromosomes (X), 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 differs from how dosage compensation is achieved in *Drosophila melanogaster*, where the single X chromosome in the male is twice as active as the two X chromosomes in the female (Mukerjee and Beerman, 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; Hodgkin, 1987a; Madl and Herman, 1979). 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 duplication or transgenic arrays), should feminize XO animals or cause them to die due to a lowering of transcript levels on the single X. Likewise, if numerator elements are removed from an XX animal through deletion, the X/A ratio would be lowered, causing masculinization and possible lethality due to a higher than normal level of X-linked transcripts. Akerib and Meyer (1994) found that duplications and deficiencies mapping to the left end of the X chromosome influence the phenotype in the manner described above. Three different regions on the left end of the X chromosome cause partial XO lethality when present in extra dose (figure 4). Although not all XO animals die when

any single region is duplicated, XO lethality is highest when an extra copy of all three regions are present, suggesting that numerator elements exist in all three of these regions and work additively to specify the X/A ratio. Hodgkin *et al.* (1994) identified a potential numerator element they called *fox-1* (feminizing locus on X) within region three on the left end of the X chromosome (figure 4). Multiple copies of this 30 kb region, in the form of a transgenic array, cause XO lethality, presumably due to an increase in the X/A ratio. Nicoll *et al.* (1997) identified two mutations in the *fox-1* region 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 center of the chromosome, and encodes a member of a nuclear hormone receptor family (Carmi *et al.*, 1998) (figure 4). 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) (figure 4). 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.

Signaling 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) (figure 2) (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 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 sequences in this region also inappropriately express the *xol-1::lacZ* reporter. XX animals homozygous for *fox-1(lf)* mutations, or hemizygous for sequences in regions two and three, do not show this inappropriate expression, therefore they are likely not involved in regulating *xol-1* transcription (Nicoll *et al.*, 1997). However, high levels of *fox-1* activity are able to reduce the levels of a GFP::XOL-1 fusion protein in XX and XO animals, suggesting a post-transcriptional level of regulation (Nicoll *et al.*, 1997). It is currently unknown how the elements in region two and three specifically regulate *xol-1* expression.

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 (figure 2). Loss-of-function mutations in *sdc*-1 and *sdc-2*, and certain mutations in *sdc-3*, cause XX animals to become masculinized, and to show increased X-linked transcript levels (DeLong *et al.*, 1993; Nusbaum and Meyer, 1989; Villeneuve and Meyer, 1990). 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(+)* 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 also acts by binding to nucleic acids (Nonet and Meyer, 1991).

Genes involved in sex determination, but not dosage compensation

All genetic elements discussed thus far have roles 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 (figure 2). I will discuss only those involved in sex determination (for a review on dosage compensation, see Lucchesi, 1998). First, I 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, I 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.

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) (figure 2). 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 nonautonomously 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 nonautonomy would help to ensure that adjacent cells adopt the same sexual fate. If an XO cell were to mis-read the X/A ratio, and start a signaling cascade promoting the female cell fate, HER-1 from neighboring 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) (figure 5). 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 extracellular 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 region (3'UTR) (Goodwin *et al.*, 1993), and prevent hermaphrodites from making sperm (Doniach, 1986; Schedl and Kimble, 1988). The strongest *tra-2(gf)* 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(lf)* 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 ß-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(lf)* heterozygous background, ß-GAL expression is increased. The increase in ß-GAL expression in *laf-1(lf)* 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 cytosolic 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 (figure 2) (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; Hodgkin, 1986; Kimble *et al.*, 1984). *fem-1* 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 is post-translational (Gaudet *et al.*, 1996). It is possible that TRA-2 inactivates the FEM proteins by binding to one or more of them, and prevent 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). *fem-2* will be discussed in more detail later in this Introduction.

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). 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.

Therefore, in order for a male soma to form, *tra-2* is repressed by *her-1*, presumably through a ligand binding mechanism (figure 5). *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* (figure 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 (figures 2 and 5)

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.

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 in the XO germ line (Hodgkin, 1987b; Schedl *et al.*, 1989). The three *fem* genes are the apparent terminal regulators of sex determination in the germ line (figure 3). 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). In this thesis, I will refer to *fem-1*, *-2*, *-3*, *fog-1* and *-3* collectively as the *tgf* (terminal germ line *fems* and *fogs*) genes. In order for spermatogenesis to occur, the *tgfs* must be active. Their activity is dependent upon the inactivity of *tra-2*. Active *tra-2* serves to repress at least one of the *tgfs*. 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 (Schedl and Kimble, 1988). Genetic epistasis places *fog-2* upstream of the *tgfs* (Schedl and Kimble, 1988), therefore it could function by turning off *tra-2*, as mentioned, or by turning on one or more of the *tgfs* to promote spermatogenesis (figure 3). Even if *fog-2* does function through the *tgfs*, 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; Schedl 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 (figure 6) (Goodwin *et al.*, 1993). This results in increased *tra-2* activity that prevents 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). One component of DRF is GLD-1, whose binding to the *tra-2* 3'UTR was identified by a three-hybrid screen, and has been shown to repress translation by binding to the *tra-2* 3'UTR *in vitro* and *in vivo* (Jan *et al.*, 1999). Genetic analysis of *gld-1*

suggests that it has a necessary role in oogenesis, as well as in promoting spermatogenesis in the hermaphrodite germ line (Francis *et al.*, 1995a; Francis *et al.*, 1995b; Ellis and Kimble, 1995). 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 MX region (Kuwabara, 1996b). *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; Schedl and Kimble, 1988). The germ line gain-of-function phenotype could be due to a disruption of a binding site for an inhibitory molecule not used in somatic cells.

The transient down regulation of *tra-2* thereby allows the *tgfs* 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 *tgfs* 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) (figure 6) (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 (figure 6). The protein is referred to as the *fem-3* binding factor (FBF-1) and is encoded by the *fbf-1* gene. The sequencing project identified a homologue, *fbf-2*, that

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 downregulates *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, results 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) (figure 3). Analysis of double mutants has shown that oocytes still can 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.

Hodgkin and Barnes (1991) were able to demonstrate 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 wildtype, 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 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.

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 (Schedin *et al.*, 1994). This suggests that continuous *her-1* activity is necessary to maintain spermatogenesis in the male germ line. As with male somatic tissue, a model has been proposed in which HER-1 binds and inactivates TRA-2 (Kuwabara and Kimble, 1992) (figure 3). *fog-2* appears not to be involved in repressing *tra-2* in the male, since its loss-of-function phenotype feminizes only the hermaphrodite

germ line, with spermatogenesis occurring normally in males (Schedl and Kimble, 1988). Therefore, binding of HER-1 to TRA-2 could be the primary mechanism of turning off TRA-2 in the male, where inactivated TRA-2 would be unable to inhibit the function of the *tgf* genes. Loss-of-function mutations in any of the *fem* genes results in a feminization of the XO soma and germ line (Doniach and Hodgkin, 1984; Kimble *et al.*, 1984; Hodgkin, 1986), while loss-of-function mutations of *fog-1* and *fog-3* result in feminization of the XO germ line (Barton and Kimble, 1990; Ellis and Kimble, 1995). Therefore all five of these genes are needed for spermatogenesis in males and hermaphrodites. No genes epistatically downstream of the *tgf* genes are known to exist, therefore these five could be the terminal regulators of male germ-line development. This could be accomplished by promoting spermatogenesis or inhibiting oogenesis, or both. *tra-1* also is involved in spermatogenesis in the male. *tra-1(null)* XO animals have a male soma, however in their germ line they first produce sperm, then oocytes (Hodgkin, 1987b; Schedl and Kimble, 1989). This demonstrates that *tra-1* is not necessary for spermatogenesis to occur, but rather for the maintenance of spermatogenesis. It is an unexplained paradox that *tra-1* could have a feminizing role in the soma and a masculinizing role in the germ line.

Overall, then, there are a number of differences between germ-line and somatic sex determination. For example, *tra-1* is not the terminal regulator in the germ line, rather the *tgf* genes appear to directly promote spermatogenesis. Also, the *mog* and *fog* genes primarily function in germ line, with the *mogs* turning off the *tgfs* in the hermaphrodite to allow for oogenesis. The *fogs* promote spermatogenesis, either by repressing *tra-2* in the hermaphrodite germ line (*fog-2*), or by working with the *fems* in both sexes (*fog-1* and *fog-3*). The regulation of some of the genes appears to differ in the germ line, with translational repression (*tra-2* and *fem-3*) being a common theme, although recent evidence suggests that 3'UTR mediated translational repression may also be found in the

soma (Goodwin *et al.*, 1997; Gallegos *et al.*, 1998). Though there are differences between the control of germ-line and somatic sex determination, both involve epistatic relationships consisting of step-wise negative interactions.

GENETIC ANALYSIS OF *fem-2*

b245, the first *fem-2* allele isolated

The first allele isolated of *fem-2* was the temperature sensitive allele *b245* (Kimble *et al.*, 1984). At the permissive temperature (<20°), XX and XO animals show no mutant phenotype, however at the restrictive temperature (>20°), both show some feminization (table 1) (Kimble *et al.*, 1984). XX animals fail to make sperm, therefore are transformed into functional females. XO animals produce fewer sperm and then switch to the production of oocytes. They also develop an intersexual somatic gonad, however no other somatic feminization is apparent (Kimble *et al.*, 1984). The first *fem-1* allele isolated (*hc17*), is also temperature sensitive and its phenotype is very similar to that of *fem-2(b245)* described above (Nelson *et al.*, 1978). However, feminization is more extreme when animals are homozygous for the temperature sensitive alleles of both genes, with XO animals raised at 25° being completely feminized and animals raised at lower temperatures showing less extensive feminization (Kimble *et al.*, 1984). This suggests that both *fem-1* and *fem-2* have masculinizing activity in the soma, even though the single mutant phenotypes do not reflect this.

Temperature sensitive period of *fem-2*

Since *b245* is a temperature sensitive allele, Kimble *et al.* (1984) used it to determine when in development *fem-2* activity is needed. It is possible that the temperature sensitive period (TSP) reflects a period of protein synthesis rather than protein function, depending on how the *b245* mutation hinders *fem-2* function. They found that the TSP

for the hermaphrodite germ line includes the first and second larval stages (L1 and L2) (figure 7). This differs from the TSP of the male germ line, which starts at L2 and continues throughout adulthood (figure 7). It is not surprising that the TSP is short in the hermaphrodite germ line and extended in the male germ line since spermatogenesis occurs for a brief time in the hermaphrodite and throughout the adult life of the male. The TSP for the male somatic gonad is during L1 (Kimble *et al.*, 1984). The TSP in other somatic tissues was determined in *fem-1(hc17)* and *fem-2(b245)* double mutants since either of the mutations on their own do not affect somatic tissues. XO double mutant animals raised at 25° developed as males, providing they were shifted to 16° prior to L3 (figure 7). Alternatively, XO animals raised at 16° developed as males providing they were not shifted to 25° prior to L2. If they were shifted to 25° before L2, then they developed as females (Kimble *et al.*, 1984). These TSP periods suggest *fem-2* is required early in larval development, as well as throughout adulthood in the male germ line.

Isolation of stronger alleles of *fem-2*

Because the *fem-1(hc17);fem-2(b245)* double mutant animals are more feminized than either single mutant (Nelson *et al.*, 1978; Kimble *et al.*, 1984), these alleles may not be null. In order to obtain alleles of *fem-2* that are more severe, Hodgkin (1986) performed a mutagenic screen that used the gene *tra-3(e1767)*, which is epistatically upstream from *fem-2*. *tra-3(e1767)* XX animals are masculinized, however the masculinization is repressed by *fem-2(b245)* at 25° (the *b245* restrictive temperature). At 20°, *fem-2(b245)* is unable to repress *tra-3(e1767)*, rendering the XX animals sterile. Double mutant animals were mutagenized at 25°, and progeny that were fertile at 20° were selected. The theory was that stronger *fem-2* alleles would not be temperature sensitive, therefore would still suppress *tra-3(e1767)* at 20°. From this screen, four new *fem-2* alleles were isolated; *b245e1982, b245e1985, b245e2005* and *b245e1963* (Hodgkin, 1986). These new alleles showed stronger feminization of XO animals than *b245* (table 1), and each

were thought to contain two molecular lesions in the *fem-2* locus (i.e. *b245* and the new mutation) (Hodgkin, 1986). *b245e2005* was used in a non-complementation screen to isolate *fem-2* alleles that showed the same severe feminization, but contain only one mutation. From this screen, six new *fem-2* alleles were isolated (*e2101-6*) (Hodgkin, 1986). This screen will be discussed in more detail in the discussion.

Phenotype of *fem-2(e2101-6)* alleles and maternal rescue

e2105 homozygous XX animals still produce sperm if their mother was heterozygous (m+z-), however if both mother and daughter are homozygous for *e2105* (and no cross fertilization), the daughter completely lacks sperm production at all temperatures (table 1) (Hodgkin, 1986). XO animals homozygous for *e2105* are also rescued by a wild-type maternal contribution. However, m-z- XO animals are partially feminized in the soma and germ line, the severity of which is dependent upon temperature (table 1) (Hodgkin, 1986). At 25°, m-z- XO animals are cross fertile females, at 15° they are sterile, partly feminized males and at 20° they are sterile intersexes. Similar results were obtained with the other five alleles, therefore even with these stronger alleles, the phenotype is still temperature sensitive (table 1) (Hodgkin, 1986). This raised the possibility that these alleles may not represent null alleles, and may differ as to their strength. In order to determine the relative strength of the newly isolated alleles, Hodgkin (1986) relied on the brood sizes of XX animals homozygous for *fem-2(e210n)*, whose mothers were heterozygous for the alleles (i.e. m+z-). It was found that even though m+z- XX animals are self-fertile, they produce fewer progeny than wild-type animals, due to fewer sperm being produced (table 1) (Hodgkin, 1986). Brood sizes of m+z- animals were measured for all six of the new alleles and it was found that the broods of *e2105* animals were the smallest, suggesting that it is the strongest *fem-2* allele.

Potential *fem-2* open reading frame shows similarity to PP2C enzymes

Pilgrim (1993) aligned the physical and genetic maps of the left end of linkage group III and found that *fem-2* mapped between two Restriction Fragment Length Polymorphisms that were approximately 50 kbp apart (figure 8). Cosmid and lambda clones in the region were injected into *fem-2(b245ts)* animals and scored for their ability to restore fertility to the animals at 25° (Pilgrim *et al.*, 1995). Two overlapping subclones narrowed the minimal rescuing region to ~3.0 kbp in size, and these subclones were used to probe a cDNA library (Pilgrim *et al.*, 1995). Sequencing of the positive cDNAs revealed an open reading frame predicted to encode a protein similar to protein phosphatases of the type 2C (PP2C) (figure 8) (Pilgrim *et al.*, 1995). PP2C enzymes have been identified in numerous organisms, including plants (Leung *et al.*, 1994; Meyer *et al.*, 1994), yeast (Maeda *et al.*, 1994) and humans (Nomura *et al.*, 1994). PP2C enzymes seem to have diverse roles in various organisms. For example, the PP2C enzyme Ptc1p in yeast regulates a MAP kinase pathway that is activated by both osmotic and heat stress (Maeda *et al.*, 1994). In *Arabidopsis*, PP2C enzymes have been shown to be involved in transducing a signal stimulated by the hormone abscisic acid (Leung *et al.*, 1994; Meyer *et al.*, 1994). Thus far, no PP2C enzymes have been identified that have a role in sex determination.

The hypothesis that I am testing in this thesis is that FEM-2 is a protein phosphatase whose dephosphorylating activity is necessary for its masculinizing function. Testing this hypothesis includes confirming that the ORF encoding the protein similar to PP2C enzymes is *fem-2*. It is possible that *fem-2* is located in another portion of the rescuing fragment. This also includes directly testing the ORF encoded protein, and a mutant form of the protein, for phosphatase activity. The results of these experiments then allow for the identification of regions of the regulatory regions and protein that are necessary for proper function.

MATERIALS AND METHODS

5' RACE of *Ce-fem-2*:

Confirmation of the 5' end of the *fem-2* transcript used the rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988) with a Life Technologies (Faithersburg, MD) 5' RACE kit, following the directions of the manufacturer. The primer DHA9 (table 2) was used for first strand synthesis, and AMC1 (table 2) was used for PCR amplification. The RACE product was cloned into the vector pGEM-T (Promega, Madison, WI) before sequencing using Sequenase v. 2.0 following the manufacturers instructions (Amersham).

Confirmation of *fem-2* deletion in *sDf124* and *wcDf1* deficiencies:

The deletion of at least part of the *fem-2* locus by the deficiency *sDf124* was noticed by D. Collins and H. Stewart (personal communication) and confirmed by myself. Heterozygous *sDf124(s2670)*/+ hermaphrodites were placed on a plate and allowed to lay eggs for 24 hours at 20°. The adults were removed, and after a further 24 h, unhatched embryos were isolated for PCR analysis. Wild-type embryos were also isolated to be used as controls. DNA from single embryos was prepared for PCR as described (Williams *et al.*, 1992). Primers DHA4, DHA17, DHA19 and SAD2 (table 2) were used to test for DNA at the *fem-2* locus, while primers MMA1 and MMA2 (table 2), which amplify DNA near the *unc-119* locus on the right end of Linkage group III (Maduro and Pilgrim, 1995), were used as positive controls. A similar analysis was performed for the *wcDf1* deficiency.

Sequencing of mutant alleles of *fem-2*:

DNA from the mutant alleles of *fem-2* was amplified as follows: for *b245ts* and *q117ts*, homozygous strains were grown at 20°, and genomic DNA was isolated as described in

Pilgrim (1993). For *e2103* and *e2105*, individual L4 hermaphrodites (either *fem-2/+* or *fem-2/fem-2*, which are morphologically indistinguishable) from a *fem-2/+* mother were set up on a plate at 20° and allowed to produce an F1 brood. Six animals were selected from broods that consisted entirely of female adults (*fem-2/fem-2*), combined in one tube, and prepared as for the single worm PCR method described (Williams *et al.*, 1992). A portion of the DNA was amplified by PCR using the primers DHA3 and DHA4 (table 2), and cloned into the pGEM-T vector (Promega). For each mutant allele, clones from at least three independent PCR reactions were sequenced. For *e2101, e2102, e2104, e2106* and *b245e2005*, genomic DNA was amplified by PCR using the primers DHA3 and DHA4 (table 2), and the PCR product was directly used as the template for sequencing.

Plasmid construction; GST-fusion proteins:

For the production of GST-FEM-2 (Glutathione S-transferase) fusion proteins in bacteria, the following plasmids were constructed (table 3). pDP#DH14: pGEX-1λT (Pharmacia) vector was digested with *Eco* RI and ligated with primer PJA1 (table 2), which introduces a *Sal* I site at the location of the original *Eco*RI site to make pGEX-Sal. Full-length *fem-2* cDNA was amplified by PCR using primers PJA2 and PJA3 (table 2) and a 20:1 mixture of Taq polymerase to Pfu DNA Polymerase (Stratagene). The amplified product was gel purified and digested with *Xho*I, which cuts at the sites introduced by the two primers. The product was then cloned into the introduced *Sal*I site of the pGEX-Sal vector and orientation was confirmed by restriction digestion. pDP#DH19: The pDP#AMc001 'minigene' described previously (Pilgrim *et al.*, 1995), was mutagenized *in vitro* using primer DHA17 (table 2) to introduce a mutation into the FEM-2 coding region identical to that found in the temperature-sensitive *b245* mutation. The clone was then digested with *Sal* I and *Sst* I, and the insert containing the *b245* mutation was gel purified and cloned into pDP#DH14 that had been digested with the same enzymes and separated from the corresponding insert. pDP#PJB1 was a gift of Petra Jäckle-Baldwin and

consists of a fragment of the *fem-2* cDNA from the *Sal* I site to the 3' end (figure 9) cloned into the pGEX-Sal plasmid. This results in a GST-FEM-2 fusion protein which is missing 87 amino acids at the amino-terminus of FEM-2. This truncated form of FEM-2 is referred to as FEM-2ΔN1 (figure 9)

Isolation of the FEM-2 protein produced in *E. coli*:

GST fusion vectors pDP#DH14, pDP#DH19 and pDP#PJB1 (table 3) were transformed into *E. coli* strain BL21 DE3, then grown in LB-ampicillin to an OD_{550} of ~0.9 at 30°C. Induction of the fusion protein was achieved by the addition of isopropyl-ß-D-thiogalactoside to a final concentration of 400 micromolar as described by the supplier (Pharmacia) for an additional 2-3 hours. The cells were pelleted and resuspended in lysis buffer (100 mM NaCl, 2.5 mM EDTA, 0.1% Tween-20, 50 mM Tris-HCl pH 8.0). Cells were lysed using a Sonifier 450 (Branson) by sonication on ice with 4 bursts of 60 seconds each on power setting 3. The debris was removed by centrifugation at 20,000 x g for 15 minutes in a Sorvall SS-34 rotor and the supernatant was added to 0.5 ml bed volume of washed Glutathione Sepharose 4B (Pharmacia) and allowed to bind at 4° for two hours with inversion. Beads were washed three times with Phosphate Buffered Saline (PBS) and protein was eluted with two washes each of 10 and 15 mM glutathione in washing buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0). The eluant was dialyzed against washing buffer for two hours, then overnight against 50 mM NaCl, 20 mM HEPES-KOH pH 7.2, 20% glycerol, 10% Polyethylene Glycol 20,000. Isolated proteins were quantified using the method of Bradford (Bradford, 1976).

Phosphatase assays:

Bovine milk casein (Sigma C-4765) was radioactively labeled using 7000 Ci/mMol [γ-^{32}P] ATP (ICN) with the catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma P-2645) as described by the supplier. The labeled protein was then

precipitated with 20% trichloroacetic acid/ 20mM NaH_2PO_4 and washed four times with the same solution. The pellet was dried and resuspended in 0.2 M Tris-HCl (pH 8.0) to a final concentration of ~10^5 cpm/µl. The phosphatase assay was performed as described (Maeda, *et al.*, 1993) at 22° and similar results were obtained in at least two separate reactions. All reactions contained 50 mM $MgCl_2$ unless otherwise noted.

Plasmid construction; yeast vectors:

pDBL, containing the 2 µm origin of replication and ADH1 promoter, has been described previously (Milne and Weaver, 1993), and was obtained from T. Milne (Harvard Medical School, Cambridge MA) (table 3). This plasmid was used as the backbone for the construction of pDP#1 (directs the synthesis of FEM-2 under the control of the ADH1 promoter in yeast) and pDP#DH29 (same as pDP#DH1 but contains a deletion in the region encoding the amino terminal domain of FEM-2) (table 3). For pDP#DH1, a cDNA, previously shown to contain the entire *Ce-fem-2* open reading frame and upstream sequences including the first few bases of the SL1 spliced leader cloned into the *Eco*RI site of pBluescript (Pilgrim *et al.*, 1995), was liberated from the plasmid by digesting with *Not*I and *Eco*RV. *Not*I linkers were added to the blunt end, the insert was redigested with *Not*I and then cloned into the *Not*I site of pDBL. For pDP#DH29, the insert from pDP#DH1 was cloned into the *Not*I site of pBluescript II (SK-) and subjected to *in vitro* mutagenesis (Leatherbarrow and Fersht, 1986), using mutagenic primers DHA22 and DHA23 (table 2). These created in-frame *Bgl*II restriction sites at positions 396 and 1187 of the *fem-2* sequence (Pilgrim *et al.*, 1995), which were digested and then religated, removing the sequence between the two sites (figure 9). This resulted in a truncated open reading frame and the resulting protein is referred to as FEM-2ΔN2 (figure 9). The engineered insert was then removed by digesting with *Not*I and ligated into the *Not*I site of pDBL.

Yeast strains, media and transformation:

Saccharomyces cerevisiae strain TM126 (*MATa leu2 ura3 his3 ptc1::URA3*) was obtained from H. Saito (Maeda *et al.* 1994). Transformation of plasmids into strain TM126 was carried out as described (Schiestl *et al.*, 1993) following protocol number 3 in that reference. Transformation of pDP#DH1 into TM126 yielded strain sDH2, pDBL yielded sDH3, and pDP#DH29 yielded sDH5 (DNA constructs described above and Table 3). For growth curves, cells were grown overnight at 30° with constant shaking in selective (SC-leu) liquid media. The next day, 5 µl of each overnight culture was added to 5 ml of fresh media. These samples were grown with constant shaking at 30° or 37° at 300 rpm and the OD_{550} was measured at specified intervals in a Bausch and Lomb Spectronic 20 spectrophotometer. Growth was assayed for five independent cultures of each strain at both temperatures. For growth on solid media, transformed strains were grown to saturation in liquid SC (minus leucine and uracil). Serial ten-fold dilutions of the cultures were made in saline and four microliters of each dilution was dropped onto duplicate SC (minus leucine and uracil) plates and grown overnight at 30° or 37°.

Detection of FEM-2 in yeast:

Protein extracts of the yeast strains sDH2, sDH3 and sDH5 were obtained following overnight growth at 30° in liquid SC (minus leucine and uracil) media. The cells were pelleted and resuspended in 1.5 ml of water. 0.24 ml of 1.85M NaOH, 7.4% β-Mercaptoethanol was added to the cells and incubated at 4° for 10 minutes. 0.24 ml of 50% trichloroacetic acid was added to the solution and incubated again at 4° for 10 minutes. The solution was centrifuged and the pellet was resuspended in 1.5 ml of acetone. The acetone was removed after centrifugation and the dried pellet was resuspended in 0.1 ml of 2X sample buffer (Sambrook *et al.* 1989).

SDS-PAGE was performed as described (Sambrook *et al.* 1989) using a 12% resolving gel. Western blotting and detection were performed using the ECL kit

(Amersham) following the suppliers instructions using a 1:2500 dilution of a polyclonal antibody made against Ce-FEM-2 (Jäckle-Baldwin 1996).

Plasmid construction; truncated amino terminus:

pDP#DH28 (table 3) was made by *in vitro* mutagenesis (Leatherbarrow and Fersht, 1986) of a wild-type genomic *fem-2* clone (pDP#DH11) that can completely rescue *fem-2* mutant defects (data not shown; insert contains the minimal rescuing region shown in Pilgrim *et al.* (1995)) (table 3). Primers DHA22 and DHA23 (table 2) introduced in-frame *Bgl*II restriction sites (as described for plasmid pDP#DH29 (figure 9)). The plasmid was digested with *Bgl*II and religated after removal of the intervening sequence. The resulting protein is the same as FEM-2ΔN2 expressed in yeast strain sDH5.

Attempted rescue of *fem-2* animals with truncated FEM-2:

pDP#DH28 (table 3) was co-injected with pRF4 into strains DP51 *(fem-2(e2105) unc-45(r450ts) / sC1* [*dpy-1(s2171)*];*him-8(e1489)*) and DP53 (table 4). DP53 is similar to DP51 except that it carries a lethal allele *(s2683)* on the balancer *sC1* chromosome (gift of H. Stewart and D. Baillie). Analysis of the transformed worms was performed at 21° as described in the Materials and Methods section entitled 'Partial rescue of *fem-2* animals with *C. briggsae* clone'.

Cloning of the *C. briggsae* homologue:

Southern blotting was performed using *C. briggsae* genomic DNA digested with various restriction endonucleases and probing with a full length *C. elegans fem-2* cDNA. Hybridization was performed at 50° with three subsequent 15 minute 50° washes of 2XSSC, 0.1% SDS. Autoradiography revealed a single 3.7 kbp band when the *C. briggsae* DNA was digested with *Xba* I (figure 10). A genomic mini-library was made by gel purifying *Xba* I digested *C. briggsae* DNA that was 3.2-4.0 kbp in size. These

fragments were ligated into *Xba* I digested pBluescript II SK-, transformed into bacteria, and screened using colony hybridization as described (Sambrook *et al.* 1989). Hybridization and washing conditions for colony hybridization were the same as those used for the Southern blot mentioned above. Analysis of the sequence of a positive clone (pDP#DH42) showed that all of the predicted coding region was contained in the 3.7 kbp *Xba* I fragment, however not all of the predicted 3' untranslated region (3' UTR) and only 414 bp of upstream sequence was contained in the clone (figure 11). In order to get a larger clone, another minilibrary was constructed by digesting *Hin*D III digested genomic DNA and isolating fragments between 10 and 20 kbp. These were cloned into pBluescript II SK- and transformed into bacteria. pDP#DH42 was used to probe colony lifts. The blots were hybridized at 65° and then, at the same temperature, washed twice with 2XSSC (10 minutes), once with 2XSSC, 0.1% SDS (15 minutes) and once with 0.1XSSC (5 minutes). One positive clone was isolated (pDP#DH53) and confirmed to completely contain pDP#DH42 region by restriction mapping, PCR and partial sequencing (figure 11). The sequence of *Cb-fem-2* can be obtained from GenBank (accession number AF054982)

Obtaining a genomic clone of *Cr-fem-2*:

Genomic *C. remanei* DNA was digested with various enzymes and electrophoresed on an agarose gel. The DNA was transferred to a nylon membrane and probed with a radioactive DNA copy of the insert from plasmid pDP#PSr1, which contains a genomic portion of *Cr-fem-2* (table 3). Autoradiography revealed a band approximately six kbp in size, corresponding to the DNA digested with *Sst*I. A mini-library was then constructed by gel purifying *C. remanei* genomic DNA five to seven kbp in size that had been digested with *Sst*I and ligating into pBluescript II (SK-) (Stratagene), which had also been digested with *Sst*I. These DNA clones were transformed into *E. coli* and the resulting colonies were transferred to nylon membrane and screened using a radioactive copy of

the insert from pDP#PSr1. The positive clone, pDP#DH150 (table 3), was partially sequenced revealing that it did not contain the entire coding region (figure 11), therefore another mini-library was constructed as outlined above, but using the restriction enzymes *Bam*HI and *Hin*DIII. Restriction mapping of pDP#DH150, and genomic Southern analysis had revealed that a *Bam*HI and *Hin*DIII genomic fragment would overlap with the pDP#DH150 insert, as well as extend the amount of DNA cloned in the region by 4.4 kbp. A positive clone, pDP#DH160 (table 3), was obtained by probing the transformed library with a radioactive copy of the insert from pDP#DH150 (figure 11). Restriction digestion and sequencing confirmed that pDP#DH160 overlapped with pDP#DH150, and that they corresponded to the gene identified by pDP#PSr1. In order to obtain a genomic clone that completely contained *Cr-fem-2*, both pDP#DH150 and pDP#DH160 were digested with *Hin*DIII. This cuts pDP#DH160 into a single linear fragment and pDP#DH150 into two (figure 11). Digested pDP#DH150 was gel electrophoresed and the band corresponding to the region of DNA not overlapping, but rather flanking, pDP#DH160 was isolated and ligated to the linear pDP#DH160, resulting in clone pDP#DH161 (table 3). Correct orientation of the insert was confirmed by sequencing.

C. briggsae mRNA transcript analysis:

Total RNA was isolated from hermaphrodite (XX) worms which were grown in liquid culture as described (Sulston and Hodgkin, 1988). Four volumes of TRIZOL (Life Technologies) were added to packed worms and resuspended by vortexing, then incubated at 20° for 5 minutes. The solution was centrifuged at 16000 xg (4°) for 10 minutes and liquid was transferred to a new tube. One fifth volume of chloroform was added to the solution and vortexed for 15 seconds. Centrifugation was performed at 16000 xg (4°) for 15 minutes and the aqueous phase was transferred to a new tube containing one volume of isopropanol and incubated for 10 minutes at 20°. The RNA

was recovered by centrifuging at 16000 xg for 10 minutes (4°) and the pellet was washed with 75% ethanol and suspended in DEPC treated water.

A DNA copy of the 5' end of the *Cb-fem-2* transcript was obtained by Rapid Amplification of cDNA Ends (RACE) using a 5' RACE Kit (Life Technologies), following the manufacturers instructions. Primer DHB.4 (table 2) was used for first strand synthesis and primer DHB.3 (table 2) was used for PCR amplification (figure 11). The PCR product was cloned into pGEM-T (Promega). Sequencing revealed the presence of a *trans*-spliced leader with the sequence 5'GGTTTAATTACCCAAGTTGAG3', although the number of G's at the 5' end cannot be precisely determined because the RACE protocol incorporates a string of G's at this location. The leader splices into the sequence UUUCAG*__AUG__, with * being the site of splicing. The AUG shown (bold) is predicted to encode the initiator methionine. Therefore, as seen with some *C. elegans* transcripts (Blumenthal and Steward 1997), there is no sequence between the *trans*-spliced leader and the coding region. Since the RACE product contained only one intron splice site, RT-PCR was used to obtain a cDNA containing all putative intron splice sites. Primer DHB.4 was used for first strand synthesis and primers DHB.4 and DHB.5 (table 2) were used for amplification (figure 11). The product was cloned into pGEM-T (pDP#DH113; table 3) and sequenced.

3' RACE was used to obtain a DNA copy of the 3'end of the transcript using a 3'RACE kit (Life Technologies), following the manufacturers instructions. Primer DHB.5 (table 2) was used for the PCR reaction and the product was gel purified and ligated into vector pGEM-T (Promega). A total of 16 clones were isolated and each were sequenced in the region of the DNA corresponding to the poly-A tail.

RNA gel electrophoresis and Northern blotting were performed as described (Sambrook *et al.* 1989) Ten micrograms of the total RNA described above was electrophoresed on a 1.3% agarose gel and transferred to GeneScreen Plus nylon membrane (NEN). The RNA was hybridized to a ^{32}P-labeled DNA probe made from the

insert of clone pDP#DH113 (table 3). Hybridization was performed at 65° overnight using Hybridsol II hybridization solution (Oncor). The blot was washed at 65° twice with 2XSSC for 15 minutes, once with 2XSSC, 0.1% SDS for 30 minutes and once with 0.1XSSC, 0.1% SDS for 10 minutes then exposed to film at –80° for eight days.

Northern analysis of *Cr-fem-2*:

Gel electrophoresis of the RNA and Northern blotting were performed as described (Sambrook *et al.*, 1989). A 1.1% agarose gel was used to electrophorese five micrograms of total RNA (isolated by Paul Stothard by the method described above for *C. briggsae* RNA isolation), which was then transferred to Genescreen plus nylon membrane (New England Biolabs, Beverly, MA). A ^{32}P-labeled DNA probe was made from the insert of clone pDP#PSr1 and was hybridized to the RNA using Hybridsol II hybridization solution (Oncor) overnight at 65°. The blot was washed twice with 2X SSC for 15 minutes each, once with 2X SSC, 0.1% SDS for 30 minutes, and once with 0.1X SSC, 0.1% SDS for ten minutes. The blot was then exposed to film for five days at –80°.

Partial rescue of *fem-2* animals with *C. briggsae* clone:

pDP#DH53 was co-injected with pRF4 (*rol-6(su1006dm)*) (Kramer *et al.*, 1990), into the gonads of hermaphrodites of strain DP51 (*fem-2(e2105) unc-45(r450ts) / sC1* [*dpy-1(s2171)*];*him-8(e1489)*). pDP#DH53 was injected at concentrations of 50 ng/µl and 100 ng/µl as described (Mello *et al.*, 1991). F1 progeny with the rolling phenotype were confirmed to also be transmitting pDP#DH53 by single worm PCR using primers DHB.3 and DHB.5 (table 2) as described (Pilgrim *et al.* 1995; Williams *et al.* 1992). Single rolling Unc hermaphrodites were transferred onto plates and allowed to self fertilize at 21° and 25°. F2 hermaphrodite (XX) and male (XO) rolling animals were scored for their ability to self fertilize, as well as somatic sexual phenotype, compared to control DP51 animals grown at the same temperature.

Plasmid construction; 3'UTR deletion vectors

Deletions in the *Ce-fem-2* 3'UTR were made through *in vitro* mutagenesis (Leatherbarrow and Fersht, 1986). For plasmid pDP#DH126 (table 3), primer DH3DEL (table 2) was used to mutagenize rescuing plasmid pDP#DH11. The mutagenesis removes 40 bases and replaces them with an *Xba*I site (TCTAGA) (figure 12). Mutagenizing pDP#DH11 with primer 3AvrII (table 2) resulted in plasmid pDP#DH96, which deletes 58 bases and inserts three (AGG). The insertion of the three bases, along with three bases not deleted, results in an *Avr*II restriction site. In figure 12, the region of the 3'UTR that is deleted consists of the 58 bases that are closest to the polyadenylation site and are doubly underlined. A larger deletion plasmid was made by combining the two deletions in pDP#DH126 and pDP#DH96. In order to construct this plasmid (pDP#DH163), pDP#DH126 was digested with *Xba*I and *Kpn*I. pDP#DH96 was digested with *Avr*II and *Kpn*I Each of these digestions resulted in the plasmids being cut twice, once in the 3'UTR, and once in the polylinker of pBluescript II. The digested DNA was electrophoresed and the resulting bands were gel purified as described (Sambrook *et al.*, 1989). The DNA fragment from the pDP#DH126 digestion that contained the *fem-2* gene was then ligated to the opposite fragment from the pDP#DH96 digestion that contained the rest of the 3'UTR and pBluescript II. Since *Xba*I and *Avr*II have compatible cohesive ends, this resulted in a plasmid similar to pDP#DH11 (the original rescuing plasmid), but lacked the portions of the 3'UTR correlating to the pDP#DH126 andpDP#DH96 deletions, as well as the sequence between the two deletions (figure 12).

Plasmid construction; heat shock vectors

pDP#DH145, pDP#DH148, pDP#DH153 and pDP#DH154 (table 3) are all derivatives of pPC16.41 (kindly provided by Dr. Peter Candido via Dr. Elizabeth Goodwin), differing

only in their 3'UTRs. For pDP#DH145, a wild-type copy of the *fem-2* 3'UTR (from plasmid pDP#DH11) was PCR amplified using primers DH3.1 and DH3.2 (table 2). Each of these primers contains an *Eag*I restriction site, therefore the PCR product and pPC16.41 were each digested with *Eag*I and ligated. After transformation into *E. coli* and subsequent isolation of the plasmids, orientation was confirmed by restriction digestion. pDP#DH153 and pDP#DH154 were constructed similarly, however the PCR reactions were performed using pDP#DH126 and pDP#DH163 as a templates respectively (table 3). The *fem-2* 3'UTR in pDP#DH126 contains a 40 bp deletion and pDP#DH163 contains a 124 bp deletion. For pDP#DH148, plasmid pPD96.04 (kindly provided by Dr. Andrew Fire) was digested with *Eco*RI and *Eag*I, which liberated the *unc-54* 3'UTR and a small portion of the *lacZ* gene. pPC16.41 was also digested with the same enzymes, which removes the 3'UTR cloning site as well as the same portion of the *lacZ* gene described above. The vector containing fragment of pPC16.41 and the *unc-54* 3'UTR containing fragment of pPD96.04 were ligated, resulting in plasmid pDP#DH148.

ß-GAL expression of various 3'UTR constructs:

Plasmid constructs pDP#DH145, pDP#DH148, pDP#DH153 and pDP#DH154 (table 3) were each injected into N2 worms at a concentration of 50 ng/µl as described (Mello *et al.*, 1991) (co-injected with pRF4 (*rol-6(su1006dm)*)). Each of the constructs contains the heat shock promoter *hsp16-41* (Stringham *et al.*, 1992), as well as the *lacZ* gene and one of several differing 3'UTRs. Transmitting worms were grown on XL1-B (*lacZ-) E. coli* (Statagene). Heat shocking was performed at 33° for two hours, then a 30 minute recovery period at 20°, followed by another heat shock at 33° for two hours. Worms were transferred into water and spun at ~3000 xg for one minute, followed by removal of most of the liquid. They were then flash frozen in liquid Nitrogen and lyophilized in a vacuum for 45 minutes. Cold (-20°) acetone was added to the pellet and incubated at –20° for three minutes. Most of the acetone was removed, then the pellet of worms was

placed in a vacuum until dry. ß-GAL staining solution (200 mM Na-Phosphate, pH 7.5; 1 mM $MgCl_2$; 0.004% SDS; 5 mM Potassium Ferricyanide; 5 mM Potassium Ferrocyanide; 0.75 mg/mL kanamycin; 2 µg/mL DAPI; 0.04% X-Gal) was added to the pellet and incubated overnight at room temperature. Worms were analyzed by Nomarski microscopy for ß-GAL expression.

fem-2 3'UTR partially deleted plasmid in N2 worms

pDP#DH11 and pDP#DH163 (table 3) were each injected into N2 worms each at a concentration of 20 ng/µl as described (Mello *et al.*, 1991). They were co-injected with marker plasmid pRF4 pRF4 (*rol-6(su1006dm)*) at 100 ng/µl. Transmitting lines were grown at 25° and examined using Nomarski microscopy for phenotypic characterization.

RESULTS

Most of the results in this chapter have been published in similar form elsewhere (Pilgrim *et al.*, 1995; Hansen and Pilgrim, 1998)

Null phenotype of *fem-2*:

The original *fem-2* mutation isolated was the temperature sensitive *b245* allele (Kimble *et al.*, 1984; see introduction). Hodgkin (1986) then reported six other alleles (*e2101-e2106*), which have a much stronger phenotype than *b245*, but the severity of feminization was still dependent upon temperature. They showed complete feminization of the XX and XO germ line at all temperatures, but somatic feminization of XO animals was minimal at 15° and virtually complete at 25°, and intersex at temperatures in between (table 1) (Hodgkin, 1986). It is possible that *e2101-6* are hypomorphic alleles, where part of the *fem-2* activity is retained at 15°, but absent at 25°. Another possibility is that *e2101-6* are, in fact, null alleles, but that *fem-2* activity is dispensable in the soma at low temperatures, and more stringently required at higher temperatures. In order to test these possibilities, we tested the phenotypes of animals that are hemizygous for these alleles, and compared them to the phenotypes displayed when the alleles are homozygous. If these alleles retain some *fem-2* activity, then it would be expected that having only one copy (hemizygous to a deficiency in the region), would result in a stronger (more feminized) phenotype than homozygous. If, however, these are null alleles, the phenotype would not be expected to be more severe when in only one copy. In order to test this, it was necessary to identify deficiencies that completely delete *fem-2* sequences. Two deficiencies that map near *fem-2* are *wcDf1* and *sDf124*, both of which are lethal when homozygous. To determine if one or both of them lack *fem-2* coding sequence, I used PCR to amplify two sub-regions at either end of the gene using either DNA isolated from dead embryos homozygous for one of the deficiencies, or wild-type DNA (figure 13). The absence of a band in deficiency embryos confirms that these sequences were

absent in the deficiency chromosome. I also amplified a region corresponding to the unlinked *unc-119* gene in the same PCR reaction in order to show that a lack of a band in deficiency embryos is not due to a lack of template DNA. I found that both *wcDf1* and *sDf124* lack *fem-2* coding sequence corresponding to the regions containing the primers (see figure 13). This result allowed us to test *e2101-6* as described above. We found no apparent difference in phenotype when the alleles are homozygous versus hemizygous, suggesting that these are null alleles (Pilgrim *et al.*, 1995).

Identification of lesions associated with *fem-2* mutant alleles:

In order to confirm that *fem-2* was, in fact, the gene cloned through plasmid rescue, I sequenced the putative coding region from all known *fem-2* alleles that resulted from a single mutational event (i.e. excluding the *b245exxx* doubly mutated alleles). The first *fem-2* allele obtained was *b245* (Kimble *et al.*, 1984). Comparison of the sequence obtained from *fem-2(b245)* animals to that from wild-type animals revealed a missense mutation at codon 339 (figure 14). Consistent with the use of EMS in the mutagenesis (Kimble *et al.*, 1984), the mutation was identified as a G-C to A-T transition that results in a glycine residue (GGA) changed to arginine (AGA). The other temperature sensitive allele, *q117(ts)*, is also due to a missense mutation. This occurs at codon 160 (figure 14), and causes a change from a glycine (GGA) residue to glutamic acid (GAA). Six other mutant alleles (*e2101-e2106*), were generated in a non-complementation screen by Jonathan Hodgkin (1986). *e2102* has a premature stop at codon 167 (CAA to TAA), which results in a truncated protein completely lacking the domain with similarity to PP2C enzymes (figure 14). Surprisingly, five of these six alleles (all but *e2102*), contain the mutation associated with the *b245* allele. Also, each of the five contain an identical mutation that results in a premature stop at codon 218 (TGG to TGA) (figure 14). As mentioned previously, *e2101 – e2106* were isolated from a non-complementation screen (Hodgkin, 1986). The *fem-2* allele used in the screen was *b245e2005*, which was

generated by mutagenizing *b245* animals and screening for those that are still able to suppress a *tra-3* allele at the permissive temperature (Hodgkin, 1986). The *e2005* mutation was linked to *b245*, therefore it most likely caused another mutation in the *fem-2* gene. I sequenced the *b245e2005* allele and found that it contains the *b245* mutation, as expected, as well as the premature stop codon found in the other five alleles (figure 14). The simplest explanation for this result is that *e2101, e2103, e2104, e2105* and *e2106* are all re-isolates of *b245e2005* (see Discussion for possible explanation).

fem-2 mRNA is _trans_-spliced to an SL1 leader:

A number of *fem-2* cDNAs have been isolated and sequenced (Pilgrim *et al.*, 1995), however it was not clear if any of them contained the entire 5' end. In order to obtain a DNA copy of the 5' portion of the *fem-2* mRNA, I used the 5' RACE (Rapid Amplification of cDNA Ends) PCR based approach following the manufacturers instructions (BRL Life Technologies). I found that none of the cDNAs were full length, and that the mRNA is *trans*-spliced to the well characterized SL1 leader sequence. Approximately 57% of *C. elegans* genes are *trans*-spliced to SL1 (Zorio *et al.*, 1994), and its function is not yet clear. Not including SL1, the 5'UTR is 55 bases in length, and contains no obvious regulatory sequences.

FEM-2 has phosphatase activity *in vitro*:

The Open Reading Frame (ORF) encoding the FEM-2 protein showed similarity to phosphatases of the type 2C (PP2C) (Pilgrim *et al.*, 1995). In order to determine if FEM-2 has phosphatase activity that is consistent with its classification of a PP2C enzyme, it was tested *in vitro*. The FEM-2 protein was synthesized in *E. coli* as a Glutathione-S-transferase (GST) fusion. The fusion was constructed to contain the entire FEM-2 open-reading frame, including the initiator methionine (figure 9). The fusion protein was purified from bacterial cell lysates by affinity chromatography, as described in Materials

and Methods. GST-FEM-2 exhibits phosphatase activity using ^{32}P labeled casein as a substrate. The release of phosphate from labeled casein is linear with time, and is dependent upon magnesium ions (figure 15). No phosphatase activity above background is observed when GST alone is added to the reaction, or when magnesium ions are omitted. This demonstrates that FEM-2 is a Mg^{+2}-dependent protein phosphatase, consistent with its sequence similarity to PP2C enzymes, and the addition of the GST peptide at the amino terminus does not prevent phosphatase activity. Phosphatase activity of FEM-2 was shown independently by Chin-Sang and Spence (1996), with very similar results.

Rescue of yeast *ptc1* mutation:

There may be some overlap in function between the various PP2C enzymes within a yeast cell (Maeda *et al.*, 1994; Shiozaki and Russell 1995). Ce-FEM-2 has 50% similarity with *Saccharomyces cerevisiae* Ptc1p in the six conserved motifs of the phosphatase domain (figure 9). Since a phenotype has been described for *S. cerevisiae ptc1* mutant cells (Huang and Symington 1995; Jiang *et al.* 1995; Maeda *et al.*, 1993; Robinson *et al.* 1994), I tested whether the similarity between the two proteins is sufficient for Ce-FEM-2 to complement the yeast defects. ABI2 from *Arabidopsis*, another FEM-2 homologue, has recently been shown to rescue *ptc1* cells (Leung *et al.*, 1997). *ptc1* mutant cells show a temperature-dependent growth defect, where growth is significantly slowed at 37°, but not at 30°. I transformed *ptc1* yeast cells with a plasmid containing the Ce-FEM-2 coding region, under the control of the constitutive yeast ADH1 promoter. In transformants containing Ce-FEM-2, the growth arrest is not observed, but growth arrest is observed in transformants containing vector alone (figure 16). The rescue is evident in experiments using both liquid and solid media.

Amino terminus not necessary for phosphatase activity:

FEM-2 is predicted to have a longer amino terminus than either *S. cerevisiae* Ptc1p or its *S. pombe* homologue (figure 9) (Pilgrim *et al.* 1995). As mentioned, GST-FEM-2 exhibits phosphatase activity using ^{32}P labeled casein as a substrate (figure 15). I tested the phosphatase activity of a truncated form of FEM-2 that lacks the first 87 amino acids (FEM-2NΔ1) (figure 9). The deleted protein retains the six conserved motifs found in all PP2C enzymes (Pilgrim *et al.* 1995). GST-FEM-2ΔN1 was purified and tested for phosphatase activity in a similar fashion to that described for GST-FEM-2. FEM-2ΔN1 has indistinguishable phosphatase activity from intact FEM-2 (figure 15). Therefore, an intact amino terminus is not required for phosphatase activity *in vitro*.

As an independent *in vivo* assay for the effect of the amino-terminus on FEM-2 phosphatase activity, a second truncated form of Ce-FEM-2 was tested. FEM-2-ΔN2 contains an in-frame deletion of 127 amino acids, which includes the majority of the amino terminal extension (figure 9). When this truncated form of Ce-FEM-2 is expressed in yeast, it is also able to rescue the temperature dependent growth defect (figure 16), with no apparent difference from the rescue achieved by intact Ce-FEM-2. Strains sDH2 and sDH5 were confirmed to be expressing Ce-FEM-2 and Ce-FEM-2ΔN2 respectively through Western analysis (figure 17). Thus, the conserved motifs of FEM-2 are sufficient to rescue the growth defect caused by lack of Ptc1p PP2C activity in yeast. This further confirms that the amino terminus is not necessary for FEM-2 phosphatase activity.

Lack of rescue using truncated *fem-2* in worms:

In order to determine if the amino terminal domain is necessary for proper FEM-2 function in worms, a clone carrying an in-frame deletion of this region of the protein was injected into *Ce-fem-2(null)* worms (pDP#DH28). The portion of the protein deleted is identical to that deleted in FEM-2-ΔN2 (figure 9), which is able to rescue a PP2C

deficient yeast strain, suggesting that the phosphatase activity is still intact (discussed above). XO m-z- *Ce-fem-2(null)* animals (n=33) carrying the array showed the same degree of somatic feminization as those not carrying the array. Germline rescue was not observed in m-z- XX *Ce-fem-2(null)* animals carrying the array (n>40), as scored by self sterility. Western blotting was used to confirm that worms carrying the array were expressing the amino truncated form of FEM-2 (figure 17). Strain DP51 shows a band at the predicted size for FEM-2 (51kD), however strain DP151 (same as strain DP51 but carrying a transgenic array with plasmid pDP#DH28), shows a band at both 51kD and 36kD (the expected size of Ce-FEM-2ΔN2). In order to maintain the line carrying the pDP#DH28 plasmid, heterozygous animals for *fem-2(e2105)* are used, and homozygous offspring are tested for rescue. For the Western blot, a population of worms consisting of homozygous and heterozygous animals, were used to obtain the cell lysate. Therefore, the lysate contained both full length Ce-FEM-2 and Ce-FEM-2ΔN2, resulting in two bands (figure 17). Yeast strain sDH5, which also expresses Ce-FEM-2ΔN2, shows a band of the same size (figure 17). This suggests that the amino terminus is necessary for FEM-2 to function properly in *C. elegans* sex determination, and that phosphatase activity alone is not sufficient.

Loss of phosphatase activity with *b245(ts)* mutation:

The *fem-2* gene was first discovered due to a temperature-sensitive mutation, *b245* (Kimble *et al.* 1984). I have shown that the molecular lesion associated with *b245* is a missense mutation in conserved motif IV (figure 14) (Pilgrim *et al.*, 1995). This base pair change was introduced into the FEM-2 coding sequence on a plasmid containing GST-FEM-2 (pDP#DH19), and this fusion protein (FEM-2-*b245*) was expressed and purified. As predicted from its mutant phenotype, little phosphatase activity above background could be detected (figure 15). Since even null alleles of *fem-2* show temperature dependent phenotypes (Pilgrim *et al.* 1995), the low level of phosphatase

activity produced from FEM-2-*b245* may be sufficient for FEM-2's masculinizing function at lower temperatures, but not the higher restrictive temperature. Alternatively, GST-FEM-2-*b245* may not have been folded properly in bacteria, but is in worms at permissive temperatures.

C. briggsae homologue of *fem-2*:

A common method of identifying protein or regulatory sequences necessary for proper function is to compare them to homologous genes from other species and look for regions of conservation. The amino terminal domain of the Ce-FEM-2 protein shows no significant similarity to any other known protein, making this type of analysis difficult. I cloned the *fem-2* homologue from the closely related nematode in the hope that its amino terminus would show similarity to that from *C. elegans*, as well as to identify possible regulatory regions. The sex determination genes that have been cloned from *C. briggsae* to date (*tra-1* and *tra-2*) are surprisingly divergent from their *C. elegans* homologues, making it difficult to use conventional cloning methods (deBono and Hodgkin, 1996; Kuwabara, 1996b). In fact, for *tra-2* it was necessary to clone syntenic genes and sequence overlapping clones in order to identify *Cb-tra-2* (Kuwabara and Shah, 1994). *Cb-fem-2* was cloned using low stringency hybridization (figure 10), and the entire putative coding region, covering approximately 3.0 kbp, was sequenced (figure 11). Northern blotting shows a single band at approximately 1.9 kb, suggesting that *Cb-fem-2* makes only one transcript, or more than one with very similar sizes (figure 18). Comparison of the genomic sequence of *Cb-fem-2* to that obtained through 5' RACE, 3'RACE and RT-PCR reveals the presence of five introns (figure 11), three of which are at the same location as those in *Ce-fem-2* (figure 19). The sequence of the 5' RACE product contains a leader with the sequence 5'GGTTTAATTACCCAAGTTGAG3', confirming that the *Cb-fem-2* transcript is also *trans*-spliced. The leader is similar to SL1 to which the *Ce-fem-2* transcript is spliced (described above).

The predicted Cb-FEM-2 protein consists of 502 amino acids, 53 amino acids longer than Ce-FEM-2, due to an even longer domain amino terminal to the conserved phosphatase motifs (figure 9). Over 449 amino acids, the two proteins are 63% identical and 85% similar. The degree of similarity is not constant over the entire lengths of the proteins (figures 9 and 19). In the regions common to PP2C enzymes (the carboxy 279 amino acids), the two proteins are 72% identical and 90% similar. The first 170 amino acids of Ce-FEM-2 and the equivalent region of Cb-FEM-2 are 49% identical and 80% similar (figure 19).

Comparison of ~400 bp of *Cb-fem-2* promoter sequence with the *Ce-fem-2* promoter revealed no significant similarity except for the nine bp sequence 5'TCTGCATTA3'. This sequence may be significant because it is at approximately the same location in both genes. This element starts 105 bp and 104 bp upstream of the *Ce-fem-2* and *Cb-fem-2* respective *trans*-splice sites. However, the significnace of this sequence was not tested.

Cloning of genomic region of *fem-2* from *C. remanei*

Paul Stothard cloned a small portion of a *fem-2* homologue from the male/female nematode species, *C. remanei,* using degenerate PCR. He also used RT-PCR, 5' RACE and 3' RACE to obtain the entire coding region of the gene. In order to identify intron positions and sizes, as well as to obtain the promoter and down-stream regions of *Cr-fem-2*, I cloned a genomic fragment of DNA containing the entire *Cr-fem-2* gene. I also sequenced the entire coding region and introns. Comparison of the genomic sequence to the cDNA sequence obtained by Paul Stothard revealed the presence of seven introns, two more than that found in *Cb-fem-2* (figure 11). I originally cloned the *Cr-fem-2* gene in two pieces (pDP#DH150 and pDP#DH160), using two different mini-libraries (see Materials and Methods). I then combined the two clones to obtain a single clone

containing the entire gene (pDP#DH161) (figure 11). Northern analysis shows hybridization at only one band, ~1.8 kb in size, consistent with the size of the transcript predicted through RT-PCR, 5' RACE and 3' RACE performed by Paul Stothard (figure 18). This suggests that, as seen in *C. elegans* and *C. briggsae*, only one size of transcript is made. A comparison of the Cr-FEM-2 protein with Ce-FEM-2 and Cb-FEM-2 is being prepared (Stothard, Hansen and Pilgrim, manuscript in preparation).

Partial rescue of *Ce-fem-2(null)* with *Cb-fem-2*:

While the sequence identity of the phosphatase domains between Ce-FEM-2 and Cb-FEM-2 is high, the level of identity in the amino termini is much lower than is typically seen between these two species (deBono and Hodgkin, 1996), suggesting that these domains may be rapidly evolving. In order to determine if the two genes are functionally interchangeable, I introduced *Cb-fem-2* into *Ce-fem-2(null)* animals. Wild-type XX animals are normally self-fertile hermaphrodites, while wild-type XO animals are male. XX *Ce-fem-2(null)* animals, which receive neither maternal nor zygotic *fem-2* product (m-z-), are self sterile at both 21° and 25°, due to their inability to make sperm (Hodgkin 1986). XO m-z- *Ce-fem-2(null)* animals are feminized in both the germ line and the soma. The degree of somatic feminization is temperature dependent, with the somatic tissue of XO m-z- animals being intersexual at 21° and female at 25° (Hodgkin 1986) (table 1). Transgenic *Cb-fem-2* is unable to rescue the germline feminization defect of either XX or XO animals. XX m-z- animals carrying the *Cb-fem-2* array (n>60) were unable to self-fertilize. After self sterility was confirmed, two of these worms were mated to *+/fem-2* males and produced progeny, confirming that oogenesis occurred normally in these worms. At 21°, XO m-z- *Ce-fem-2(null)* animals have an intersexual soma that includes a partially formed vulva, a hermaphroditic bi-lobed gonad and an incomplete male tail (Hodgkin 1986). XO m-z- *Ce-fem-2(null)* animals, either carrying the array or lacking it, were examined for somatic phenotype. It was found that all

animals carrying the array lacked any obvious vulval tissue, had a single-lobed gonad and an incomplete male tail at both 21° (n=34) and 25° (n=24) (figure 20). The germ line was still feminized as evidenced by the presence of oocytes. Therefore, the conserved sequences in *Cb-fem-2* are sufficient to rescue, at least partially, the somatic feminization caused by *Ce-fem-2* mutations.

C. briggsae 3'UTR:

In order to obtain a DNA copy of the 3' end of the *Cb-fem-2* mRNA, as well as to identify the extent of the 3'UTR, 3'RACE was performed on total *C. briggsae* RNA. The amplified products were cloned into pGEM-T (Promega), and sixteen individual clones were sequenced. Surprisingly, many of the clones differed in the location of polyadenylation (figure 21). Indeed, from the sixteen clones, eight different polyadenylation sites were identified. The polyadenylation site nearest the stop codon results in a 3'UTR 68 bases in length. The longest 3'UTR identified is 314 bases long, with the other six ending between these two (figure 21). None of the polyadenylation sites contain the canonical upstream AAUAAA signal sequence. It is possible that there is only one polyadenylation site and that these different products are artifacts of mispriming, however two lines of evidence argue against this. First, the primer used in 3'RACE contains 17 'T' residues attached to the Universal Amplification Primer. If mispriming were to occur, then you would expect a maximum run of 17 T's at the putative site of polyadenylation (those found in the primer). The clone with the shortest 3'UTR had a run of 26 T's, and clones with larger 3'UTRs had runs as large as 103 T's. The second line of evidence suggesting that these clones do reflect sites of polyadenylation is that when 3' RACE was performed on the *Ce-fem-2* gene, only one product was obtained, which correlates with the site of polyadenylation identified through examination of the cDNAs (results not shown).

***C. elegans fem-2* 3'UTR can direct expression of a reporter construct:**

Two other *C. elegans* sex determining genes, namely *fem-3* and *tra-2*, have been shown to be regulated by their 3'UTRs (Goodwin *et al.*, 1993; Ahringer and Kimble, 1991). Since there is some sequence similarity between the *Ce-fem-2* and *Cb-fem-2* 3'UTRs (figure 21), I decided to test the *fem-2* 3'UTR to see if it could regulate expression. In order to do this, the *Ce-fem-2* 3'UTR was fused to a reporter construct that consisted of the *hsp16-41* heat shock promoter and the *lacZ* coding region (figure 22) (pDP#DH145). As a control, a similar construct was made that contained the *unc-54* 3'UTR (pDP#DH148), which is the standard 3'UTR used in the most commonly used expression vectors in the field, those constructed by Dr. Andrew Fire (Fire *et al.*, 1990). Most heat-shocked animals with the *unc-54* 3'UTR show ß-GAL expression in the pharynx, embryos, tail and other somatic cells (figure 23; table 5). Virtually no animals with the *fem-2* 3'UTR show expression in somatic tissues, except for the pharynx, where roughly half of the animals show ß-GAL expression (figure 23; table 5). This shows that the *fem-2* 3'UTR is capable of reducing the expression levels of the reporter construct. Two deleted forms of the 3'UTR were also tested for their ability to direct expression of ß-GAL. The first deleted 3'UTR (plasmid pDP#DH153) is 34 bases smaller than the full length 3'UTR (figures 12 and 22). The deletion is the same as in plasmid pDP#DH126, and removes a segment of RNA that may be capable of forming a large hairpin loop (figure 12). The number of animals showing ß-GAL expression in the tail and other parts of the soma significantly increased when the deleted form of the *fem-2* 3'UTR was used instead of the full length version (table 5). The other deleted form of the *fem-2* 3'UTR (plasmid pDP#DH154), contains the same deletion as pDP#DH153, but extends further towards the poly-adenylation site (same as in pDP#DH163). In total 124 of the 395 bases of the 3'UTR are deleted (figure 12). Again, significantly more animals show ß-GAL expression when carrying the construct with this form of the 3'UTR as compared to the number of animals when the full version is used (table 5). The number of animals

expressing ß-GAL is also increased above the number showing expression with the smaller 3'UTR deletion.

Phenotypic effect of deletion in 3'UTR of rescuing *fem-2* plasmid:

If the deletion of a portion of the 3'UTR causes inappropriate expression of FEM-2, then it is possible that this would result in some masculinization. In order to determine if this occurs, animals carrying either plasmid pDP#DH11, which contains a wild-type copy of *fem-2*, or a similar plasmid with part of the 3'UTR deleted (pDP#DH163), were examined for possible phenotypic differences. Although subtle, a significant number of the animals carrying arrays with pDP#DH163 had a shorter than wild-type tail spike (table 6, figure 24). Roughly 4% of the animals carrying a wild-type copy of *fem-2* had underdeveloped tail spikes, as compared to over 60% of the animals carrying plasmid pDP#DH163. These underdeveloped tail spikes could reflect partial masculinization due to inappropriate expression of FEM-2.

DISCUSSION

Re-isolation of *b245e2005* allele

The sequence of the set of the *fem-2* alleles revealed that most of them were actually re-isolates of the original allele used in the non-complementation screen (Hodgkin, 1986). In order to explain how this may have occurred, I will first describe the pertinent aspects of the screen. *tra-3* XO males were mutagenized and crossed with *fem-2(b245e2005) dpy-1*; *tra-3* 'females' (figure 25) (Hodgkin, 1986). From this cross, all progeny will have the genotype *fem-2 dpy-1/++*; *tra-3*. Since *fem-2* is able to suppress the masculinizing phenotype of *tra-3* only when homozygous, these animals will be masculinized and self-sterile. Therefore, no self progeny will be produced unless a new *fem-2* mutation comes from the mutagenized parental male, resulting in animals with the genotype *fem-2(b245e2005) dpy-1/ fem-2(*) +*; *tra-3*, where '*' represents a newly generated *fem-2* mutant allele (figure 25). If the new allele does not complement *fem-2(b245e2005)*, then the lack of *fem-2* activity will suppress *tra-3*, resulting in self-fertile animals. Therefore, the screen involves identifying crosses that result in self-fertile animals. The new allele can subsequently be distinguished from *fem-2(b245e2005)* by the lack of the *dpy-1* marker. I believe the reason why five of the six alleles generated from this screen are actually re-isolates of *fem-2(b245e2005)* has to do with the fact that the parental 'females' used in the original cross (i.e. *fem-2(b245e2005) dpy-1*; *tra-3*), are not 'females', but rather hermaphrodites capable of self-fertilization. Indeed, the basis of this screen, as well as the screen used to originally isolate *fem-2(b245e2005)*, is that *fem-2* ;*tra-3* animals are self-fertile (Hodgkin, 1986). This raises the possibility that some animals resulting from self fertilization (with the genotype *fem-2(b245e2005) dpy-1*; *tra-3*), were amongst the expected cross progeny describe above (i.e. *fem-2 dpy-1/++*; *tra-3*). Hodgkin (1986) states that he let the parental 'females' mature into 'healthy adult

females' before crossing with the males. Therefore some self-fertilization could have occurred prior to mating with the males. Alternatively, some animals may have avoided mating with the males for a period of time, allowing for their own sperm to be used for fertilization. This would result in animals of two different genotypes existing in this generation; (1) *fem-2 dpy-1/++; tra-3* and (2)*fem-2(b245e2005) dpy-1; tra-3*. Group (1) animals are masculinized and are able to mate with group (2) animals (figure 25). Since *dpy-1* is about eight map units from *fem-2* (Hodgkin, 1986), some of the sperm from *fem-2 dpy-1/++; tra-3* males would contain a *fem-2(b245e2005)* allele that is not marked with *dpy-1*. Fertilization by these sperm will result in animals that are *fem-2(b245e2005) dpy-1/fem-2(b245e2005)+; tra-3*. These animals would look identical to those resulting from a new mutational event as described above, therefore would be considered new alleles of *fem-2*, even though they are actually re-isolates of *b245e2005*. Since so many animals were screened, this need only happen very infrequently for re-isolates to be obtained. Although there could be other reasons why five of the six alleles are re-isolates of *b245e2005*, I believe this to be the most probable explanation.

Null phenotype of *fem-2*

The strongest *fem-2* alleles isolated to date are variable in their phenotype depending on the temperature at which the worms are grown (Hodgkin, 1986). *fem-2(b245e2005)* XO animals grown at 15° show very little feminization, while those grown at 25° are completely feminized. The following are two possible reasons why these alleles are conditional. 1) The alleles are not null, but rather retain some *fem-2* activity, and the mutation involved is affected by temperature (for example, a mutation that prevents proper protein folding at higher temperatures). 2) The alleles are null, however the requirement for *fem-2* is more stringent at high temperatures, and partially dispensable at low temperatures. Two lines of evidence suggest that the latter is the more plausible explanation. First, animals hemizygous for one of the alleles (*e2105*) have the same

phenotype as animals homozygous for the same allele at both 20° and 25° (Pilgrim *et al.*, 1995). If the allele retained partial *fem-2* activity, resulting in incompletely feminized animals, then it is likely that animals that have only one copy of the allele would show a more severe phenotype than animals that have two. Since hemizygous and homozygous animals show the same phenotype, this suggests that *e2105* is a null allele. The second line of evidence supporting the hypothesis that these are null alleles has to do with the location of the molecular lesions associated with the alleles. *b245e2005* (i.e. *e2101, e2103-6*) and *e2102* both contain premature stop codons that eliminate the phosphatase domain (figure 14). *fem-2's* phosphatase activity is necessary for its masculinizing function since the *b245* allele lowers its dephosphorylating ability and results in a feminizing phenotype (figure 15). Also, Chin-Sang and Spence (1996) induced a mutation in a *fem-2* transgene that eliminated phosphatase activity and showed that it was no longer able to rescue *fem-2(lf)* XO animals. Since *b245e2005* and *e2102* lack the phosphatase domain, and phosphatase activity is necessary for *fem-2's* masculinizing activity, the phenotype associated with these alleles is most likely due to a complete loss of *fem-2* activity. As mentioned, this could mean that *fem-2* is dispensable in some tissues at lower temperatures. Perhaps another PP2C enzyme is able to compensate for the loss of *fem-2* at lower temperatures. Indeed, the genome sequencing project has identified three Open Reading Frames (ORFs), that encode likely PP2C enzymes (Wilson *et al.*, 1994; Pilgrim *et al.*, 1995). It has been suggested that there is some overlap in function between the various PP2C enzymes in yeast (Maeda *et al.*, 1994; Shiosaki and Russel, 1995), therefore it is possible that this is also true in *C. elegans*. It is also possible that no other proteins are directly compensating for the loss of *fem-2* at lower temperatures, but rather the lack of complete feminization is due to an inherent temperature sensitivity in the sex determination mechanism. In order to determine if the other PP2C enzymes are compensating for *fem-2*, they could be inactivated both in wild-

type and *fem-2(null)* backgrounds, possibly through RNA interference (Fire *et al.*, 1998), to look for a sex determination phenotype.

FEM-2 has phosphatase activity (PP2C)

Although somewhat longer at the amino terminus, the predicted FEM-2 amino acid sequence showed strong similarity to known serine-threonine phosphatases of the type 2C (PP2C). I undertook two methods in order to determine if the similarity reflects actual dephosphorylating activity. First, I showed that bacterially expressed FEM-2 dephosphorylates radioactively labeled casein in a manner consistent with its classification as a PP2C enzyme (e.g Mg^{2+} dependent). Second, I demonstrated that FEM-2 is able to complement mutations in the canonical PP2C enzyme, pPtc1, in *Saccharomyces cerevisiae*. PP2C enzymes have diverse functions in a number of organisms (reviewed in Barford, 1996), however a common general role in eukaryotes is the regulation of signaling pathways associated with stress response. For example, two genes encoding PP2C enzymes in Arabidopsis (*ABI1* and *ABI2*), are necessary for the plants' response to Abscisic acid (ABA) (Leung *et al.*, 1994; Meyer *et al.*1994; Leung *et al.*, 1997). ABA is involved in the plants' tolerance to a number of environmental stresses (summarized in Leung *et al.*, 1997). In yeast, overexpression of *ptc1* was able to suppress the lethality caused by hyper-excitation of the PBS2/HOG-1-MAP kinase pathway, which is involved in response to both osmotic and heat stress (Maeda *et al.*, 1994). Loss of *ptc1* activity in yeast cells causes no discernable phenotype at 30°, however, cells with this mutation grow very slow at 37° (Maeda *et al.*, 1993). Also, *ptc1* is synthetically lethal with *ptp2*, a Protein Tyrosine Phosphatase gene, suggesting that these genes may have overlapping functions (or regulate redundant pathways) (Maeda *et al.*, 1993). As mentioned, FEM-2 is able to rescue the slow growth defect of *ptc1* in yeast. This confirms that it is able to function as a phosphatase, but also suggests that it does not have a high degree of target specificity. It is unlikely that FEM-2 and pPtc1

dephosphorylate the same protein since they are involved in such different processes, but even if they do, the targets would likely be quite diverged between the two organisms. Since other PP2C enzymes have been identified in *C. elegans*, and they are likely involved in varied processes, it is logical that the dephosphorylating ability of FEM-2 would be exerted on only its sex determination target(s). Perhaps this specificity is partially accomplished by its long amino terminal extension, although some overlap in function would help to explain the temperature sensitive null phenotype, at least at lower temperatures.

Role of the FEM-2 amino terminus

FEM-2 is part of a distinct class of PP2C enzymes (Class A), based primarily on its long amino terminus (Pilgrim *et al.*, 1995). Other Class A PP2C homologues include ABI1, ABI2 and KAPP from *Arabidopsis* (Leung *et al.*, 1994; Meyer *et al.*, 1994; Stone *et al.*, 1994; Leung *et al.* 1997) and a predicted protein of unknown function from the random sequencing of a human cDNA library (Nomura *et al.*, 1994). KAPP has been shown to bind a serine/threonine receptor kinase through its amino terminal domain, although the physiological role of either the phosphatase or the kinase homologue remains obscure (Stone *et al.*, 1994). As mentioned, it is possible that the FEM-2 amino terminus is involved in regulating the phosphatase activity. It is unlikely that the amino terminal domain is directly involved in dephosphorylation because FEM-2 proteins lacking it show no detectable difference in dephosphorylating casein *in vitro* or in its ability to rescue the slow growth defect in yeast (figures 15 and 16). It is certain that this domain is necessary for FEM-2's masculinizing activity since proteins lacking it are unable to rescue *fem-2(null)* animals. As mentioned above, the amino terminus may be necessary for proper regulation of the phosphatase activity. Regulation could be accomplished in a number of ways, however it is unlikely that this domain serves as a negative regulator of phosphatase activity since an increase in activity would have been expected when the

domain is removed, and none was seen. It is possible that the amino terminus interferes with its association with other potential targets, preventing dephosphorylation, however, since full length FEM-2 is able to function in yeast, most likely on a target quite dissimilar from its natural target, this is unlikely. Perhaps the amino terminus binds to another protein that confers specificity to the reaction. This protein may be the target itself, or another protein that binds the target. The amino terminus, or a protein that it binds, could also determine the sub-cellular localization of FEM-2, removing it from other potential targets and placing it in proximity to its intended target. Chin-Sang and Spence (1996) have shown that FEM-2 and FEM-3 interact, and the amino terminus may be involved in fostering this association.

Rapid divergence of sex determination proteins

FEM-2 contains a large domain that is very similar to PP2C enzymes and is responsible for FEM-2's phosphatase activity. It also contains a long amino terminus that is necessary for the masculinizing activity of FEM-2, but is not directly involved in dephosphorylation (discussed above). One method to predict the possible role of a protein or domain is to identify similar proteins or domains of known function. Since the amino terminus shows no significant similarity to known proteins or domains, I cloned the *fem-2* homologue from a closely related nematode species (*C. briggsae*), hoping that similarities in its amino terminus yields information as to regions of functional significance. In collaboration with Paul Stothard, I also cloned the *fem-2* homologue from the male/female species *C. remanei*. It is estimated that *C. briggsae*, *C. remanei* and *C. elegans* diverged 20-50 million years ago (Heschl and Baillie, 1990; Lee *et al.*, 1992; Kennedy *et al.*, 1993; Fitch and Thomas, 1997). Many *C. elegans* homologues have been cloned from *C. briggsae*, and often show very high degrees of similarity (see deBono and Hodgkin, 1996). In fact, many are so similar that they yield very little information as to which amino acids are functionally important (e.g. Maduro and Pilgrim,

1996). This is not so for *fem-2*. The FEM-2 protein from *C. briggsae* (Cb-FEM-2) is not only 53 amino acids larger than Ce-FEM-2, but the overall amino acid similarity and identity is much lower than what has been reported for other *C. briggsae* homologues of *C. elegans* genes, suggesting that *fem-2* could be rapidly evolving, although this seemingly high degree of divergence could just be due to variablility (figure 27). However, *fem-2* is not the only gene that shows striking divergence between the two organisms. *tra-1* and *tra-2* also appear to be rapidly evolving, having only 44% and 43% amino acid identity to their *C. briggsae* homologues respectively (figure 27) (de Bono and Hodgkin, 1996; Kuwabara, 1996). Interestingly, *tra-1* and *tra-2*, as well as *fem-2*, are all part of the sex determination signaling cascade. This could suggest that genes involved in sex determination are evolving faster than genes involved in other developmental processes. Certainly, this has been suggested for other organisms. For example, the mammalian testis determining *SRY* gene has been cloned from a number of species, including many primate species (Whitfield *et al.*, 1993) and Old World mice and rats (Tucker and Lundrigan, 1993). Both studies compared the SRY amino acid sequence between closely related species and found a high level of divergence of the regions outside of the highly conserved HMG box. Tucker and Lundrigan (1993) suggest two possibilities for the rapid divergence. 1) The regions of the proteins that have diverged rapidly are not functionally constrained. 2) The proteins could be under species-specific directional selection. To address the two possibilities, Simon *et al.* (1993) compared the number of synonymous versus non-synonymous changes in the coding sequence of *SRY* genes from closely related species. They found a high level of non-synonymous changes, suggesting that the changes in DNA sequence that result in an amino acid changes are selected. It was further suggested that since changes to *SRY* can result in sex reversal, changes to *SRY* in a population could cause reproductive isolation (thus speciation).

If a pressure towards reproductive isolation is the cause of the rapid divergence of sex determination proteins, then it is possible that proteins involved in processes other than sex determination, but that could still result in reproductive isolation upon mutation, could also be rapidly evolving. Civetta and Singh (1998) compared the coding sequence of 51 genes and their homologues from closely related species. Three *Drosophila* species were used, as well as *C. briggsae* and *C. elegans*. They found that genes involved in sex-related behaviors (mating behavior, fertilization, spermatogenesis and sex determination) diverged much faster than genes involved in other processes (Civetta and Singh, 1998). They also found that the sex-related genes had a larger frequency of non-synonymous changes, arguing for directed selection. This supports the hypothesis the that rapid divergence of proteins involved in sex determination is involved in reproductive isolation.

Indeed, sex determination mechanisms between genera can be quite different, further supporting the idea that they are rapidly evolving. Although there are some general similarities between *Drosophila* and *C. elegans* sex determination mechanisms, none of the known sex determining genes have homologues with similar roles in the other organism, with the notable exception of *mab-3*. *mab-3* (male abnormal) is a male specific gene downstream of the *C. elegans* gene *tra-1* (Shen and Hodgkin, 1988). Cloning of the gene revealed that its putative protein product is related to the sexual regulatory gene *dsx* (*doublesex*) from *D. melanogaster* (Raymond *et al.*, 1998). The male form of DSX is even able to rescue *mab-3* mutants, suggesting a similar functional role (Raymond *et al.*, 1998). It is interesting that some sex determination genes are evolving very rapidly (*fem-2, tra-1, tra-2*), while *mab-3* and *dsx* have similar functions suggesting that they are quite old. Marin and Baker (1998) have proposed that genes at the top of regulatory hierarchies can accommodate changes (rapid divergence) easier than genes at the bottom of the hierarchy (figure 2). This is due to 'higher' genes generally having

fewer targets than 'lower' genes. 'Lower' genes, such as *dsx* and *mab-3,* are thought to control the expression of a number of sex specific genes, whereas 'higher' genes are thought to have fewer targets. If a 'higher' gene were to acquire a change that results in a new protein conformation, the change only needs to accommodate the ability of the protein to regulate one other gene (or few). A 'lower' gene, whose protein product presumably interacts with and regulates many genes or gene products, is much more constrained as to the number of changes that can occur, and still properly regulate all of the many genes.

The FEM-2 example of rapidly diverging sex determination proteins differs from TRA-1 and TRA-2 because it has two very distinct domains that seem to be evolving at different rates. The Cb-FEM-2 amino terminus is 32% longer than Ce-FEM-2's, and the region that is common between the two (170 aa) is only 49% identical. The Ce-FEM-2 PP2C domain, which comprises ~60% of the protein, is 72% identical to the corresponding region of Cb-FEM-2 (figure 9). As discussed above, a possible role of the amino terminus is binding to another protein. Perhaps domains that are involved in protein-protein interactions are less constrained in their abilities to evolve. A changed amino acid in one protein could put pressure on an interacting protein to also change in order to maintain the strength of the interaction (figure 26). Thus, domains of protein-protein interaction, although functionally important, may not show a high degree of conservation between species. Domains that bind to molecules with more static structures, such as DNA (TRA-1) or a phosphate group (FEM-2), may not be able to change amino acids as readily and still maintain proper function. Indeed, I have shown that the Ce-FEM-2 amino terminus is necessary for masculinizing activity, even though it is quite diverged from Cb-FEM-2, raising the possibility that it is involved in protein-protein interaction. As mentioned, Chin-Sang and Spence (1996) have demonstrated a FEM-2/FEM-3 interaction *in vitro*. It will be very interesting to see what portions of the proteins are

involved in this interaction, and to see the relative divergence of the protein binding domains in Ce-FEM-3 and Cb-FEM-3.

Even though the Ce-FEM-2 and Cb-FEM-2 phosphatase domains are more similar than the amino termini, they are still diverging much faster than most other proteins for which we have data. Using degenerate PCR, Paul Stothard cloned a region encoding a very conserved portion of PP2C enzymes in *C. remanei* from FEM-2 (Cr-FEM-2), as well as from another PP2C enzyme whose *C. elegans* homologue was identified by the genome sequencing project (T23F11.1). The non-sex determining homologues show 95% amino acid identity in this region, while the FEM-2 homologues are only 68% identical in this same region (Stothard, Hansen and Pilgrim, manuscript in preparation). This further supports the idea that a pressure is causing genes involved in sex determination to evolve quickly, although certain domains are more constrained in the number and type of amino acid substitutions that are tolerable.

Regulation by the *fem-2* 3'UTR

One purpose of cloning *Cb-fem-2* was to compare the sequences of the possible regulatory regions with those in *Ce-fem-2*, and identify regions of similarity that could reflect conservation of regulatory elements. One region that does show some similarity between the two genes is the 3'UTR. It is difficult to determine if the similarity is significant enough to connote functional importance, however, the *Cb-tra-2* 3'UTR shows some sequence similarity to that in *C. elegans*, and functional conservation has been demonstrated (Jan *et al.*, 1997), therefore I decided that the sequence similarity of the *fem-2* 3'UTRs warranted further investigation. Translational regulation by the 3'UTR has been implicated in the control of expression of only four genes in *C. elegans*. *lin-14* is a heterochronic gene whose 3'UTR can be bound by the RNA product of the *lin-4* gene, which serves to inhibit the translation of LIN-14 (Lee *et al.*, 1993; Wightman *et al.*,

1993). *glp-1* is involved in establishing asymmetry in the early embryo (see Anderson and Kimble, 1997 and references contained therein). *glp-1* mRNA is maternally contributed, however its 3'UTR is thought to be involved in allowing translation to occur in only certain cells in the embryo (Evans *et al.*, 1994). The *glp-1* 3'UTR shows sequence similarity to the *hunchback(hb)* 3'UTR from *Drosophila*. The *hb* 3'UTR contains *nanos* response element (NRE) sequences that are thought to be bound by *nanos(nos)* as a means of repressing translation (Reviewed in Curtis *et al.*, 1995). The other two genes from *C. elegans* that have had their 3'UTRs implicated in translational regulation are *tra-2* and *fem-3* (Goodwin *et al.*, 1993; Ahringer and Kimble, 1991), which were discussed in the Introduction. It is intriguing that two of the four genes with identified 3'UTR mediated translational regulation are sex determination genes. Perhaps this type of regulation is well suited for the regulation of sex determination genes. Another possibility is that 3'UTR mediated translational regulation is involved in the control of expression of numerous other genes, but the phenotypes associated with a disruption of this regulation in *tra-2* and *fem-3* are easily identifiable. In any event, it is interesting that the *fem-2* 3'UTR also is capable of regulating expression. No genetic lesions have been identified in the *fem-2* 3'UTR, suggesting that its role may not be identical to that of *fem-3*. Specifically, the *fem-3* 3'UTR appears to function by regulating spermatogenesis in the hermaphrodite. Since no *fem-2* mutant alleles have been identified that cause an increase in spermatogenesis in the hermaphrodite (like *fem-3(gf)*), the role of the regulation may not be equivalent between the two genes. However, it should be noted that six of the nine original *fem-3(gf)* alleles isolated were generated in a mutation screen relying on the suppression of *fem-2(b245)* (Barton *et al.*, 1987). Therefore, it is unlikely that *fem-2(gf)* alleles would be identified by this screen.

The current model of 3'UTR mediated regulation of translation in *tra-2* and *fem-3* is that a repressor binds to this region preventing translation (figure 6) (Jan *et al.*, 1999; Zhang

et al., 1997), however the precise mechanism involved is far from being understood. Some insight into how this repression may be achieved comes from a study of *oskar* translation in *Drosophila.* The translation of *oskar* mRNA is temporally regulated by binding of repressor proteins that prevent translation (Kim-Ha *et al.*, 1995; Gunkel *et al.*, 1998). It is thought that in order for translation to be initiated, the 3'UTR and 5'UTR need to interact, which the repressor prevents (Gunkel *et al.*, 1998). Therefore the repressors that bind to the *fem-3* and *tra-2* 3' UTRs may prevent interaction with the 5'UTR and initiation of translation. It is possible that that the *fem-2* 3'UTR also binds a repressor protein that prevents translation. Since deletion of part of the 3'UTR seems to lessen its ability to prevent translation, it is possible that the deletion destroys part of the repressor binding site. However, it is also possible that a mechanism exists where the 3'UTR is directly involved in lowering translation levels, and the increase in expression in the 3'UTR deleted constructs is due to a removal of the inhibitory element.

It is interesting that an increase in expression is seen in the hermaphrodite tail when part of the *fem-2* 3'UTR is deleted from the reporter construct (figure 23, table 5). Another reporter construct that consists of the *fem-2* promoter, *lacZ* coding region and the *unc-54* 3'UTR shows very little somatic expression in the hermaphrodite, except for a limited number of cells in the tail (Johnson, 1995). Perhaps there are a number of levels of regulation of *fem-2* (as is seen with *tra-2*; see Introduction), which are partially redundant to ensure proper expression. Transcriptional regulation prevents expression in numerous cell types, and translational regulation by the 3'UTR adds to the regulation. This could explain why only a subtle phenotype is seen (underdeveloped tail spike) when a portion of the 3'UTR is deleted. Limited transcription has already prevented the production of *fem-2* mRNA in many of the tissues. This could also be similar for the *fem-3* gene. *fem-3(gf)* mutants show only a germ line aberrant phenotype, however expression of a reporter construct in the hermaphrodite soma is regulated by the *fem-3* 3'UTR (Gallegos

et al., 1998). Most likely, other levels of regulation (pre- or post-translational) prevent improper activity of FEM-3 in the soma. Although a study of the control of expression of *fem-2* by its 3'UTR is still in its infancy, it appears as though this may be an important, although possibly redundant, mode of regulation.

CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this thesis project was to confirm cloning of the *fem-2* gene from *C. elegans*, confirm its role as a phosphatase predicted by its sequence similarity to other phosphatases, and to identify conserved regions of the protein or regulatory regions in order to identify sequences with functional importance. In sequencing the mutant alleles of *fem-2*, I confirmed its cloning, as well as supported the hypothesis that the strongest alleles (i.e. *b245e2005* and *e2102*) are probably null alleles. I also demonstrated that FEM-2 has PP2C-type phosphatase activity that is necessary for its masculinizing role in sex determination, although its target(s) has not yet been identified. It is possible that the FEM-2/FEM-3 interaction identified by Chin-Sang and Spence (1996) represents the dephosphorylating interaction (i.e. FEM-3 is the target), however this hypothesis still needs to be tested. Alternatively, it is possible that FEM-3 is not the target, but rather regulates FEM-2's phosphatase activity. Perhaps a yeast two hybrid screen, or co-immunoprecipitation, could be used to identify its targets. Another protein that has not been identified is the kinase that phosphorylates FEM-2's target. Its predicted role in sex determination would be to feminize the XX soma and germ line, with a loss-of-function phenotype of masculinization of XX animals (Tra phenotype). Since it has not been identified by genetic means, it is possible that it has other functions, and its loss-of-function phenotype masks its role in sex determination. Another possibility is that there is more than one kinase, and a loss of one is compensated by another. If this is the case, biochemical techniques may be more useful in identifying the kinase than genetic (e.g. co-immunoprecipitation or yeast two hybrid), once the target is identified.

Cloning of the *C. briggsae* homologue of *fem-2* assisted our understanding of *fem-2* in *C. elegans*. First, it is the first protein identified to show similarity to the amino terminus of Ce-FEM-2. Some regions are more conserved than others, allowing predictions to be

made as to regions of function importance. Cloning of other homologues of *Ce-fem-2* (i.e. *Cr-fem-2*) increases the possibility for this analysis to yield useful information. Cloning of *Cb-fem-2* also revealed unusual rapid sequence divergence similar to that seen with other sex determination proteins. Since FEM-2 has two domains, which appear to be diverging at different rates, this could assist in our understanding of why and how these proteins are evolving so quickly. Also, since FEM-2 belongs to a family of enzymes (PP2C), similar regions can be compared between homologues to support the hypothesis that sex determination proteins evolve faster than proteins involved in other developmental processes. Lastly, comparison of the regulatory regions of *Cb-fem-2* and *Ce-fem-2* revealed sequences with possible functional significance. This includes the short sequence in the promoter that has not yet been tested for function. Similarity in the 3'UTR may also signify an important mechanism used to regulate expression. Indeed, I have shown that the *Ce-fem-2* 3'UTR can regulate expression of a transgene in the soma. There are many questions that still need to be experimentally addressed with respect to the 3'UTR. For example, does a negative regulator bind to the 3'UTR to prevent translation. A band shift assay could be used to determine if a protein does bind to the 3'UTR. Also, a yeast three-hybrid screen could be used to identify proteins that bind to the RNA, similar to the analysis used to identify proteins that bind to the *tra-2* and *fem-3* 3'UTRs (Jan *et al.*, 1999; Zhang *et al.*, 1997).

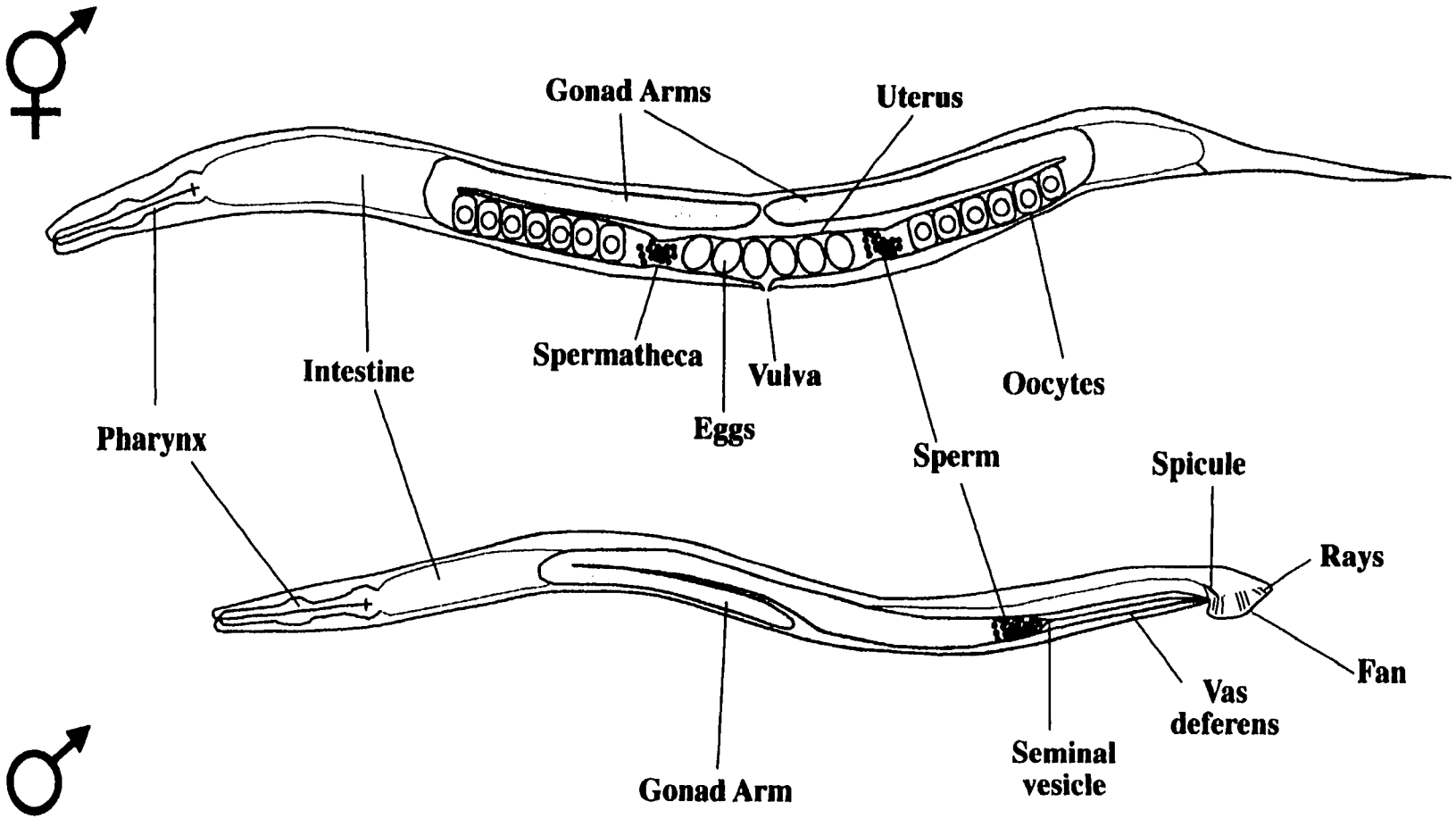


Figure 1. Basic anatomy of adult *C. elegans* hermaphrodite (top) and male (bottom). The grey portions of the animals depict their respective gonads. The hermaphrodite gonad consists of two reflexed arms, opening at the vulva in the middle of the ventral side, while the male only has one, opening at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

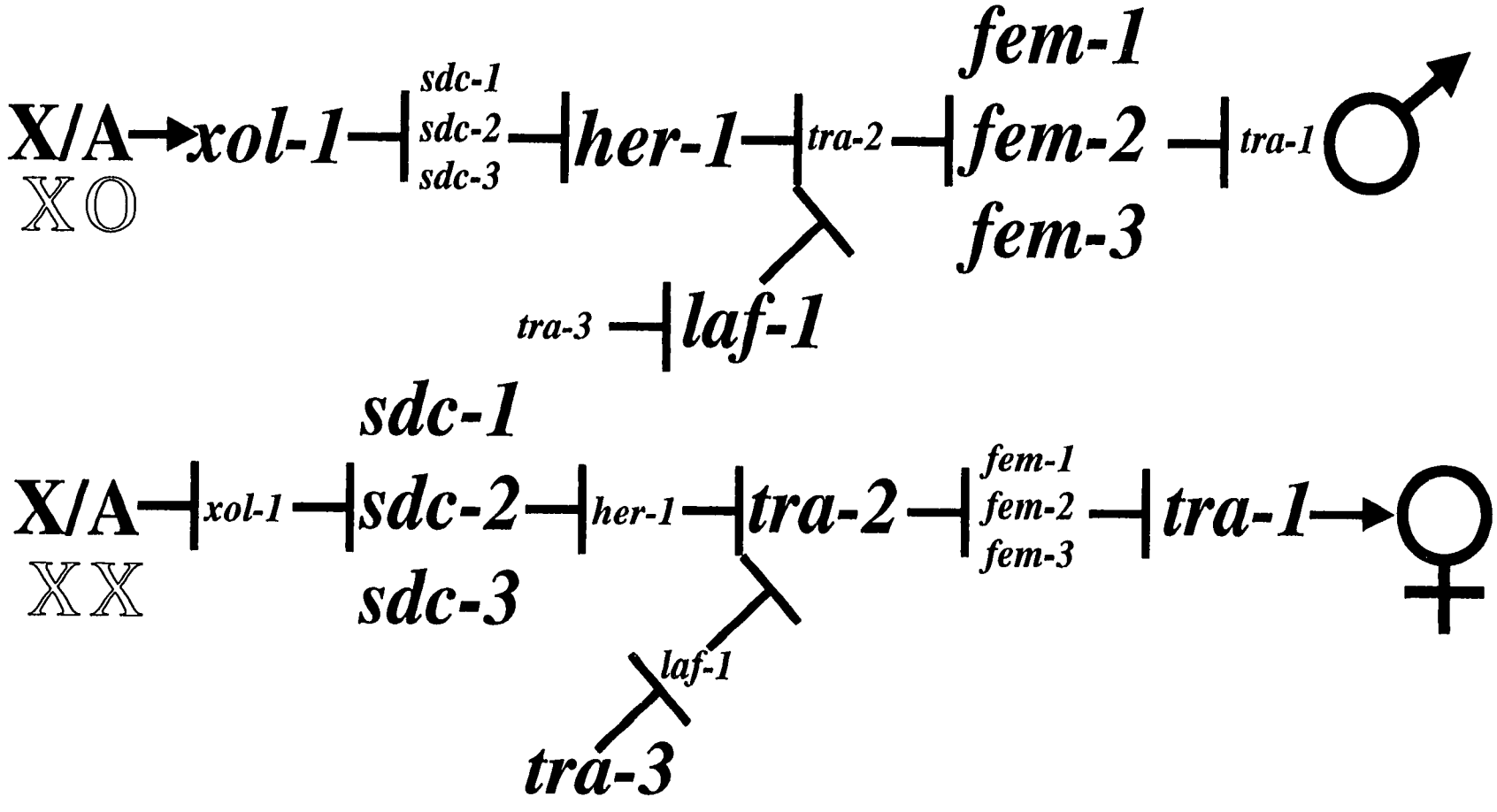
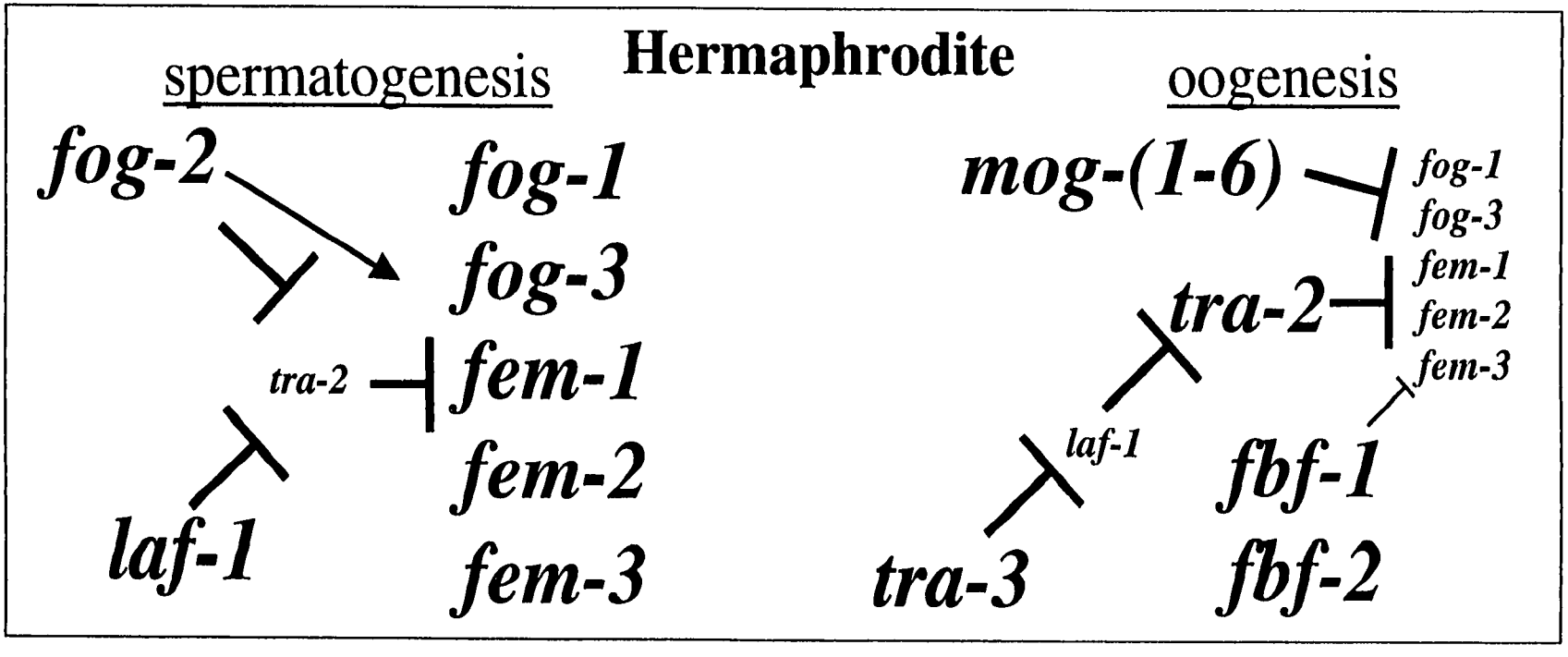


Figure 2. Genetic interactions involved in somatic sex determination. Arrowheads represent positive interactions while vertical bars represent negative or inhibitory interactions. The two pathways represent the activities of the genes in promoting the male (top) and female (bottom) fates. Gene activities which 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/laf-1* pathway receives its initial signal. The dosage compensation pathway is downstream of the *sdc* genes, separate from *her-1*.



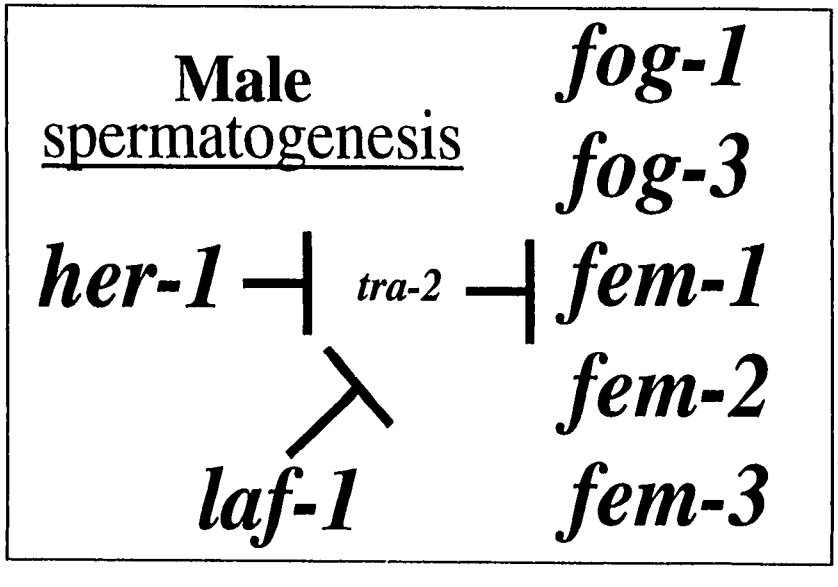


Figure 3. Some genetic interactions involved in germ-line sex determination. As in figure 2, the gene activities that are dispensable are shown in small font. The upper panel shows some of the genes that regulate germ-line sex determination in the hermaphrodite, first to promote spermatogenesis (left), then oogenesis (right). For spermatogenesis, *tra-2* is inactivated, possibly by *laf-1* and *fog-2*, in order to allow the *tgf* (terminal germline fems and fogs) to promote spermatogenesis. After a period of time, the *tgf* genes are inactivated by *tra-2* , and the *fbf* genes prevent the translation of *fem-3*, which causes a switch from spermatogenesis to oogenesis. In the male, *her-1* is active in repressing the activity of *tra-2*, therefore allowing the *tgf* genes to promote spermatogenesis. *laf-1* is also involved in repressing *tra-2*. Germline activity of *fem-1*, *-2*, *-3* and *fog-1*, *and -3* genes are referred to as the *tgf* genes (terminal germline *fems* and *fogs*). It is still unclear how *fog-2*, *mog-(1-6)* and the *tra-3/laf-1* pathway receive their primary signal.

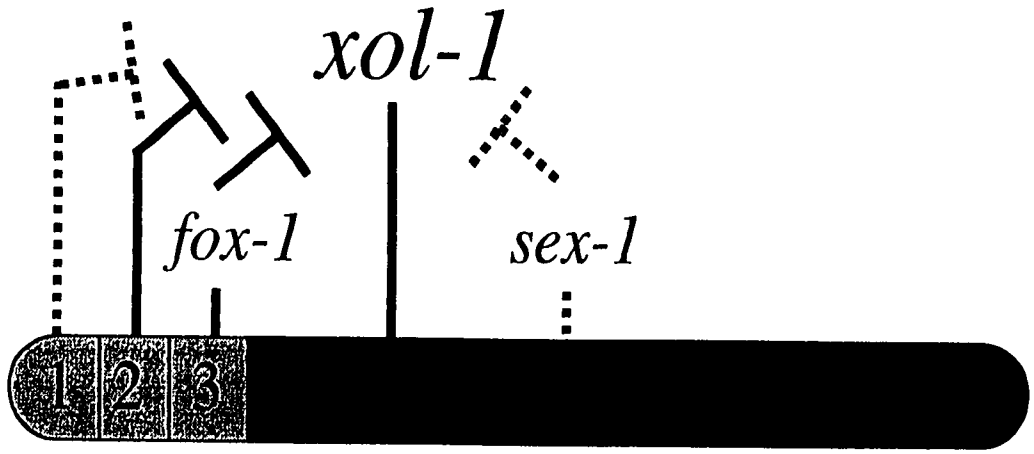


Figure 4. X/A numerator elements on the X chromosome. The regions of the X chromosome labeled one through three represent the three regions identified through duplication and deletion analysis that contain X/A ratio numerator elements (Akerib and Meyer, 1994). *fox-1* is located in Region 3 on the left end of the X chromosome, while another numerator element, *sex-1*, is located near the center of the chromosome. All of the numerator elements seem to repress the activity of *xol-1*, which is also located on the X chromosome. The dotted lines represent transcriptional repression, while the black lines represent repression that is exerted post-transcriptionally. This figure is adapted from Meyer (1997) and is not drawn to scale.

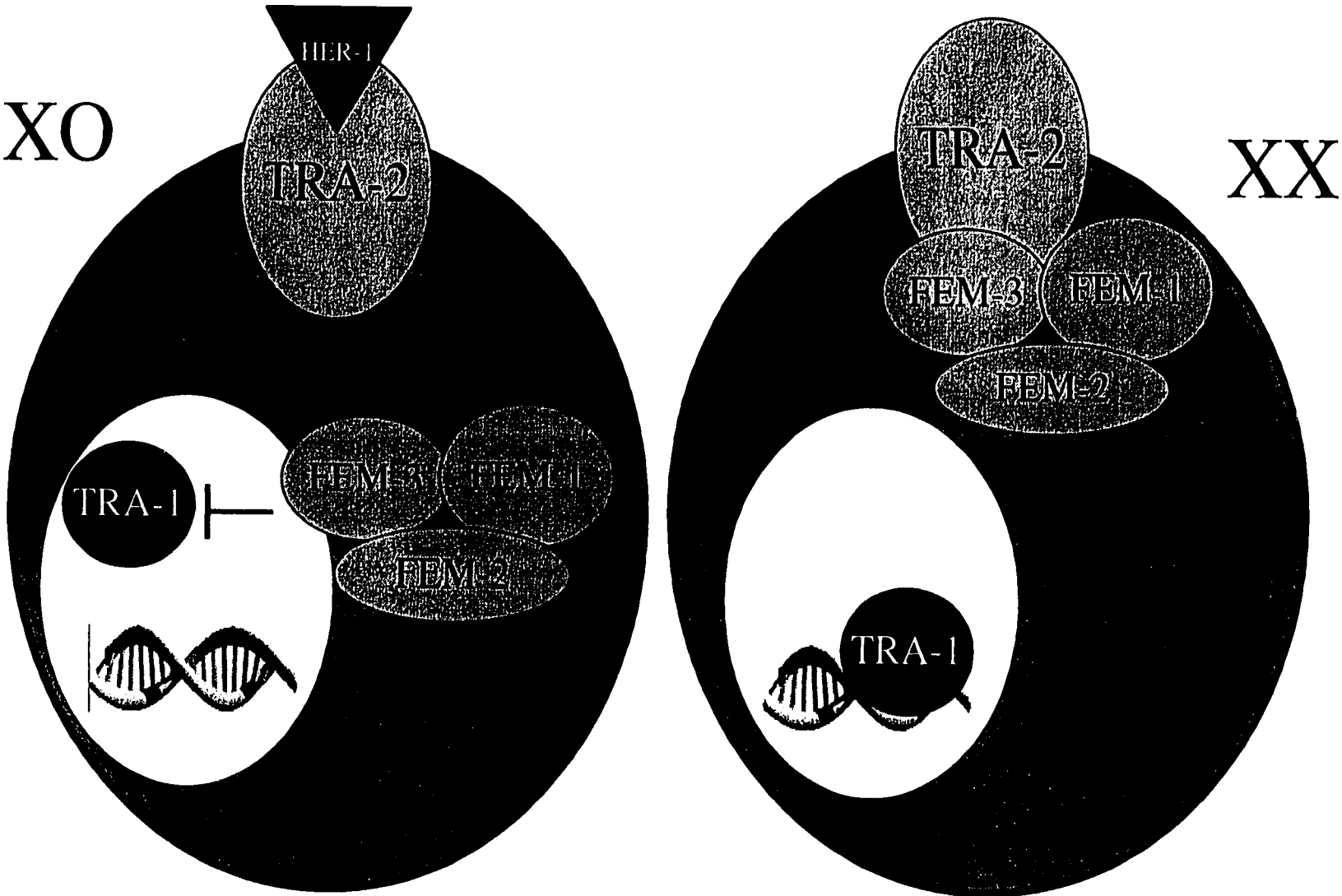


Figure 5. The current model of protein interactions involved in somatic sex determination. The large circle represents a somatic cell, while the smaller white circle represents the nucleus. In XO animals (left), HER-1 is thought to bind TRA-2, which spans the cell membrane. This inactivates TRA-2, allowing the FEM proteins to exert their inhibitory influence on TRA-1. With TRA-1 repressed, the cell is able to take on the male fate. In XX animals (right), HER-1 is not present, therefore active TRA-2 inhibits the FEM proteins, possibly by sequestering them to the surface of the cell membrane. This allows TRA-1 to exert its female promoting function, most likely by serving as a transcription factor. This figure does not include transcriptional and translational regulation of *tra-2*, however this could be reflected by an increase in the number of TRA-2 molecules spanning the cell membrane in XX animals. This figure adapted from Kuwabara and Kimble (1992).

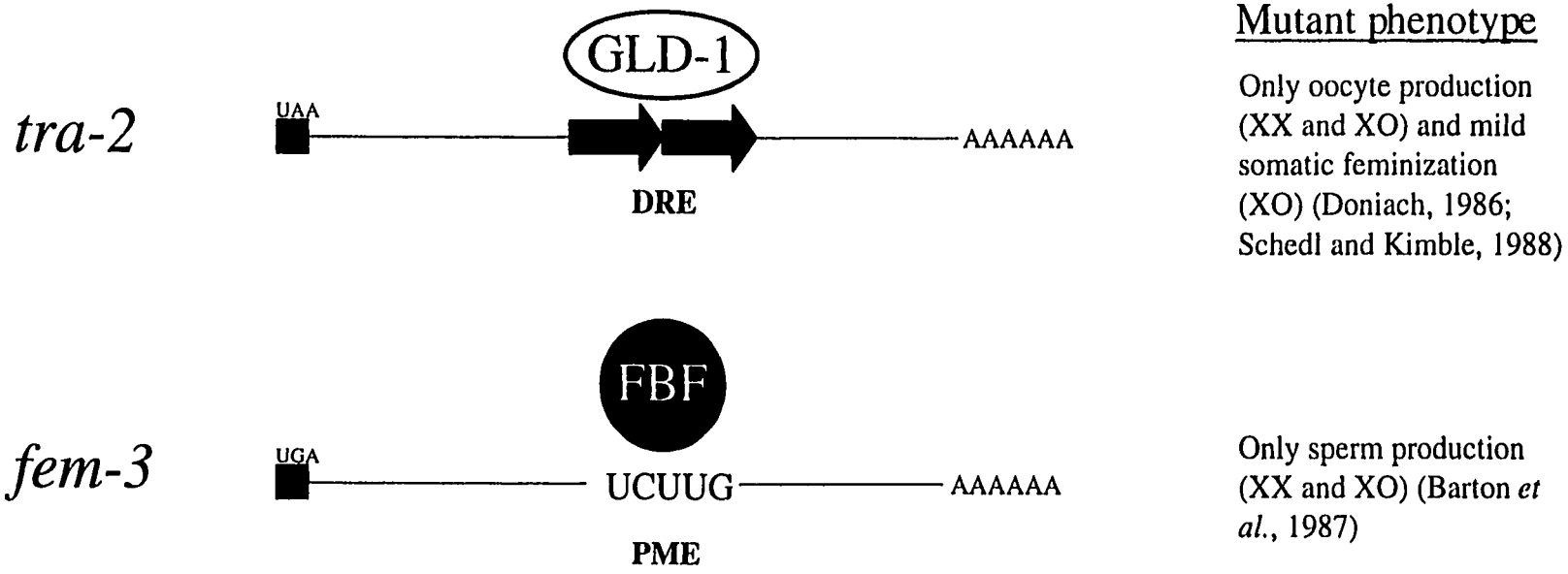


Figure 6. Translational regulation by *tra-2* and *fem-3* 3'UTRs. The *tra-2* 3'UTR contains two direct repeat elements (DRE) that result in a mutant phenotype when deleted (Goodwin *et al.*, 1993). A negative regulator (DRF) is thought to bind DRE to prevent translation. GLD-1 may be part of DRF (Jan *et al.*, 1999). The *fem-3* 3'UTR contains a five bp sequence referred to as the point mutation element (PME) (Ahringer and Kimble, 1991), which is thought to be bound by a negative regulator, FBF (Zhang *et al.*, 1997). Point mutations in the PME result in gain-of-function mutations that cause the absence of the switch from spermatogenesis to oogenesis in XX animals (Barton *et al.*, 1987; Ahringer and Kimble, 1991)).

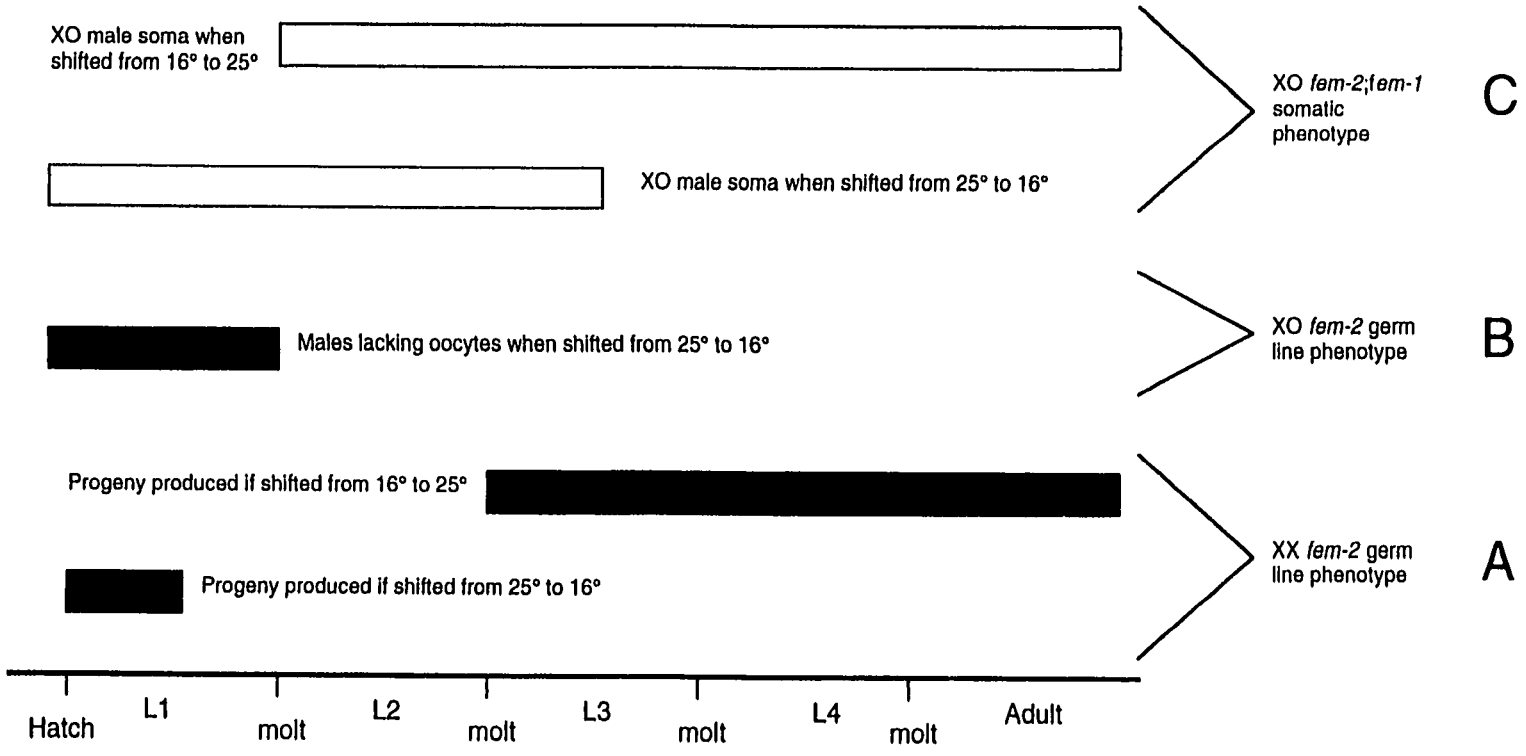


Figure 7. Summary of the temperature sensitive periods for *fem-2(b245)* (Kimble *et al.* 1984). Animals were either shifted from 16° to 25°, or from 25° to 16°. The black, grey and white boxes represent the relative times in development for the XX *fem-2* germ line, the XO *fem-2* germ line and the XO *fem-2;fem-1* soma respectively. (A) For the XX *fem-2(b245)* germ line phenotype, animals were scored based on the number of self-progeny which they produced, which reflects the amount of spermatogenesis that occurred. Animals that were grown at 25°, but shifted to 16° prior to the middle of the first larval stage (L1), produced large broods. If shifted down after the middle of L1, few if any progeny were produced. Animals raised at 16° and shifted to 25° prior to the L3 molt produced few progeny, but if shifted up during or after L3, produced large broods. (B) *fem-2(b245)* XO animals were scored based on the presence of oocytes in their germ line. Animals raised at 25°, then shifted to 16° prior to the L2 molt, did not produce oocytes. If shifted during or after L2, most XO animals showed oocyte production. XO animals raised at 16° and shifted to 25° showed oocyte production when shifted up at any stage. (C) *fem-1(hc17) fem-2(b245)* XO animals were scored for a non-feminized soma. Animals raised at 25°, then shifted down before or during early L3 developed a male soma. If shifted down in late L3 or after, they developed a female soma. XO animals raised at 16° and shifted up prior to mid L2 develop a female soma, but if shifted up after mid L2, develop a male soma.

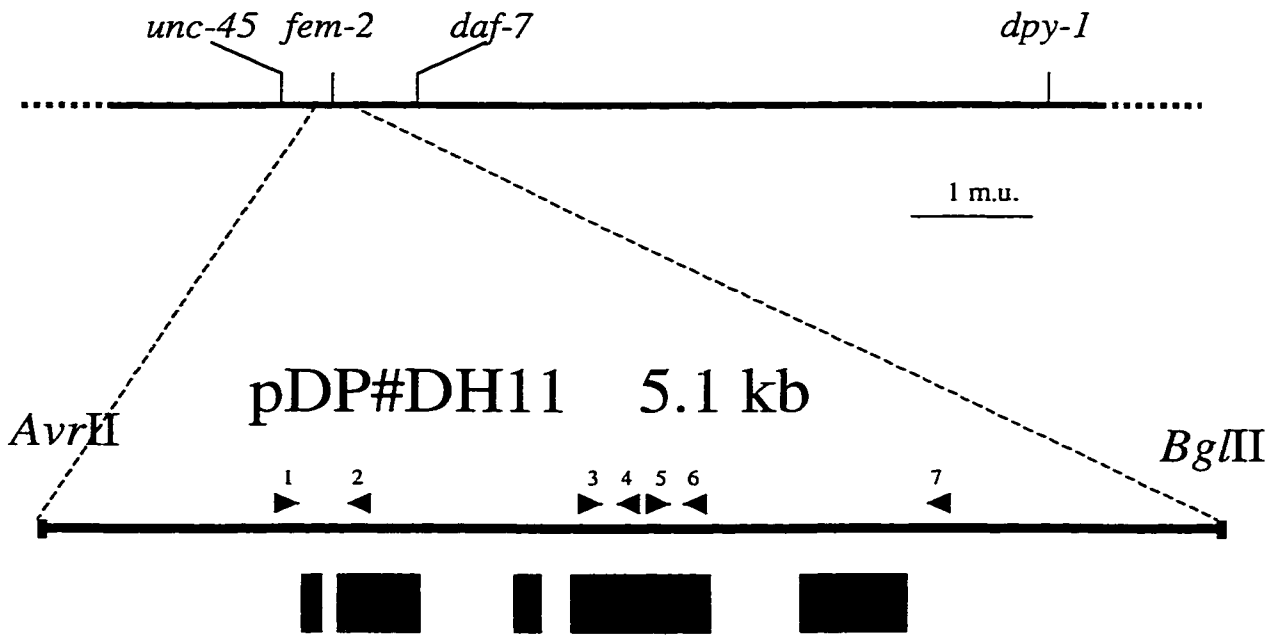


Figure 8 Genomic location of *fem-2* and primer positions. The upper horizontal line represents linkage group III with relative genetic positions of some genes located near *fem-2*. The lower horizontal line represents the genomic DNA insert in plasmid pDP#DH11, which contains the entire *fem-2* coding region. The black squares represent the coding portions of the *fem-2* exons. The arrowheads show the locations of various primers used in this study. 1=DHA3; 2=AMC1; 3=DHA19; 4=DHA9; 5=DHA17; 6=SAD2; 7=DHA4.

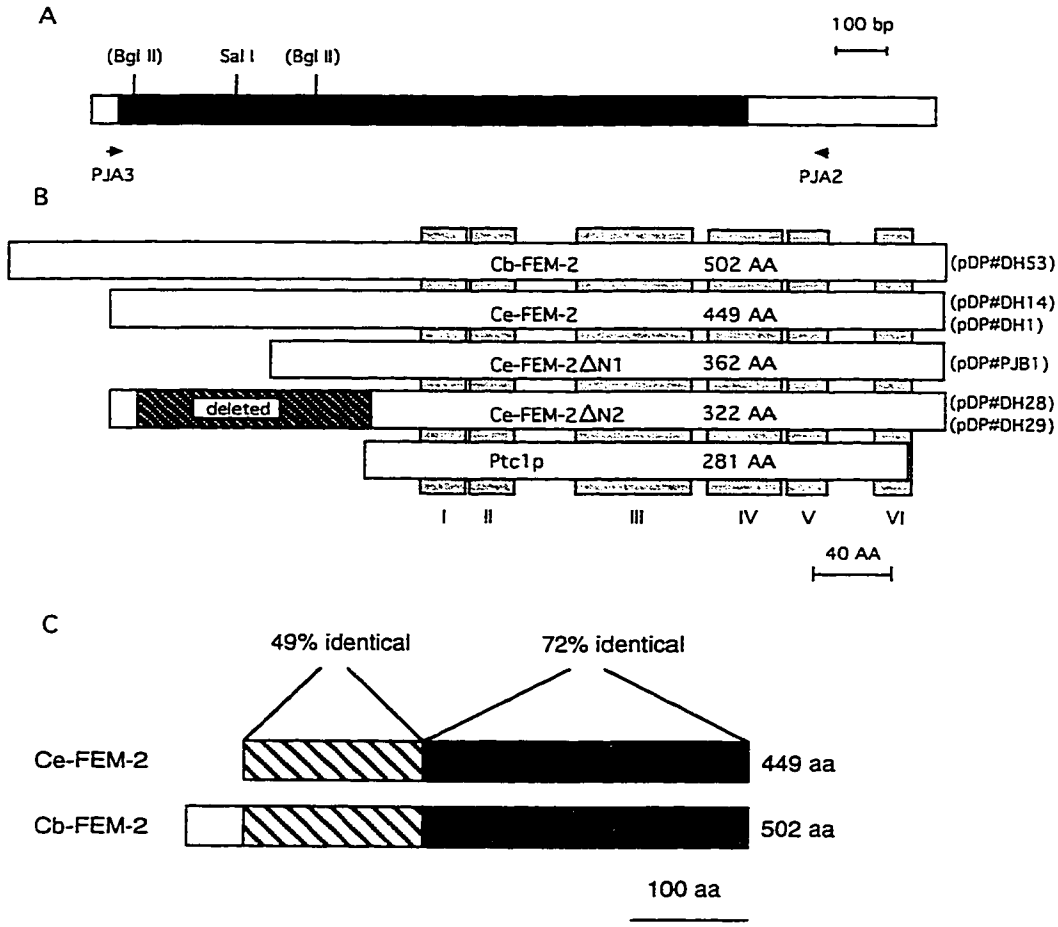


Figure 9. The *Ce-fem-2* gene, construction of fusion proteins, and summary of identity between Cb-FEM-2 and Ce-FEM-2. (A) Restriction map of the transcribed region of *Ce-fem-2* (Pilgrim *et al.*, 1995). The black box corresponds to the FEM-2 open reading frame, white boxes to the 5' and 3' untranslated regions. The *Bgl* II sites in brackets were introduced into the gene by *in vitro* mutagenesis, and were subsequently used to create an in-frame deletion that results in an amino-terminal deletion of 127 amino acids in Ce-FEM-2. Primers PJA2 and PJA3 used in the construction of pDP#DH14 and pDP#DH28 are indicated by arrows. The *Sal* I site shown was used in the construction of pDP#PJB1. (B) Alignment of the predicted Ce-FEM-2 and Ptc1p proteins, with the vertical shaded regions corresponding to the conserved sequence motifs, as described (Pilgrim *et al.*, 1995) The hatched box in FEM-2ΔN2 shows the portion of FEM-2 missing because of the deletion using the *Bgl* II sites. The names of the plasmids encoding the proteins are in parentheses beside the respective protein. Ce-FEM-2 and Ptc1p share 30% identity and 50% similarity over the six motifs. (C) The gray boxes represent the PP2C domains of Ce-FEM-2 and Cb-FEM-2. The striped boxes represent the protions of the amino-terminal domains that are common to each other.

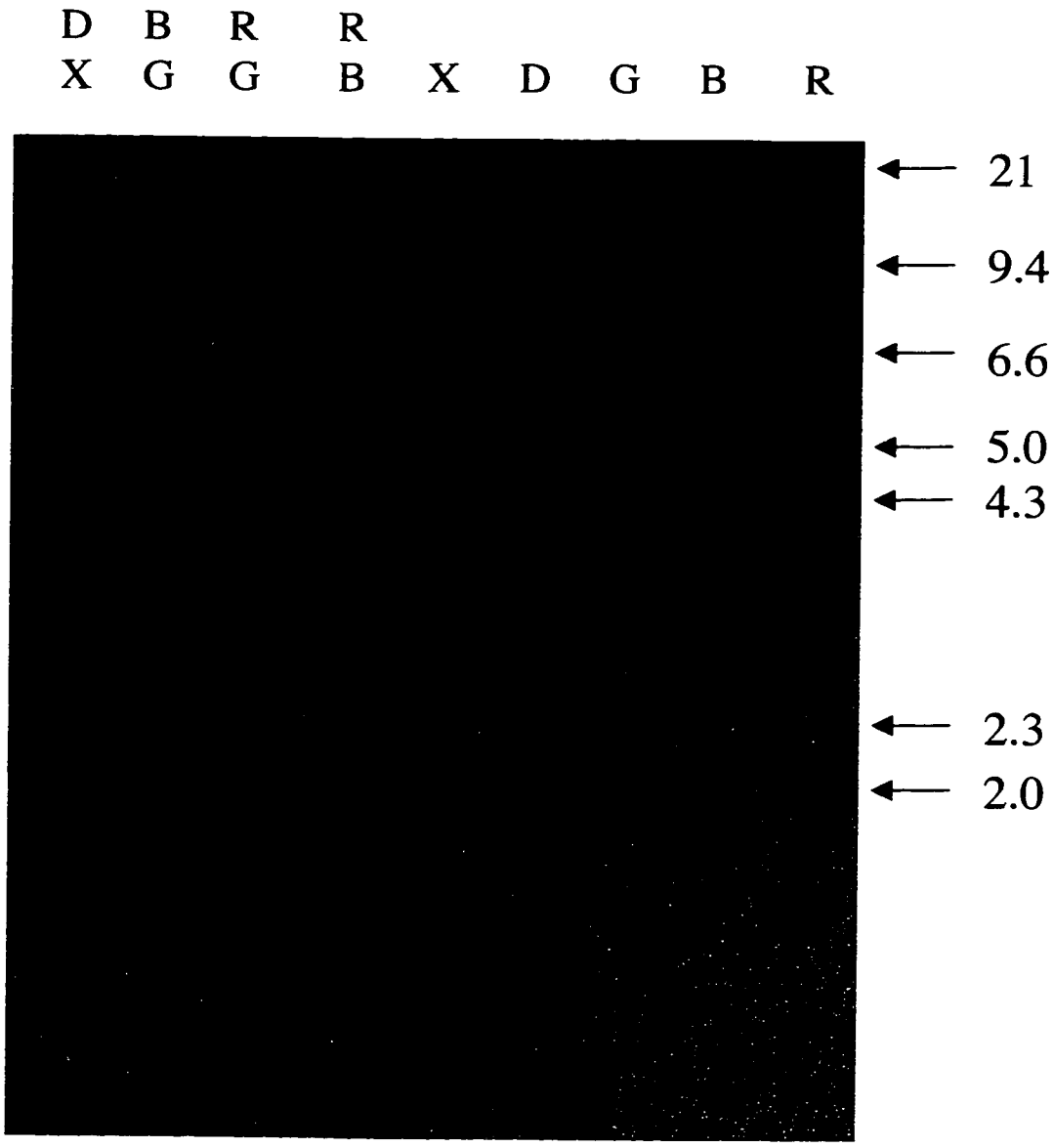


Figure 10. Southern blot of *C. briggsae* genomic DNA hybridized with ^{32}P labeled probe made from a *Ce-fem-2* cDNA clone. DNA was digested with various enzymes (D=*Hin*D III, X=*Xba* I, B=*Bam*H I, G=*Bgl* II and R=*Eco*R I). Numbers on the left show location of DNA bands from size standard (in kbp).

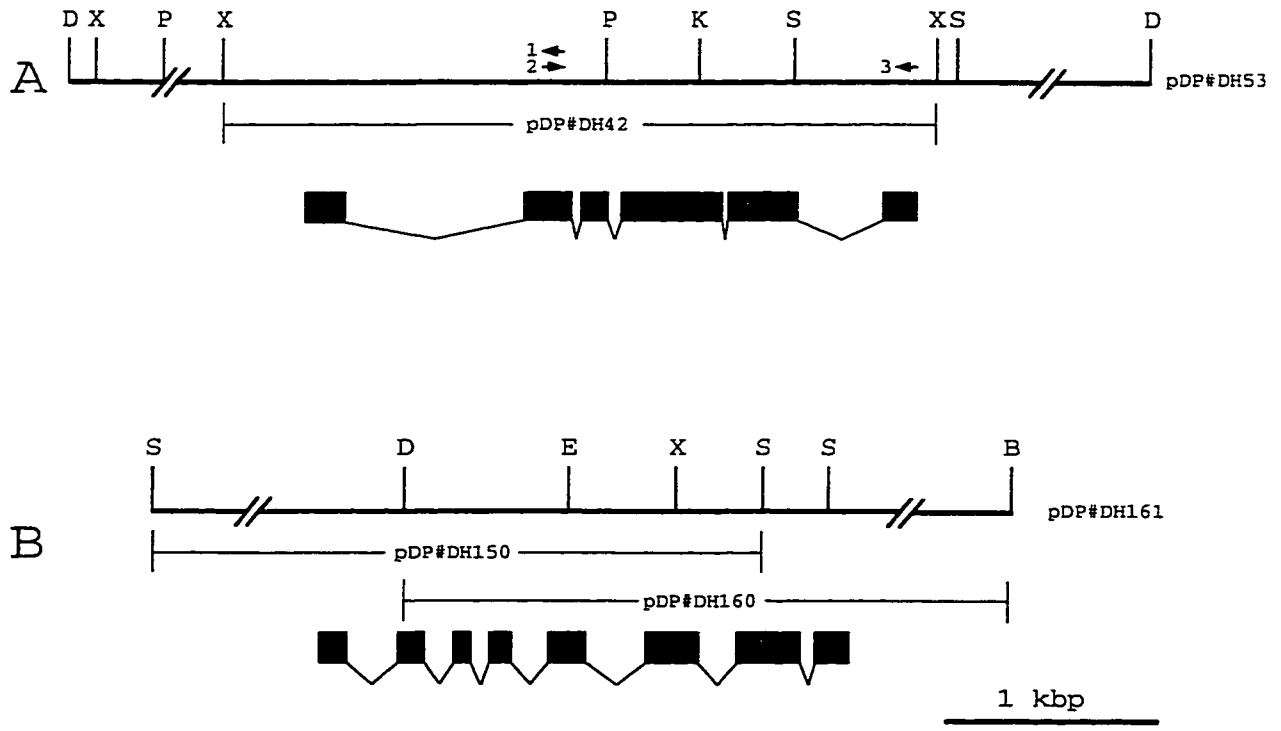


Figure 11. Restriction maps of genomic regions containing *Cb-fem-2* and *Cr-fem-2* and locations of intron and exon boundaries. Vertical lines represent restriction enzymes (B, *Bam*HI; D, *Hin*DIII; E *Eco*RI; P, *Pst*I; S, *Sst*I and X, *Xba*I). The hatch marks represent portions of DNA not included on this picture. The black boxes below the restriction maps represent the coding regions of the exons, with diagonal lines depicting the location and size of the introns. The 5' end of the mRNA is on the left. (A) The horizontal line represents the *C. briggsae* genomic DNA insert of clone pDP#DH53. The distance between the first *Hin*DIII site and the second *Xba*I is ~7.0 kbp. The numbered arrows show the location of the primers used for RT-PCR and 5' RACE (1, DHB.2; 2, DHB.5; and 3, DHB.4). (B) The horizontal line represents the *C. remanei* genomic DNA insert of clone pDP#DH161. The distance between the first *Sst*I site and the *Hin*dIII site is ~5.7 kbp. The distance between the last *Sst*I site and the *Bam*HI site is ~3.5 kbp.

A

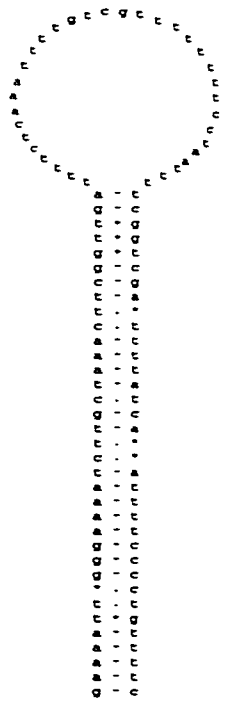


Figure 12. Possible hairpin loop in the 3'UTR of *Ce-fem-2* and 3'UTR deletions. (A) Secondary structure was predicted using the MulFold program. Dashes represent proper A-T, G-C base pairing, + represents G-T (G-U) base pairing while dots represent non-pairing. * signifies spaces in the sequence, resulting in minor loops. (B) Sequence of the *fem-2* 3'UTR. Regions underlined are those deleted in pDP#DH153 (one line), and pDP#DH154 (both one and two lines).

B

taactgcttttcggtggaaattttgcctgaaaattgggaaaattcttgct 50

aaacttcggttgattttctcaaatttttgtcgtttttttttcctaattttc 100

ggtcgattttatcaaatttttcccctgttttcctgtgtctatttgttttttt 150

gggtattccaattttctctctcctattcaccatatcgtcgtcatttctcc 200

ccgttatctagcagctttccgtgacccacacacatattttgttctcggtg 250

ttcctgtagcatttcacatttacatacgtttccaaccagttggcatccca 300

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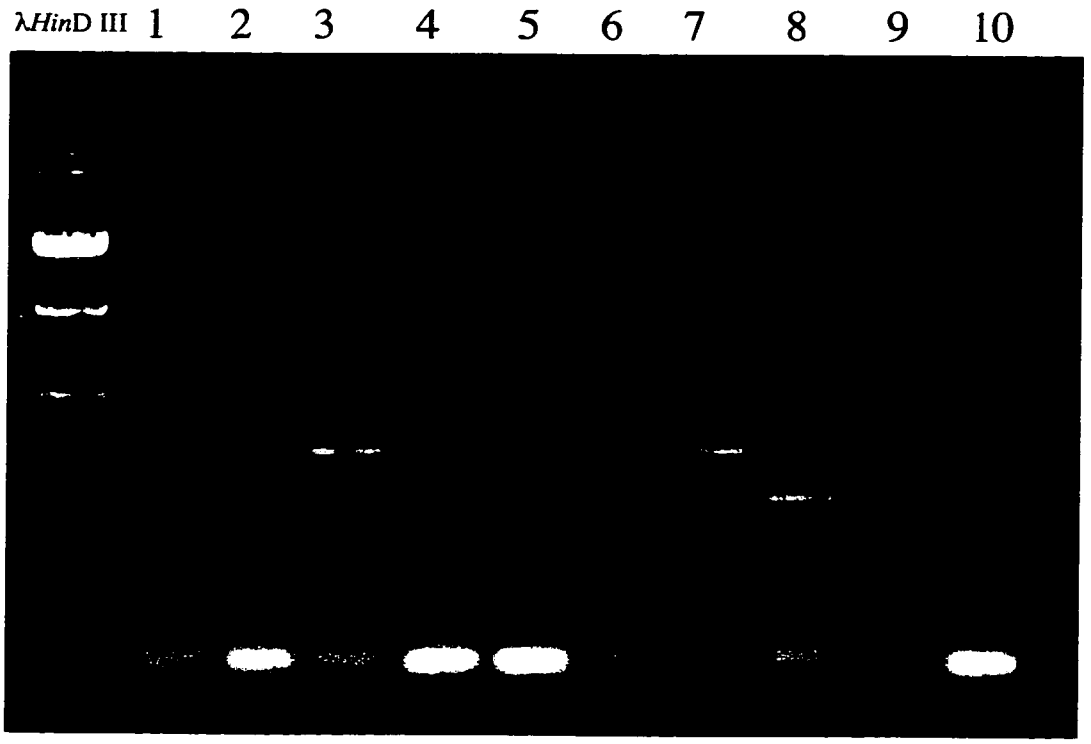
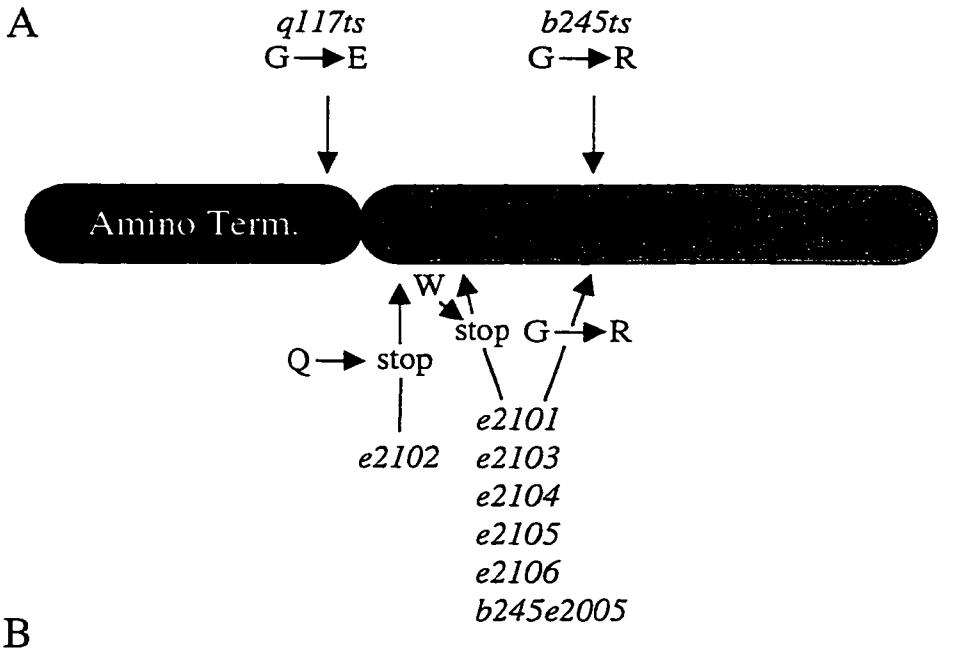


Figure 13. PCR analysis of putative deficiencies for the *fem-2* genomic region. DNA was prepared from embryos that were wild type for *fem-2* (lanes 1, 4, 7-10), homozygous for *wcDf1* (lanes 3 and 6), or homozygous for *sDf124* (lanes 2 and 5). Three primer sets were used to PCR amplify the DNA. Two sets correspond to *fem-2* (SAD2-DHA19 and DHA4-DHA17), whereas the third set amplifies a region of the unlinked *unc-119* gene (MMA1-MMA2). Lanes 1-3 show amplified products of the primer sets DHA4-DHA17 and MMA1-MMA2 with wild-type, *sDf124* and *wcDf11* embryos. Lanes 4-6 show amplified products of two primer sets (SAD2-DHA19 and MMA1-MMA2) with the three types of embryos. Lanes 7-9 show the bands amplified from each of the three primer sets alone on wild-type DNA (MMA1-MMA2, DHA4-DHA17 and SAD2-DHA19 respectively). Lane 10 contains the DNA amplified in a reaction containing only one primer from each of the three sets (DHA4, MMA1, SAD2). Each lane represents the PCR results of a single embryo.



B

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MEKVNEERDAVFEDHIGDRRRSVRSLLEEAFADEMEKTSYDVEVAD     46

TPQPHIPIRFRHPPIAGPVHDVFGDAIHDIFQKMMKRGQAVDFCHW     92

VSHLIATEIDEKFSEVAFRDVQYNPDIYVTDSTTEAKKLFNDKIWP    138

AIDKILQQNAETCPILSEKWSGIHVSGDQLKGQRHKQEDRFLAYPN    184
                  q117 ▲          ▲ e2102
GQYMDRGEDPISVLAVFDGHGGHECSQYAAGHLWETWLEVRKSRDP    230
                                          ▲ b245e2005
SDSLEDQLRKSLELLDERMTVRSVKECWKGGSTAVCCAIDMDQKLM    276

ALAWLGDSPGYVMSNIEFRQLTRGHSPSDEREARRVEEAGGQLFVI    322

GGELRVNGVLNLTRALGDVPGRPMISNEPETCQVPIESSDYLVLLA    368
                  ▲ b245
CDGISDVFNERDLYQLVEAFANDYPVEDYAELSRFICTKAIEAGSA    414

DNVSVVIGFLRPPQDVWKLMKHESDDEDSDVTDEE               449
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Figure 14. Locations of molecular lesions associated with *fem-2* alleles. (A) Cartoon depicting the approximate locations of lesions relative to amino terminal and phosphatase domains. Only the *q117* mutation is located in the amino terminus. The amino acid substitutions associated with *b245* and *q117* alleles are shown. *e2101-6* and *b245e2005* all contain premature stop codons. *e2101*, *e2103-6* and *b245e2005* all contain stop codons at the same location, as well as an amino acid substitution identical to that associated with *b245*. (B) Amino acid sequence of FEM-2 depicting the location of the molecular lesions associated with *fem-2* alleles. Black arrow heads point to the locations of the lesions.

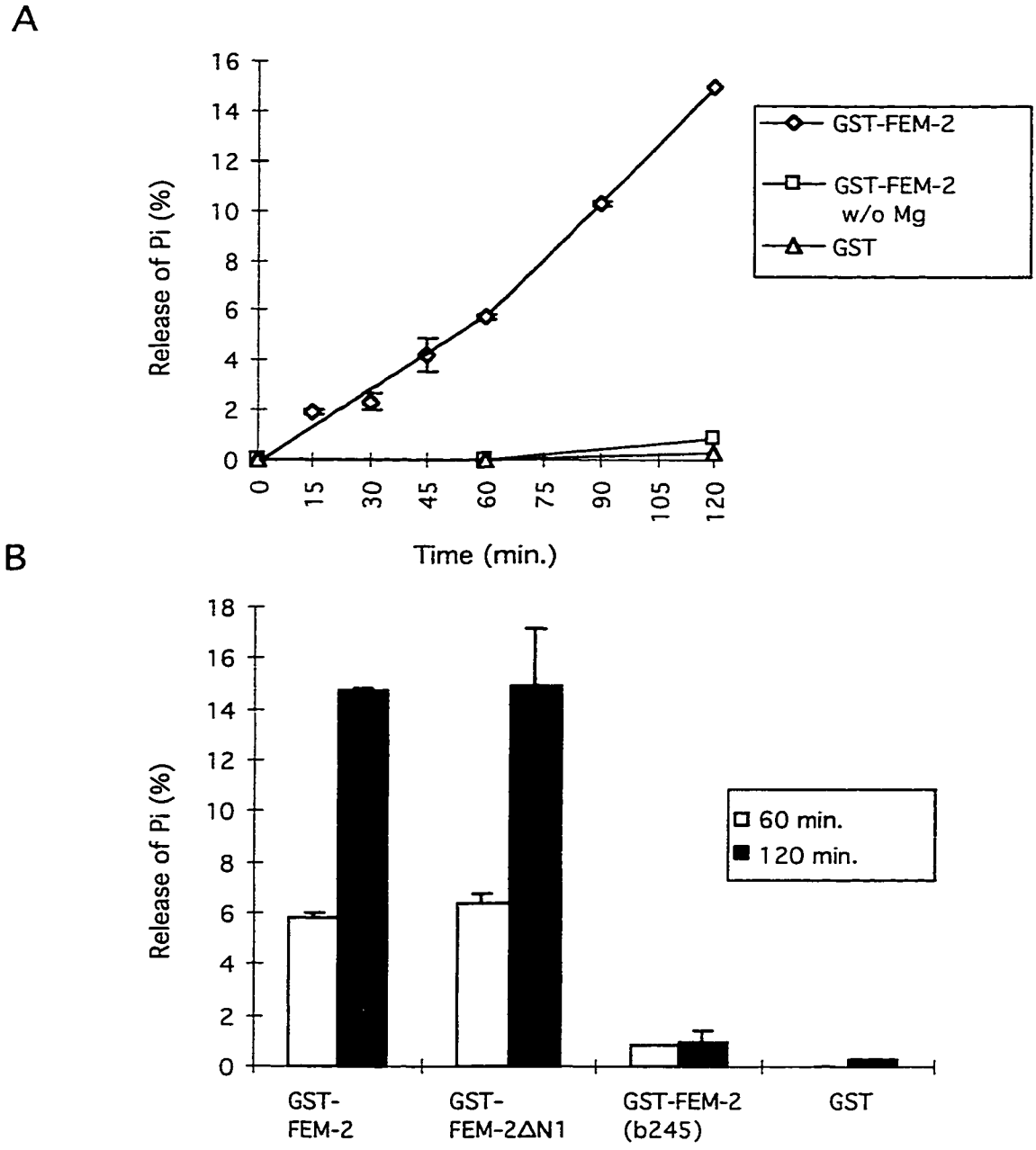

Figure 15. *in vitro* phophatase assay. GST-FEM-2 fusion proteins were purified from *E. coli* and assayed for casein phophatase activity as described in the Methods. (A) Phosphatase activity vs. time for GST-FEM-2 in the presence and absence of 50 mM Mg^{+2}. GST itself exhibits no phosphatase activity in this assay. Each point is the average of at least two independent experiments. Bars represent the range of the results of the independent trials. (B) Phosphatase activity at 60 and 120 minutes for GST-FEM-2, GST-FEM-2ΔN1 and GST-FEM-2(b245). Bars represent the range of the results of the independent trials.

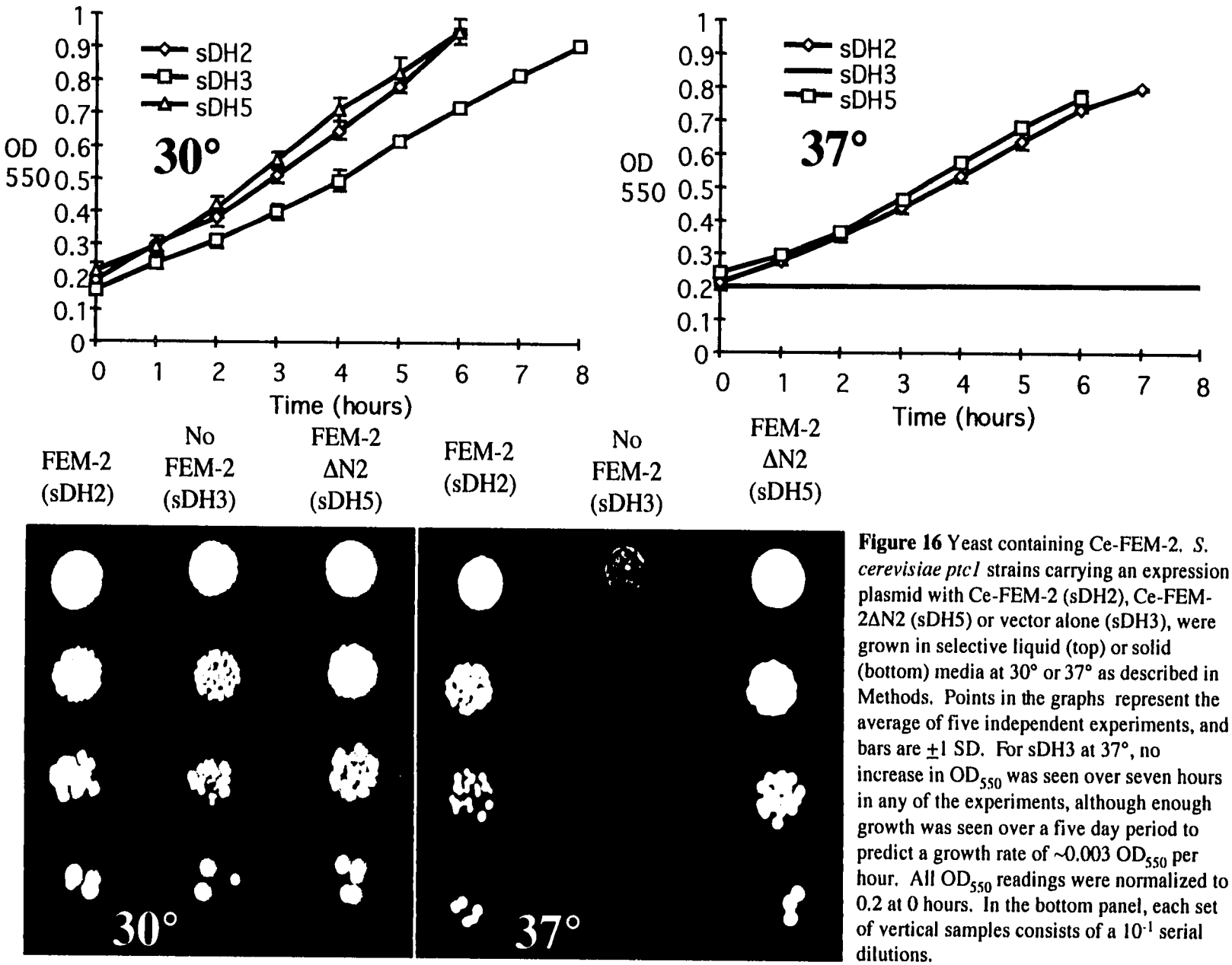

Figure 16 Yeast containing Ce-FEM-2. *S. cerevisiae ptc1* strains carrying an expression plasmid with Ce-FEM-2 (sDH2), Ce-FEM-2ΔN2 (sDH5) or vector alone (sDH3), were grown in selective liquid (top) or solid (bottom) media at 30° or 37° as described in Methods. Points in the graphs represent the average of five independent experiments, and bars are ±1 SD. For sDH3 at 37°, no increase in OD_{550} was seen over seven hours in any of the experiments, although enough growth was seen over a five day period to predict a growth rate of ~0.003 OD_{550} per hour. All OD_{550} readings were normalized to 0.2 at 0 hours. In the bottom panel, each set of vertical samples consists of a 10^{-1} serial dilutions.

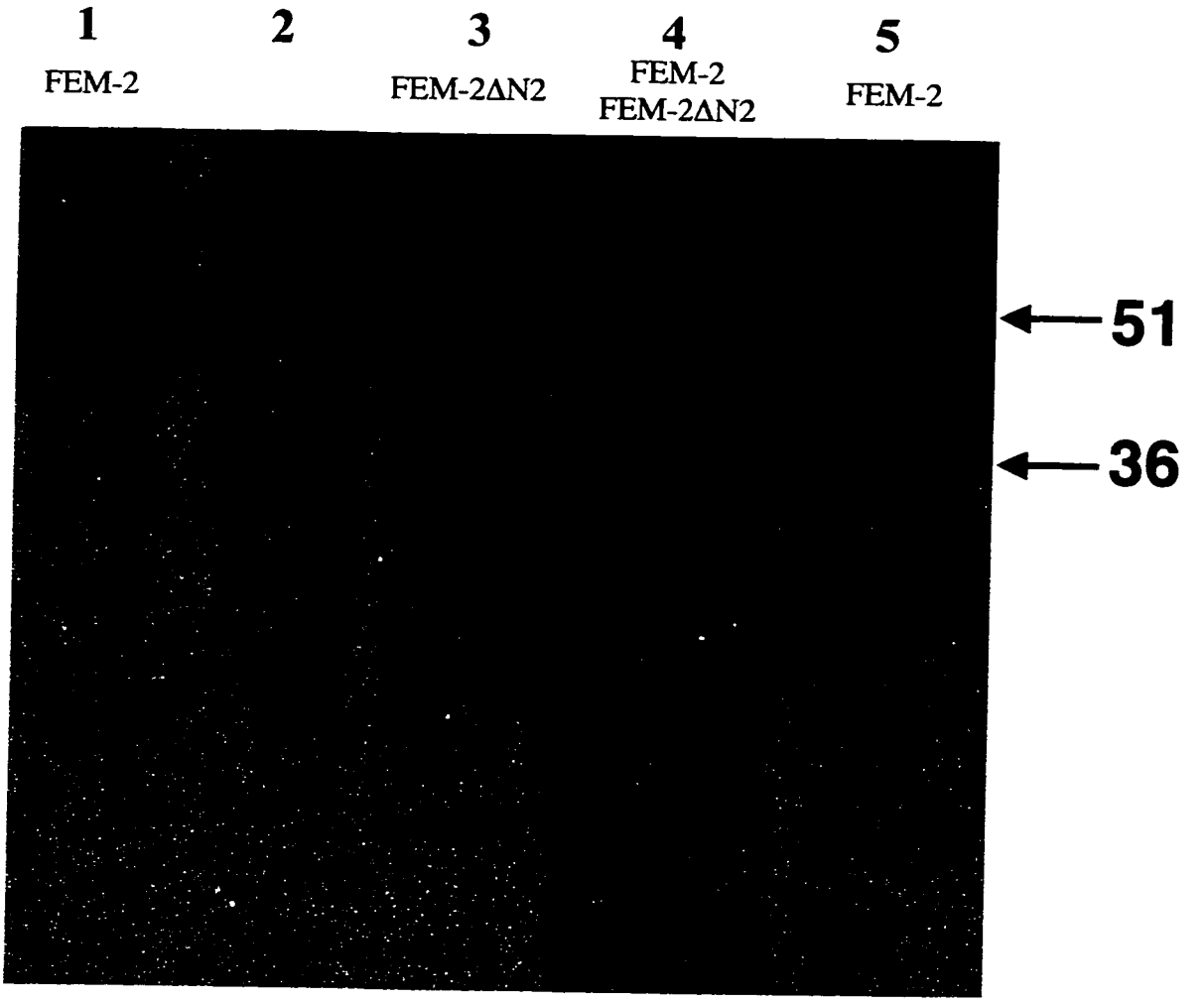

Figure 17. Western blot of yeast and worm protein extracts. Lanes 1-3 are yeast protein extracts while lanes 4 and 5 are worm extracts. Proteins were detected using a polyclonal antibody raised against bacterially expressed Ce-FEM-2. Lane 1. sDH2 which expresses full length Ce-FEM-2. Lane 2. sDH3 which does not express FEM-2. Lane 3. sDH5, which expresses Ce-FEM-2DN2. Lane 4. DP151 which expresses wild-type Ce-FEM-2 and Ce-FEM-2DN2 . Lane 5. DP51 which only expresses Ce-FEM-2.

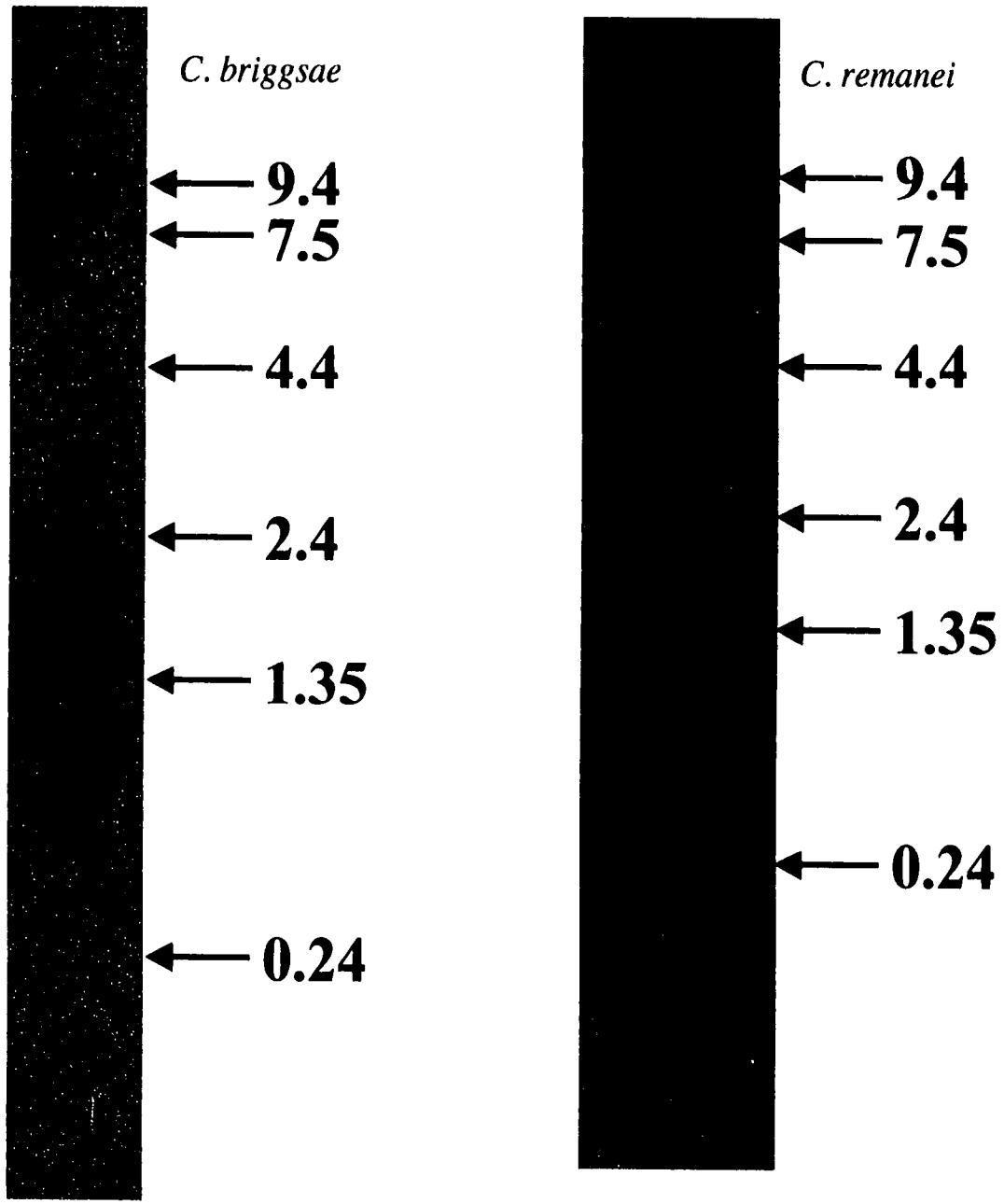

Figure 18. Northern blots of *C. briggsae* and *C. remanei* mRNA. The numbers represent relative location of RNA size standards in kb. For *C. briggsae*, ten micrograms of total RNA was hybridized with a ^{32}P-labeled probe generated from a *Cb-fem-2* RT-PCR product. Hybridization occurs only at a molecular weight of ~1.9 kb. For *C. remanei*, five micrograms of total RNA was hybridized with a ^{32}P-labeled probe from pDP#PSr1. Hybridization occurs at ~1.8 kb in size.

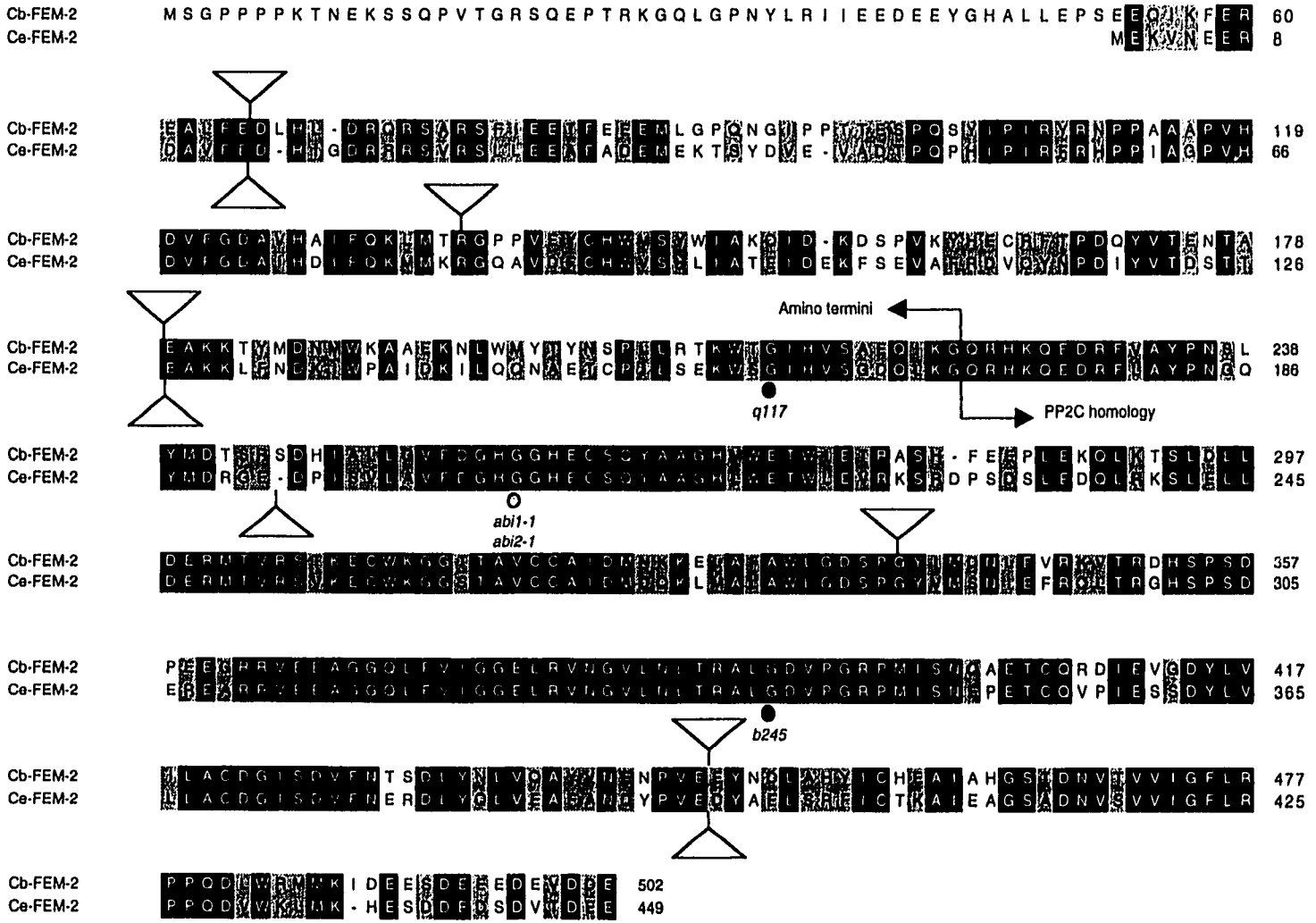

Figure 19. Alignment of amino acid sequence of Cb-FEM-2 and Ce-FEM-2. White letters on a black background identify identical amino acids while black letters on a gray background identify similar amino acids. Sequences were compared using the program ALIGN. Triangles show the locations of the predicted introns. Black circles show the locations of the lesions associated with the two temperature sensitive alleles of *Ce-fem-2* . The gray circle shows the location of the lesion associated with the Arabidopsis mutations *abi1-1* and *abi2-1* in the FEM-2 homologues (Leung *et al* . 1994; Leung, Merlot and Giraudat 1997; Meyer, Leube and Grill 1994).

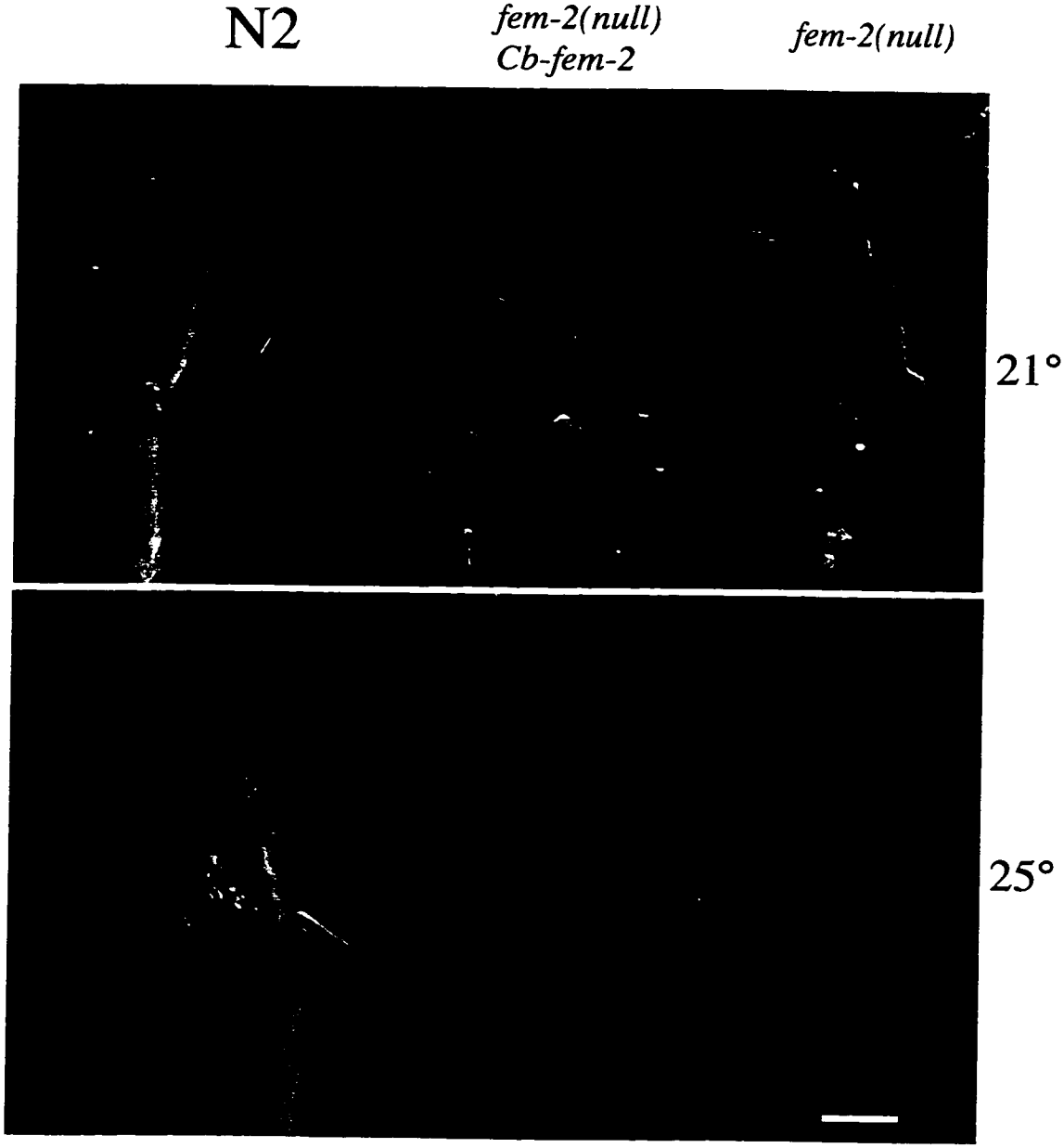

Figure 20. Comparison of the male tail in *Ce-fem-2(e2105)* and wild-type worms. The top panel shows worms grown at 21°, while those in the bottom panel were grown at 25°. The animals on the left are XO wild-type (N2), while those in the middle and on the right are m-z-*fem-2(e2105)* XO animals (the tails of XX and XO m-z-*fem-2(e2105)* animals are indistinguishable at 25°, therefore the bottom right animal could be XX or XO). The animals in the middle are also carrying a transgenic array with *Cb-fem-2*. Bar, 10 μm.

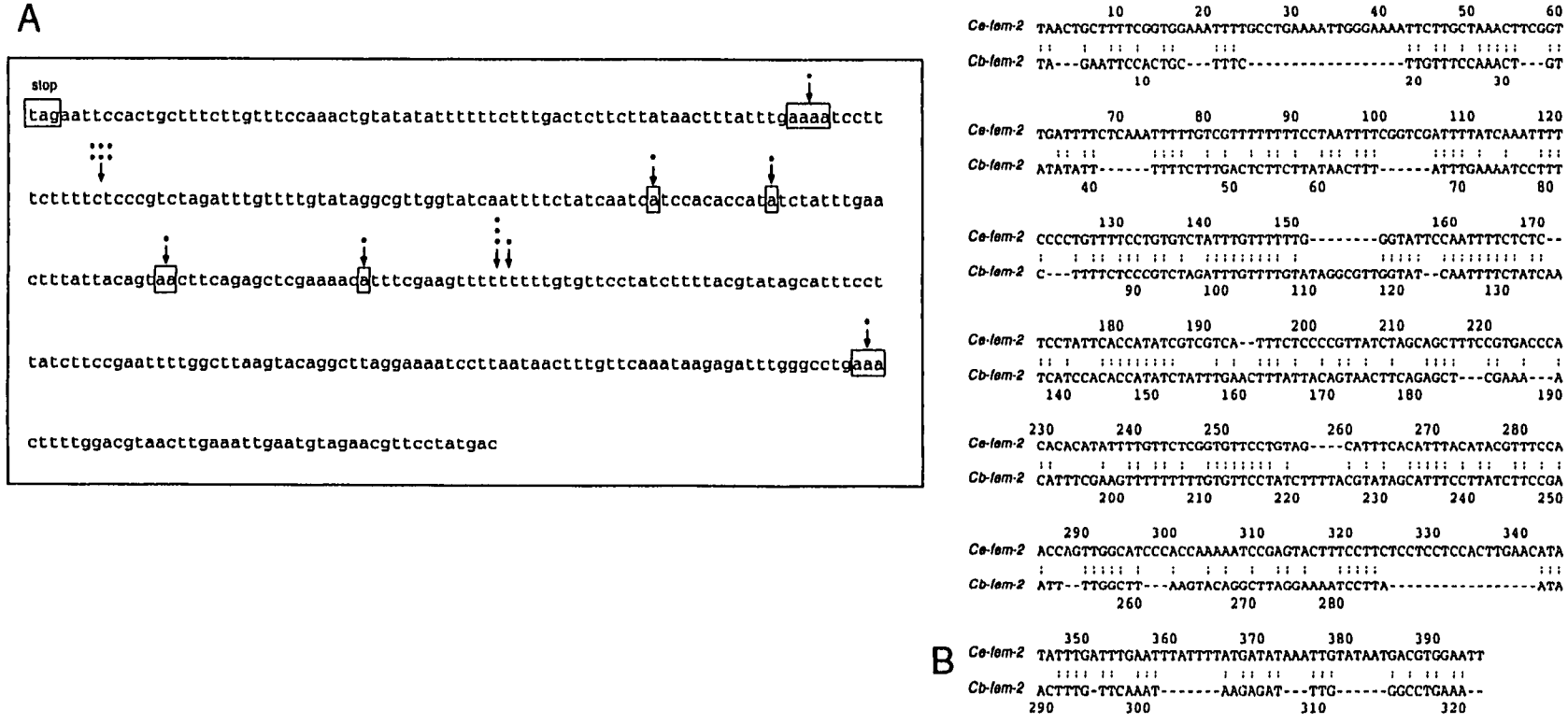

Figure 21. Location of polyadenylation sites in the *Cb-fem-2* 3'UTR and comparison of the 3'UTR sequences from *Ce-fem-2* and *Cb-fem-2*. (A) The sequence of the *Cb-fem-2* genomic DNA corresponding to the region of the 3'UTR. The first three bases constitute the stop codon at the end of the coding region. The arrows mark the locations of polyadenylation as seen through cloning and sequencing of 3'RACE products. The dots above the arrows represent the number of clones showing polyadenylation at any given location. In certain regions, the exact location of polyadenylation could not be confirmed because it occurred beside 'a' residues. These locations are marked by boxes, with polyadenylation occuring somewhere within the boxes. (B) A comparison of the 3'UTRs from *C. elegans* and *C. briggsae*. Two dots between bases represents identity. The comparison was performed using the ALIGN program at http://vega.igh.cnrs.fr/bin/align-guess.cgi. The overall identity, as scored by this program, is 48%.

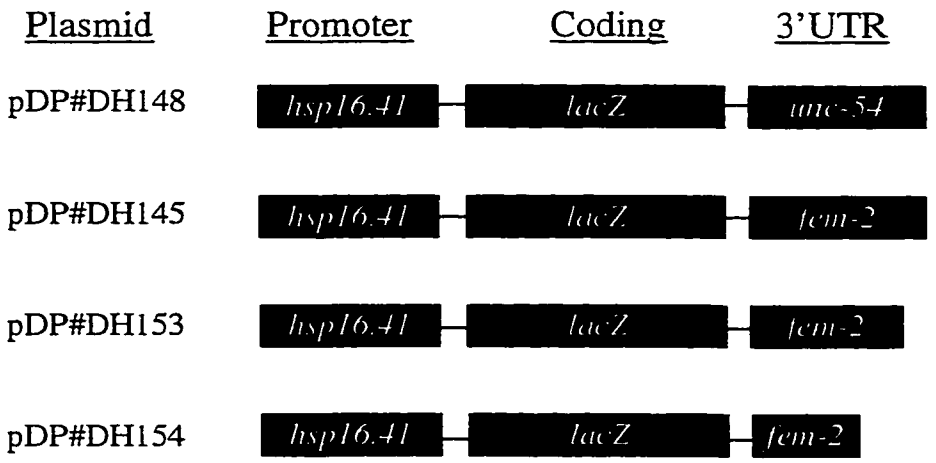

Figure 22. Cartoons depicting the composition of the plasmid constructs used in the heat-shock experiment. All of the plasmids contain the *hsp16.41* heat-shock promoter and *lacZ* coding region, but all differ in their 3'UTR. pPD#DH148 has the 3'UTR from the *unc-54* gene. pPD#DH145 contains the full length *fem-2* 3'UTR, while pDP#DH153 and pDP#DH154 contain deleted forms of the *fem-2* 3'UTR.

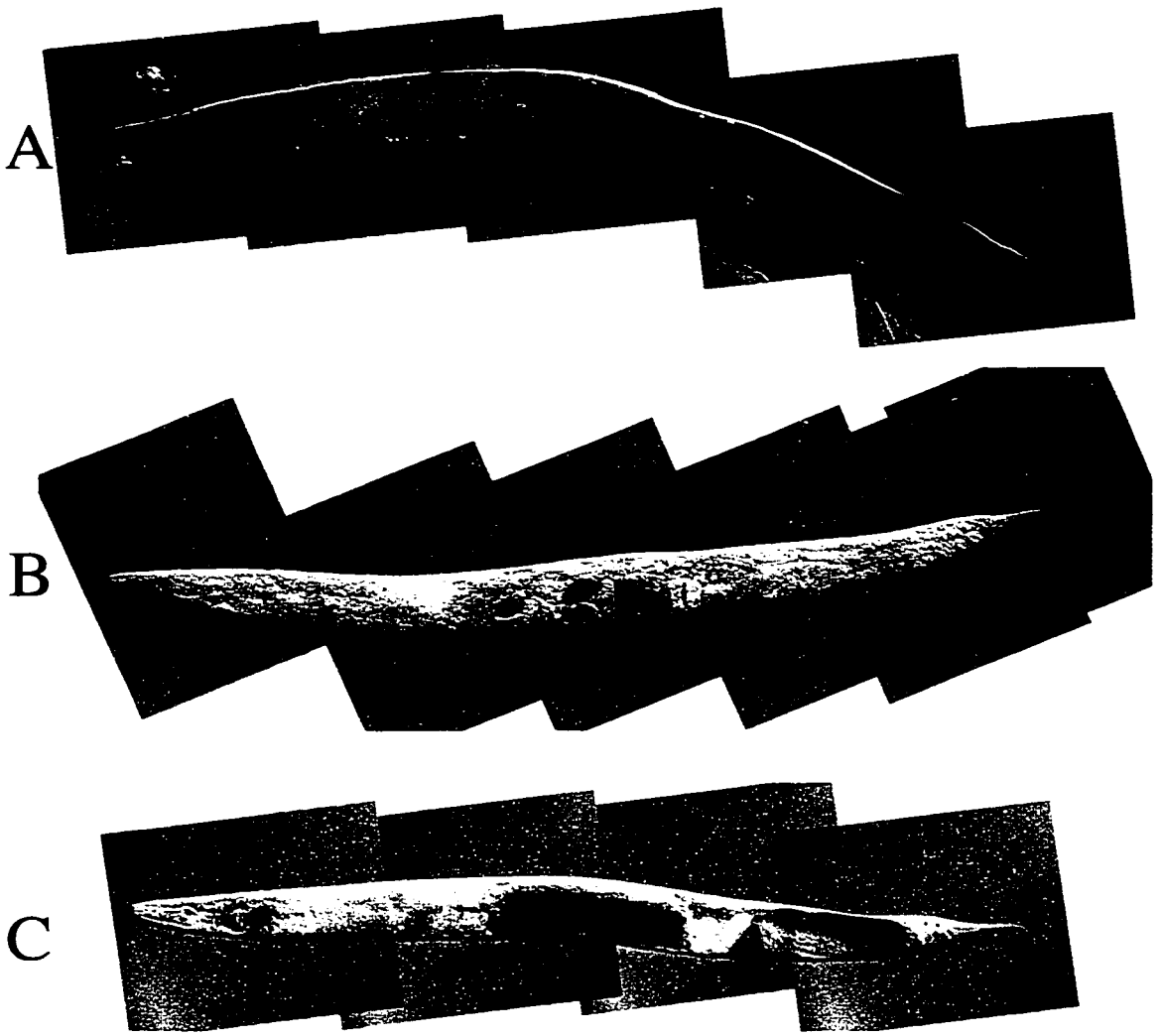

Figure 23. Typical ß-GAL expression seen in heat shocked worms carrying constructs varying in 3'UTR. All three animals carry transgenic arrays that have a plasmid with the hsp16.41 heat shock promoter (Stringham *et al.*, 1992), driving the *lacZ* gene. The plasmids in **A**, **B** and **C** differ in the 3'UTR. **A** has the *unc-54* 3'UTR (pDP#DH148), while **B** has the entire *fem-2* 3'UTR (pDP#DH145), and **C** has the deleted form (pDP#DH154).

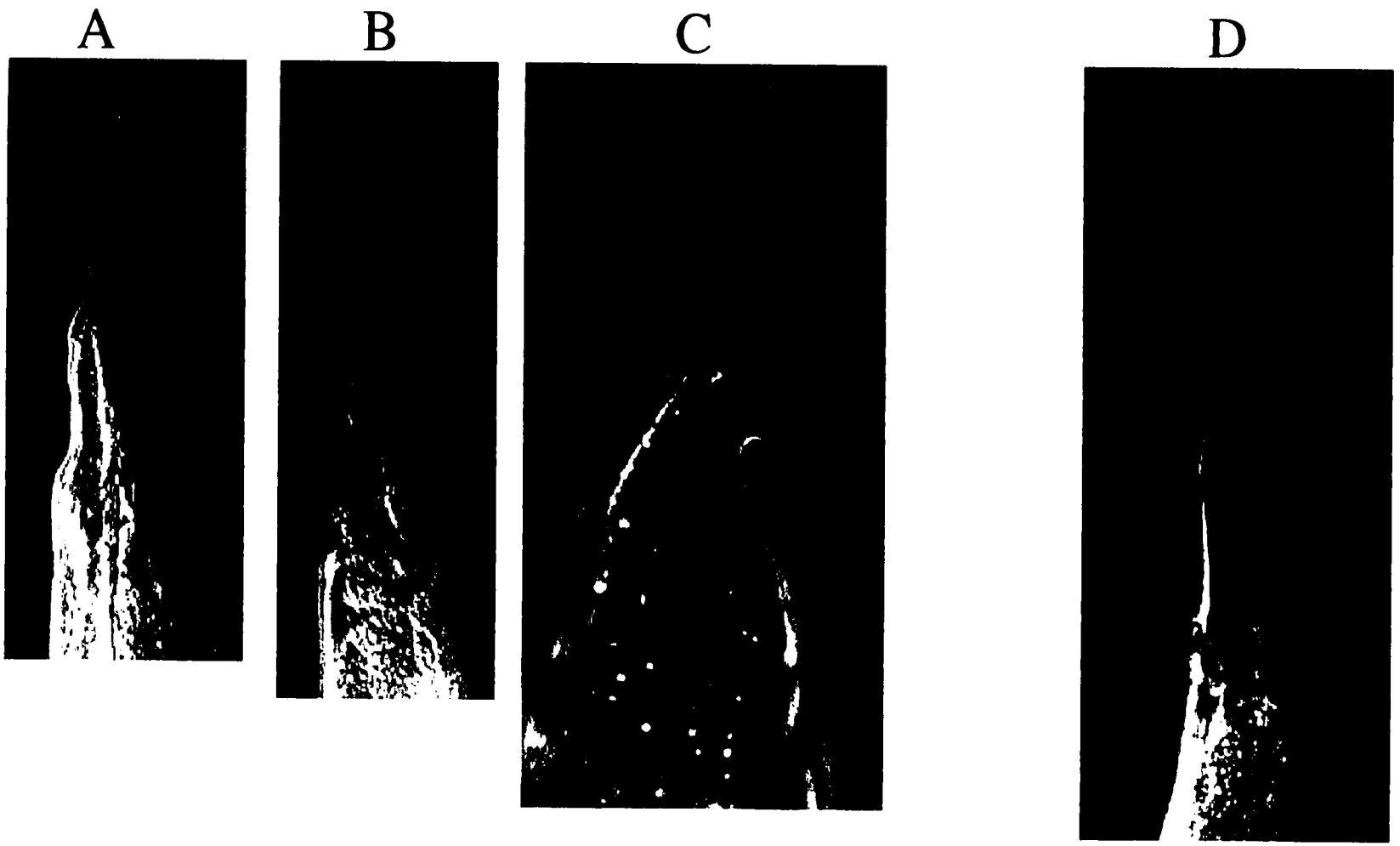

Figure 24. Underdeveloped tail spikes with *fem-2* 3'UTR deleted constructs. XX N2 animals are carrying arrays with either a plasmid with part of *fem-2* 3'UTR deleted (pDP#DH163; A,B and C) or a wild-type copy of *fem-2* (pDP#DH11; D). Above are typical examples of the tails seen in worms carrying these arrays. A,B and D are at 400X magnification while C is at 1000X.

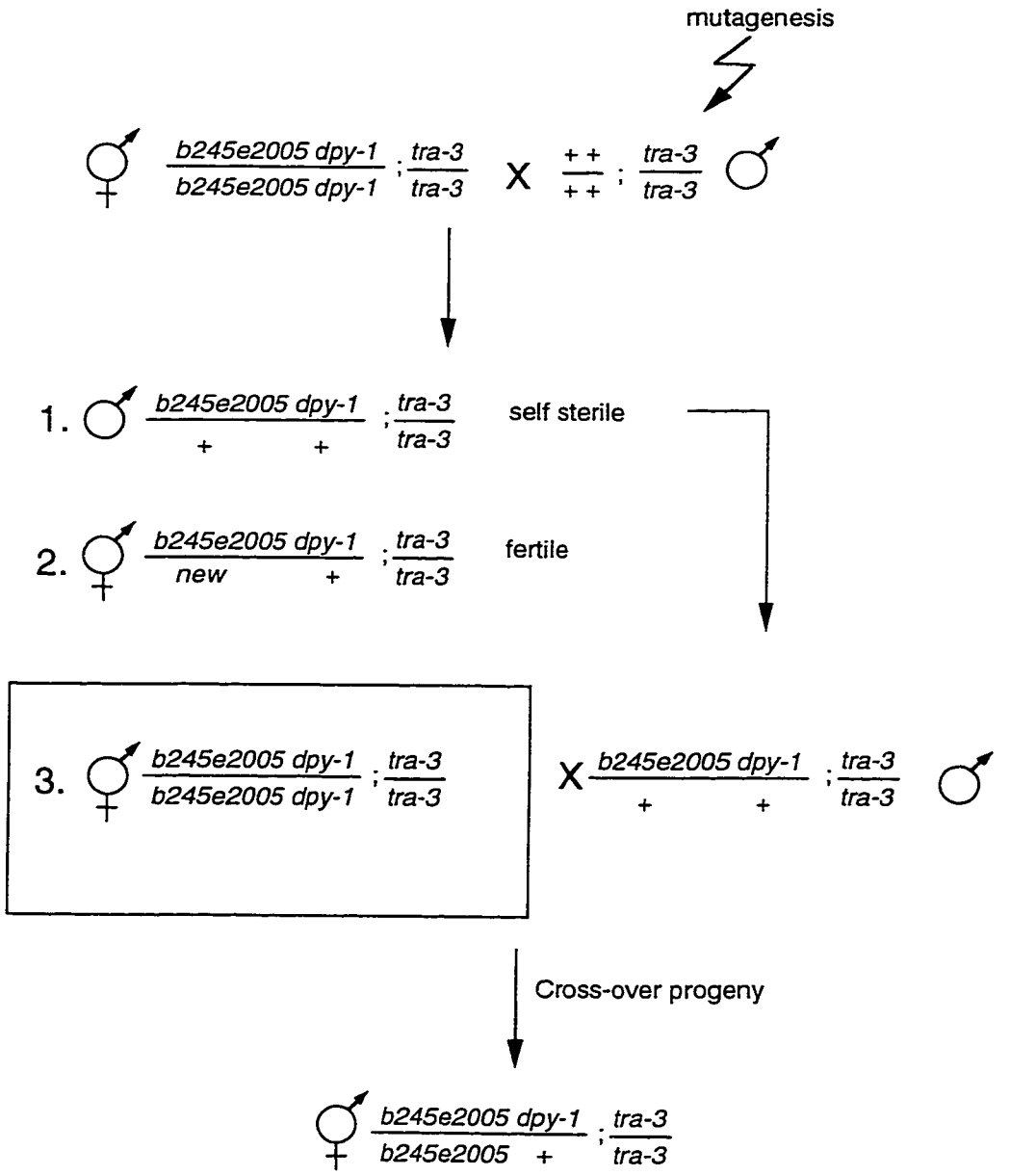

Figure 25. Re-isolation of *b245e2005* animals. Hodgkin (1986) predicted two possible offspring resulting from the cross of mutagenized males with *b245e2005 dpy-1; tra-3* hermaphrodites. These are shown as numbers 1 and 2. 1 result if no new *fem-2* mutation occurs, resulting in self-sterile males. If a new *fem-2* mutation does occur, then 2 results, which are self-fertile non-Dpy hermaphrodites. A third possible outcome results if outcrossing does not occur in the original cross, resulting in self-fertile Dpy hermaphrodites capable of mating with group 1 animals. Since *fem-2* and *dpy-1* are ~8 map units apart (Hodgkin, 1986), a cross-over could occur, allowing for the *b245e2005* allele to be linked to a wild-type copy of *dpy-1*. The resulting progeny of this cross are non-Dpy hermaphrodites, identical in appearance to the progeny of group 2 animals.

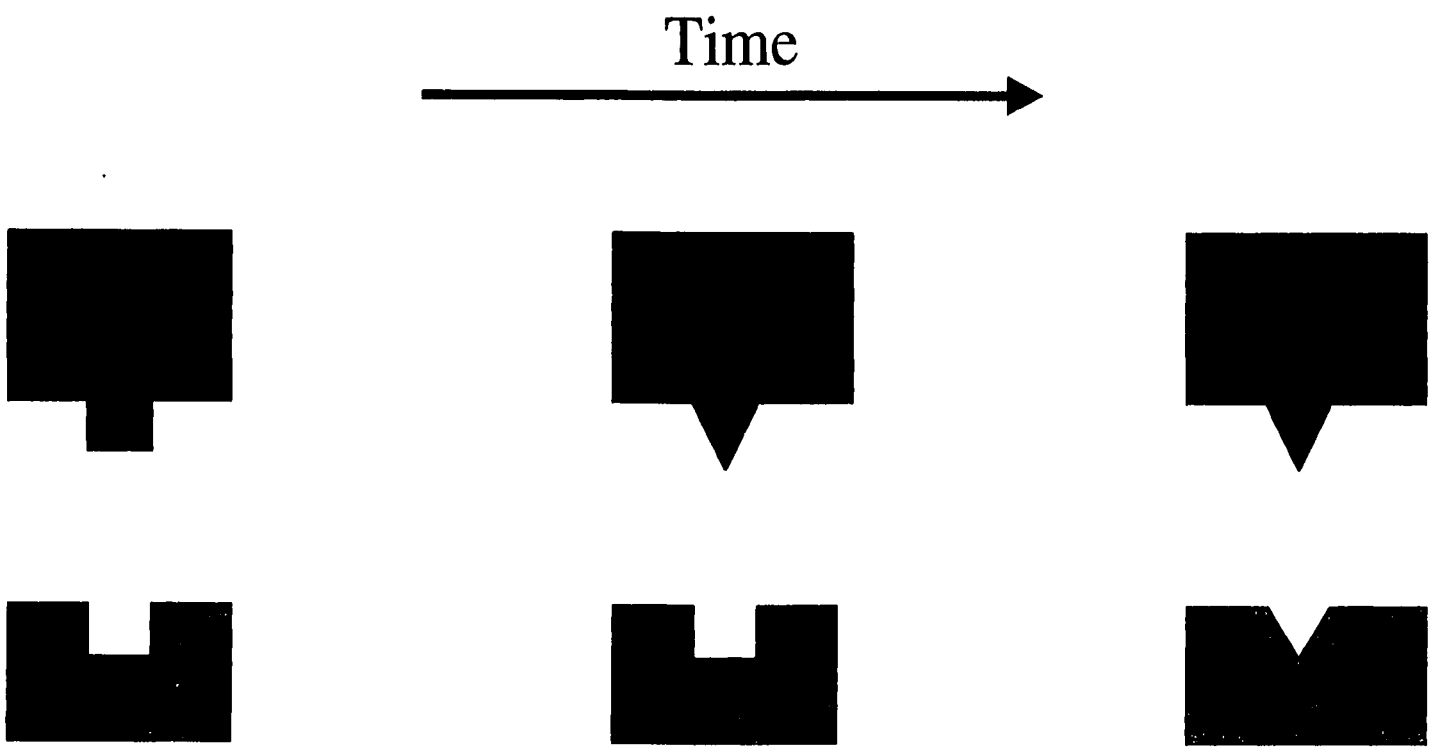

Figure 26. Model of evolution of interacting proteins. The black and gray boxes represent proteins that are evolving over time. Amino acid change is represented by a change in the conformation of the binding site. The top protein changes first, then puts pressure on the bottom protein to change in order to maintain a strong interaction.

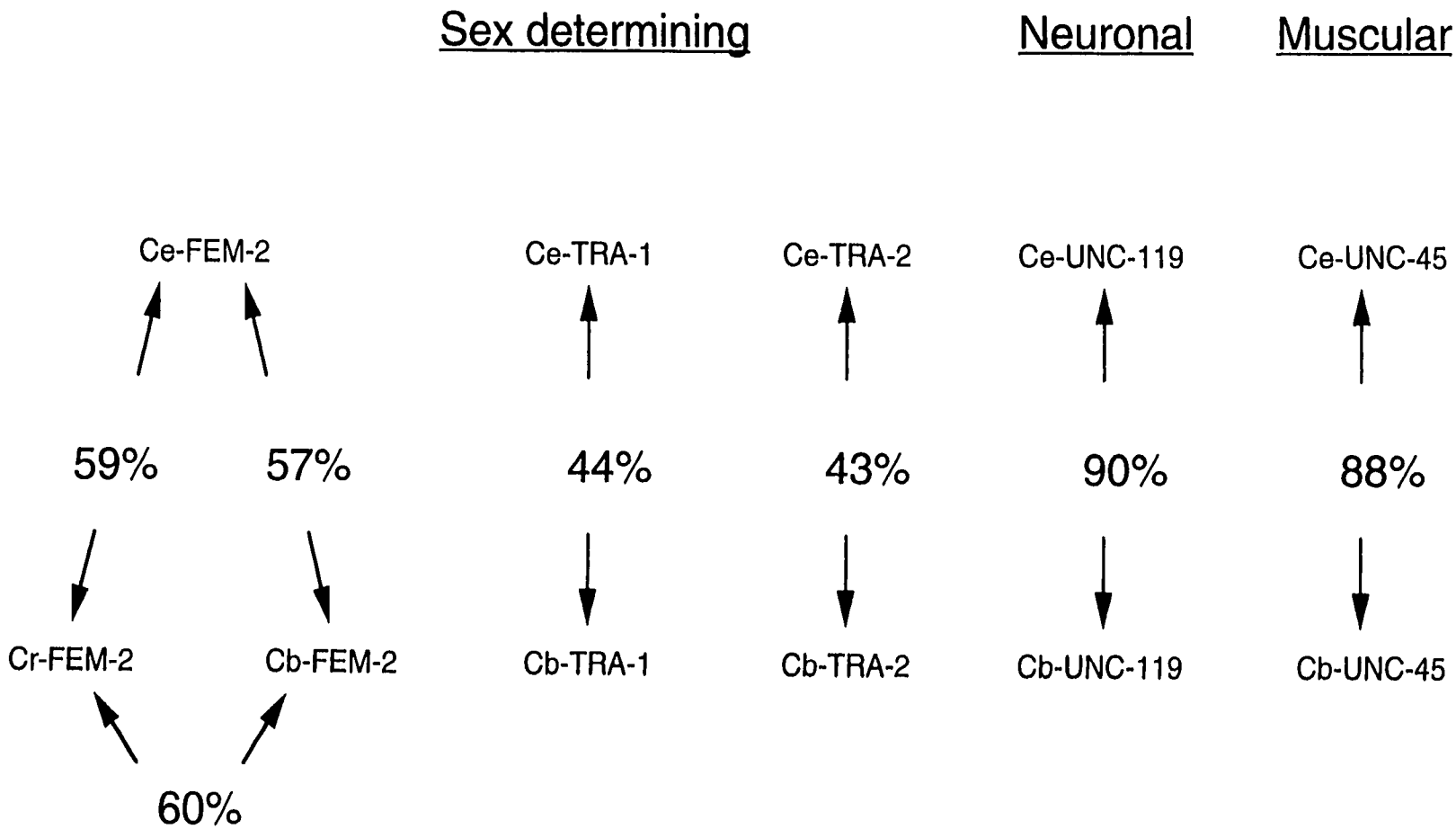

Figure 27. Summary of amino acid identity between homologues. Cb=*C. briggsae;* Ce=*C. elegans*; Cr=*C. remanei.* Per cent identity between homologues is shown for three sex determination proteins (FEM-2, TRA-1 and TRA-2), a neural protein (UNC-119) and a muscle protein (UNC-45). The non-sex determining proteins are examples of typical per cent identities for proteins involved in other developmental processes. The references for these examples are FEM-2=Hansen and Pilgrim, 1998 and Stothard, Hansen and Pilgrim, manuscript in preparation; TRA-1=de Bono and Hodgkin, 1986; TRA-2=Kuwabara, 1996; UNC-119=Maduro and Pilgrim, 1996; UNC-45=Venolia *et al.*, 1999.

Allele	XX 15°	XX 20°	XX 25°	XO 15°	XO 20°	XO 25°	Reference
b245	wild type	N/A	no sperm production	wild type	N/A	few sperm then oocytes intersex gonad	Kimble et al., 1984
q117	wild type	N/A	no sperm production	wild type	N/A	few sperm then oocytes intersex gonad	personal communication (Kimble); personal observation
b245e2005 *m-z-*	no sperm	no sperm	no sperm	partly feminized males; no sperm	intersex soma; no sperm	fertile female	Hodgkin, 1986
e2105 *m+z-*	wild type but fewer sperm	wild type but fewer sperm	wild type but fewer sperm	wild type	wild type but fewer sperm	sterile male; makes dysfunctional sperm	Hodgkin, 1986
e2105 *m-z+*	wild type	wild type	wild type	wild type	wild type	wild type	Hodgkin, 1986
e2105 *m-z-*	no sperm	no sperm	no sperm	partly feminized males; no sperm	intersex soma; no sperm	fertile female	Hodgkin, 1986

Table 1. A summary of the phenotype of some of the *fem-2* mutant alleles at various temperatures. Animals that are homozygous for the mutation, but whose mother was heterozygous, are signified by *m+z-*. Animals who are homozygous for the mutation and whose mothers were also homozygous for the mutation are signified by *m-z-*.

Primer name	Primer Sequence
AMC1	5' ACCTCGACGTCATAGCTAGT 3'
DHA3	5' CAAAGATCTTGTCCCACCGAAGCCGGTAGTGG 3'
DHA4	5' CGGTCTAGATGACGACGATATGGTGGATAG 3'
DHA9	5' CGTCTAGAAGCTCCAGCGAGCTTGCGAAGC 3'
DHA17	5' CCGAGCACTCAGAGATGTTCC 3'
DHA19	5' CGATGGTCACGGTGGTCACGAG 3'
DHA22	5' CGACAAGATCTTCCAGCAAAACGC 3'
DHA23	5' GGATCAGATCTGCGATCGCCGTC 3'
DHB.2	5' CGGATGGGAATGTATGACTGTGG 3'
DHB.4	5' GGTGGACGGAGGAATCCAATGACG 3'
DHB.5	5' CCACAGTCATACATTCCCATCCG 3'
DH3.1	5' GTCGACGCGGCCGCTGCTTTTCGGTGGAA 3'
DH3DEL	5' GCTTTTCGGTGGAAATTTTGCCTCTAGACTCAAATTTTT GTCGTTTTTTTTCCTA 3'
DH3.2	5' AGATCTGCGGCCGCCCAGTAATTCCACGTC 3'
MMA1	5' AGTCGGCCTTATTGTGCATTAC 3'
MMA2	5' AAATTGCATGCCAGCACCGGTC 3'
PJA1	5' AATTCGTCGACG 3'
PJA2	5' TCTAGATCTCGAGAGAGAAAATTGGAATACCC 3'
PJA3	5' TCTAGATCTCGAGATGGAAAAAGTAAACGAGGAG 3'
SAD2	5' AGAAATTCCATCACAAGCCAG 3'
3AvrII	5' CGATTTTATCAAATTTTCCCCTGTTTTCCTAGGCATTTCT CCCCGTTATCTAGCAGCTTTC 3'

Table 2. A list of DNA primers used in this study

Table 3. A list of plasmid constructs used in this study. Plasmid constructs are listed along with a brief description of the relevant features of the construct and its use in this study.

Plasmid	Brief Description	Purpose
pDBL	Yeast 2μ expression vector with the ADH1 promoter.	Used to express proteins in yeast.
pDP#DH1	*fem-2* cDNA (c8) in pDBL yeast vector. Has ADH1 promoter.	Used to constitutively express FEM-2 in yeast strain TM126.
pDP#DH11	Genomic *fem-2* clone containing the entire rescuing region	Used to rescue *fem-2* animals as transgenic array and used as the basis for many *in vitro* mutagenized. plasmids.
pDP#DH14	*fem-2* cDNA cloned into pGEX-Sal, which has a GST module attached for purification.	Used to express GST-FEM-2 in *E. coli.*
pDP#DH19	*fem-2* cDNA with *b245* mutation cloned into pGEX-Sal, which has a GST module attached for purification.	Used to express GST-FEM-2(*b245*) in *E. coli.*
pDP#DH28	pDP#DH27 digested with *Bgl* II, the insert removed, and religated.	Expresses FEM-2 protein lacking a large portion of the amino terminus
pDP#DH29	*fem-2* cDNA (c8), with sequence coding for amino terminus removed, in pDBL yeast vector. Similar to pDP#DH1.	Used to constitutively express FEM-2 lacking the amino terminus in yeast strain TM126.
pDP#DH42	*Cb-fem-2* genomic clone obtained from mini-library.	Initial clone isolated that contains the *C. briggsae* homologue of *fem-2.*
pDP#DH53	Larger clone of *Cb-fem-2* containing the entire coding sequence as well as approximately five kb of promoter	Used for sequencing of *Cb-fem-2* and partial rescue of *C. elegans fem-2* worms.
pDP#DH96	Same as pDP#DH11 but with deletion in 3'UTR using primer 3AvrII	Used to determine if 3'UTR is involved in regulation of *fem-2*
pDP#DH113	5' RACE product of *Cb-fem-2* cloned into pGEM-T	Sequenced to determine cDNA sequence of *Cb-fem-2* and insert used as probe for *Cb-fem-2* Northern
pDP#DH126	Same as pDP#DH11 but with deletion in 3'UTR using primer DH3DEL	Used to determine if 3'UTR is involved in regulation of *fem-2*
pDP#DH145	16-41 heat shock promoter driving *LacZ* expression with *fem-2* 3'UTR at end of transcript	Used to determine if the *fem-2* 3'UTR is capable of influencing expression.
pDP#DH148	16-41 heat shock promoter driving *LacZ* expression with *unc-54* 3'UTR at end of transcript.	Used as a control for expression seen with pDP#DH145. *unc-54* 3'UTR should not influence expression.
pDP#DH150	Genomic clone containing part of the *Cr-fem-2* gene.	Used to sequence the *fem-2* homologue from *C. remanei.*

pDP#DH153	16-41 heat shock promoter driving *LacZ* expression with *fem-2* 3'UTR with small deletion at end of transcript	Used to determine if the *fem-2* 3'UTR is capable of influencing expression. Same 3'UTR as pDP#DH126
pDP#DH154	16-41 heat shock promoter driving *LacZ* expression with *fem-2* 3'UTR with large deletion at end of transcript	Used to determine if the *fem-2* 3'UTR is capable of influencing expression. Same 3'UTR as pDP#DH163
pDP#DH160	Genomic clone containing part of the *Cr-fem-2* gene.	Used to sequence the *fem-2* homologue from *C. remanei.*
pDP#DH163	Same as pDP#DH11 but with deletion in 3'UTR including pDP#DH126 and pDP#DH96 deletions and in between	Used to determine if 3'UTR is involved in regulation of *fem-2*
pDP#DH161	Genomic clone containing all of the *Cr-fem-2* gene.	Used to obtain a clone containing the entire *Cr-fem-2* gene for future experiments
pDP#PJB1	*fem-2* cDNA, but lacking sequence for first 87 amino acids, cloned into pGEX-Sal.	Used for testing phosphatase activity of FEM-2 which lacks part of the amino terminus
pDP#PSr1	RT-PCR product of *Cr-fem-2.* Gift of Paul Stothard	Insert used to probe mini-libraries to obtain genomic clone of *Cr-fem-2*
pPC16.41	16-41 heat shock promoter driving *LacZ* expression with a polylinker for insertion of 3'UTR sequences	Used to test various 3'UTR sequences for their ability to influence expression.

Strain Name	Genotype
DP51	*fem-2(e2105) unc-45(r450ts)/ sC1(s2073) [dpy-1(s2171)]; him-8(e1489)*
DP53	*fem-2(e2105) unc-45(r450ts)/ sC1(s2073) [dpy-1(s2171) (s2683l)]; him-8(e1489)*
DP59	*unc-45(r450ts) sDf124(s2670) + / sC1(s2073)[++dpy-1(s2171)]; him-8(e1489)*
DP202	N2 *edEx78* (pDP#DH11)
DP204	N2 *edEx80* (pDP#DH11)
DP205	N2 *edEx81* (pDP#DH11)
DP210	N2 *edEx86* (pDP#DH154)
DP212	N2 *edEx88* (pDP#DH154)
DP217	N2 *edEx93* (pDP#DH148)
DP218	N2 *edEx94* (pDP#DH148)
DP219	N2 *edEx95* (pDP#DH148)
DP220	N2 *edEx96* (pDP#DH148)
DP221	N2 *edEx97* (pDP#DH163)
DP222	N2 *edEx98* (pDP#DH163)
DP227	N2 *edEx103* (pDP#DH145)
DP228	N2 *edEx104* (pDP#DH145)
DP230	N2 *edEx106* (pDP#DH145)
DP231	N2 *edEx107* (pDP#DH153)
DP234	N2 *edEx110* (pDP#DH153)
DP236	N2 *edEx112* (pDP#DH153)

Table 4. A list of strains used in this study and their genotypes. Strains containing extrachromosomal arrays contain plasmid pRF4 (*rol-6(su1006dm)*) (Dramer *et al.*, 1990), as well as the plasmid listed (table 3).

3'UTR	Plasmid	Expression in tail	Expression in pharynx	Expression in soma (exc. phar)	Expression in embryos	'n' greater or equal to	number of lines used
unc-54	pDP#DH148	89%	100%	97%	99%	143	4
fem-2 (full)	pDP#DH145	2.4%	50%	3.2%	85%	125	3
fem-2 (small deletion)	pDP#DH153	33%	96%	51%	93%	70	3
fem-2 (large deletion)	pDP#DH154	60%	96%	73%	96%	49	2

Table 5. A summary of the results obtained using heat-shock vectors with various 3'UTRs. Worms carrying extrachromosomal arrays that contain plasmid constructs with the *hsp16.41* promoter, *lacZ* gene and test 3'UTRs were heat-shocked and scored for ß-gal expression. The first column is the 3'UTR used in the experiment and the second column is the plasmid that contains the respective 3'UTR. The next four columns show the per cent of animals showing ß-gal expression in various parts of the body. Expression in the soma refers to expression somewhere in the somatic tissue other than the pharynx, but including the tail. 'n' refers to the number of animals scored, while the last column is the number of lines carrying extrachromosomal arrays. Each array gave similar results for each respective plasmid, and roughly equal numbers of animals were counted for each array.

Plasmid	Per cent short tail	Number of animals	Number of lines
pDP#DH163 (deleted 3'UTR)	61%	75	2
pDP#DH11 (wt 3'UTR)	3.9%	77	3

Table 6. A summary of the number of animals possessing underdeveloped tails. N2 animals were either carrying an array with a wild-type copy of *fem-2* (pDP#DH11) or a copy with a deletion in the 3'UTR (pDP#DH163).

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