Identification of cis-acting elements and trans-acting factors responsible for her-1 regulation in Caenorhabditis elegans

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Thesis Abstract

Expression of the her-1 gene specifies male development in Caenorhabditis elegans. her-1 is negatively regulated in hermaphrodites by the sdc genes, which are in turn negatively regulated by the xol-1 gene. I am interested in identifying cis-acting elements and trans-acting factors involved in the regulation of her-1 gene expression. Transgenic studies indicate that many regions of the her-1 locus are involved in regulating her-1. Fragments of her-1 genomic DNA cause phenotypes in transgenic XX animals that resemble sdc loss of function phenotypes. Some of these same non-coding DNA fragments can suppress the XO specific lethality of xol-1 loss of function mutations, indicating that sequestration of limiting factors necessary for dosage compensation and sex determination may be occurring.

Electrophoretic mobility shift assays show that factors in embryonic nuclear extracts bind to genetically defined regulatory regions of her-1. DNase I footprinting has identified new regions that are occupied in the her-1 promoter and may be involved in her-1 gene regulation.
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Chapter I: Introduction

1.1 Caenorhabditis elegans as a Model Organism for Developmental Studies

The free-living soil nematode Caenorhabditis elegans has become one of the favored organisms to study developmental biology. C. elegans is relatively easily propagated and maintained. The diminutive body (~1 mm in length) allows for the growth of thousands of animals on a single agar plate spotted with E.coli as an inexpensive food source. C. elegans has distinct sexual dimorphism and the transparent body of the worm allows for easy observation of morphology and screening for developmental mutations. Their life-cycle is short; worms are sexually viable approximately three days after their fertilization and they produce more than 300 progeny.

There are two naturally occurring sexes of C. elegans: hermaphrodites and males. Hermaphrodites are self-fertile somatic females, which create a limited number of sperm early in their development and later produce oocytes which can be fertilized by the sperm stored in the spermatheca. This ability to self-fertilize greatly facilitates the maintenance of clonal strains as well as isolation of recessive mutations. Infrequently (approximately 0.2% of all progeny) males are also produced by rare X-chromosome nondisjunction and
can mate with hermaphrodites where their sperm are preferentially used for insemination resulting in 50% male progeny thus allowing genetic crosses to be carried out.

The entire cell lineage of *C. elegans* has been chronologically and spatially mapped allowing for detailed analysis of cell fate decisions by genetic analysis (Sulston and Horvitz, 1977), (Sulston et al., 1983). The adult hermaphrodite has exactly 959 somatic cells, while the adult male has 1031. The hermaphrodite nervous system is made up of 302 cells, and the interneuronal wiring is for the large part completely known (White, 1986), (White et al., 1988). The male nervous system has an additional 79 neurons that are primarily sex-specific (White et al., 1976).

Transgenic strains of *C. elegans* are relatively easily created by the co-microinjection of the DNA of interest and a marker gene, into the syncitial gonad of hermaphrodites where it recombines into large concatamers that are transmitted to progeny as relatively stable extrachromosomal arrays. In addition the relatively new technique of RNA-mediated interference (RNAi) has allowed for the quick analysis of knockout (loss of function) phenotypes of genes with relative ease (Fire et al., 1998).

Recently, the entire 100 Mb *C. elegans* genome was sequenced, making it the first metazoan to enter the post-genomic era (Consortium, 1998). This information greatly facilitates the rapid analysis of homologous genes of interest from other more complex organisms in this simple well defined model organism. Bioinformatics can be used to analyze vast quantities of sequence and identify *cis*-elements important in a genomic context. In addition cDNAs identified in screens such as yeast-one-hybrid and two-hybrid screens can easily be used to identify and map the entire gene with the genomic sequence information.
Clearly Sydney Brenner made a wise decision when he chose *C. elegans* as a model organism to study the nervous system and developmental biology in general.

I.2 Sexual Dimorphism in *C. elegans*

Hermaphrodite and male animals are easily distinguishable due to the transparent body wall and distinct dimorphism of sexual structures (Figure 1). All of the tissue types have some degree of dimorphism; 30% of all adult hermaphrodite somatic nuclei are sexually specialized and 40% of all adult male somatic nuclei are sexually specialized (Sulston and Horvitz, 1977).

Many of the easily observable structures of the worm are sexually distinct. Hermaphrodites are slightly larger than males, despite having fewer cells. Their tail tapers to a whip-like point and is not specialized for a sexual function. In contrast, males possess a specialized tail structure that contains nine pairs of sensory rays in a cuticular fan, as well as copulatory structures such as the spicules and hook. The nervous system of males is more extensive and is required for specialized sensory and copulatory functions, as well as male mating behaviour.

The single-armed somatic gonad of males is easily contrasted with the large hermaphrodite bilobed gonad. The two arms of the hermaphrodite gonad meet at the vulva where egg laying as well as male-mediated insemination takes place. Hermaphrodites have an initial transient stage of spermatogenesis and the resultant limited number of sperm are stored in the spermatheca awaiting the oocytes which are subsequently produced. Germ cell proliferation takes place at the distal ends of the
hermaphrodite syncitial gonad and oocytes mature as they progress towards the uterus. Many non-sexual structures such as the intestine are also functionally distinct. For instance, hermaphrodite intestines produce yolk that is necessary for embryonic development, while the male intestine does not (Kimble and Sharrock, 1983).

I.3 General Overview of Sex Determination and Dosage Compensation in *C. elegans*

The developmental decision leading to sexual dimorphism in *C. elegans* is dictated by the ratio of the number of X chromosomes (X) to the ploidy of autosomes (A) (the X:A ratio). This is an extremely important developmental signal since it determines not only the somatic and germ line sexual fate of an animal, but also the mode of the vital developmental process of dosage compensation.

In a wild type diploid animal, males have one X chromosome and two sets of autosomes (X:A ratio = 0.5), while hermaphrodites have two X chromosomes and two sets of autosomes (X:A ratio = 1.0) (Nigon, 1951). Many of the genes involved in transmitting this X:A ratio to the downstream effector genes have been identified genetically. A bifurcated genetic pathway elucidated through epistasis experiments defines a hierarchy of primarily negative interactions that transmit the X:A ratio to the nuclei of all somatic cells (Figure 2) (Hodgkin, 1987b; Hodgkin, 1990). All of the genes involved in this pathway have been cloned and it appears that they may define a signal transduction pathway. The signal of the dose of X numerator elements relative to autosomal denominator elements is transduced through a group of genes: *xol-1, sdc-1, sdc-2, sdc-3,*
Figure 1: Sexual Dimorphism in the Soil Nematode *Caenorhabditis elegans*

Diagrammed are the two sexes of *C. elegans*. The top diagram is the XX hermaphrodite and the bottom diagram is the XO male. Because of the transparent cuticle it is easy to observe the extensive sexually dimorphic structures of the nematode. Indicated are some of these structures as well as some anatomical landmarks such as the pharynx.
Figure 1: Sexual Dimorphism in the Soil Nematode *Caenorhabditis elegans*
Figure 2: Genetic Pathway for Sex Determination and Dosage Compensation in *C. elegans*

The hierarchy that regulates both sex determination and dosage compensation begins with the dose of four known *X*-signal elements (regions 1+2, *sex-1* and *fox-1*) negatively regulating *xol-1* which in turn represses the *sdc* genes. The *sdc* genes activate the dosage compensation dumpy genes and repress *her-1* in *XX* animals. In *XO* animals *her-1* represses *tra-2* activity allowing the high activity of the *fem* genes. The *fem* genes act to repress *tra-1* and allow the male somatic fate program to be activated. There are two states to the pathway (*XX* vs. *XO*) and the level of activity for each gene is indicated below in either circumstance. The thin dotted line from *xol-1* to *tra-1* indicates the paradoxical weak feminization role that *xol-1* plays in *XX* animals. The thin curved line between *tra-1* and *tra-2* is indicative of the somatic feminizing role of the interactions between *tra-1* and *tra-2*.
Figure 2: Genetic Pathway for Sex Determination and Dosage Compensation in *C. elegans*

**Sex Determination**
- **XX**: High, Low, High, Low, High, Low, High
- **XO**: Low, High, Low, High, Low, High, Low

**Dosage Compensation**
- *dpy-21, dpy-26, dpy-27*
- *dpy-28, mix-1*

**Genetic Pathway**
- region1
- region2
- sex-1
- fox-1
- *xol-1*
- *dpy-30*
- *sdc-3*
- *sdc-1*
- *sdc-2*
- *her-1*
- *tra-2*
- *tra-3*
- *fem-1*
- *fem-2*
- *fem-3*
- *tra-1*
that regulate both sex determination and dosage compensation and are discussed below (section 1.3.2). The pathway then splits into a sex determination arm and a dosage compensation arm.

Dosage compensation is a phylogenetically diverse process necessary to equalize the expression of sex-linked transcripts between sexes which have different doses of sex chromosomes. While mammals have evolved to completely inactivate one of the two \( X \) chromosomes in females, *Drosophila* increases expression of genes on the single \( X \) chromosome in males (Cline and Meyer, 1996). In contrast, *C. elegans* employs a dosage compensation mechanism that down-regulates gene expression from both of the hermaphrodite \( X \) chromosomes by a factor of two (Meyer and Casson, 1986). The genes involved in dosage compensation include \( dpy-21, dpy-26, dpy-27, dpy-28, dpy-30, mix-1 \) as well as \( sdc-1, sdc-2, \) and \( sdc-3 \) (Hodgkin, 1983); (Hsu and Meyer, 1994); (Lieb et al., 1998). A large group of these dosage compensation proteins localize to the \( X \) chromosomes and globally repress transcription (Dawes et al., 1999); (Chuang et al., 1996); (Lieb et al., 1996); (Davis and Meyer, 1997).

There are seven genes involved in the determination of somatic sex of *C. elegans*. Named for their loss of function phenotypes, there are four that promote the male fate; \( her-1, fem-1, fem-2, \) and \( fem-3 \) and three that promote the female fate; \( tra-1, tra-2, \) and \( tra-3 \). The most upstream regulator of the somatic sex determination arm of the pathway is the male-specific gene \( her-1 \). The level of \( her-1 \) activity sets the activity state of all six genes that function downstream of \( her-1 \). The level of activity of the terminal regulator of
sex determination \textit{tra-l} ultimately decides the somatic sexual fate. This group of genes constitutes a hierarchy of negative interactions and their gene products resemble a novel signal transduction pathway.

In the following sections, I briefly describe the genes involved in the sex determination and dosage compensation pathway illustrated in figure 2.

\textbf{I.3.1 The X:A Ratio is the Determinant of Both Sexual Fate and Mode of Dosage Compensation}

\textit{C.elegans} occurs in the wild as a diploid organism and sex is determined by counting the number of \textit{X} chromosomes relative to the two sets of autosomes. As mentioned earlier, a diploid animal with two \textit{X} chromosomes becomes a hermaphrodite, while an animal with only one \textit{X} chromosome becomes a male. Experiments that altered the ploidy of the autosomes relative to the \textit{X} chromosomes revealed the presence of a counting mechanism that carefully monitors the \textit{X:A} ratio and programs the sexual fate as well as mode of dosage compensation relative to the ratio. Nematodes that had 2\textit{X}:3\textit{A} (0.67) were male, whereas a ratio of 3\textit{X}:4\textit{A} (0.75) resulted in hermaphrodite specification (Madl and Herman, 1979).

While denominator elements that count or mark autosomes have not yet been discovered, four regions of the \textit{X} chromosome that act in a dose-dependent manner to affect \textit{xol-l} activity have been identified (Akerib and Meyer, 1994); (Carmi et al., 1998). These four regions (regions 1-4) may contain “numerators” (or \textit{X}-signal elements) of the \textit{X:A} ratio to signal the relative dose of \textit{X} chromosomes. Analysis of \textit{X} chromosome
duplications and deletions in both $XX$ and $XO$ animals revealed that these signal elements act synergistically to repress the activity of the direct regulator of sex determination and dosage compensation, $xol-1$. Furthermore $xol-1$ repression occurs at two distinct levels: transcriptional and post-transcriptional. (Akerib and Meyer, 1994); (Carmi and Meyer, 1999).

Ordinarily, $xol-1$ activity is high in wild type $XO$ animals and low in $XX$. Repression of $xol-1$ activity allows the expression of the $sdc$ genes which direct the implementation of dosage compensation in $XX$ animals and the repression of the $her-1$ gene to specify the hermaphrodite sexual fate. One signal element harbored in region 3 and one in region 4 have been molecularly characterized.

$fox-1$ (feminizing locus on $X$) is a signal element that maps to region 3. $fox-1$ encodes a putative RNA binding protein, that works in conjunction with region 2 (signal elements yet to be identified) to post-transcriptionally repress $xol-1$. $fox-1$ contains a ribonuclear protein motif that when mutated in a highly conserved residue results in a null phenotype (Skipper et al., 1999); (Nicoll et al., 1997); (Hodgkin et al., 1994). Multiple copies of $fox-1$ do not affect the transcriptional levels of $xol-1$, but likely prevent an RNA-processing event critical to $xol-1$ activity. It is believed that the signal element in region 2 acts with $fox-1$ to post-transcriptionally repress $xol-1$ (Skipper et al., 1999); (Meyer, 2000).

$sex-1$ (signal element on $X$) is a signal element that maps to region 4 and encodes a nuclear hormone receptor homologue. Consistent with a role as a nuclear hormone receptor, $sex-1$ has a ligand binding domain and a DNA binding domain (Meyer, 2000). $sex-1$ and the elements in region 1 act to repress $xol-1$ expression at the transcriptional
level. Carmi et al. (1998) showed through transgenic experiments that \textit{sex-1} associates with the \textit{xol-1} promoter and therefore presumably acts to directly repress \textit{xol-1} transcription. In fact, \textit{xol-1} reporter genes were transcriptionally derepressed in a \textit{sex-1} null mutant background. In \textit{XX} embryos, homozygous null mutations in \textit{sex-1} result in lethality and masculinization, presumably due to derepression of \textit{xol-1}. Inappropriate expression of \textit{xol-1} in \textit{XX} animals results in overexpression of \textit{X}-linked transcripts due to repression of the \textit{sdc} group of genes and subsequent lack of dosage compensation. The high levels of \textit{X}-linked transcripts result in sickness and lethality. The repression of the \textit{sdc} genes in \textit{XX} animals also allows the derepression of \textit{her-1} and subsequent masculinization of \textit{XX} animals.

**I.3.2 Genes that Control Both Sex Determination and Dosage Compensation**

There are four genes that are responsible for coordinately controlling both sex determination and dosage compensation in \textit{C. elegans}; \textit{xol-1}, \textit{sdc-1}, \textit{sdc-2}, and \textit{sdc-3}.

\textit{xol-1} is the primary gene in the genetic hierarchy of \textit{C. elegans} sex determination and dosage compensation. As discussed above, the \textit{X} chromosome signal elements directly repress \textit{xol-1} activity, making it the direct target and interpreter of the X:A ratio. \textit{xol-1} acts as a developmental switch to specify the male fate; \textit{xol-1} loss of function phenotype is the opposite of the \textit{xol-1} gain of function phenotype. \textit{xol-1} is responsible for implementing the male modes of both sex determination and dosage compensation.

\textit{xol-1} prevents the implementation of dosage compensation on the single \textit{X} chromosome of \textit{XO} animals by acting to negatively regulate the hermaphrodite-specific
gene sdc-2. This is an essential process and xol-1 is named for its loss of function phenotype, \textit{XO} lethal. \textit{XX} animals are unaffected by null mutations in \textit{xol-1}, while \textit{XO} animals die as embryos. This \textit{XO} lethality is caused by underexpression of \textit{X}-linked transcripts due to the derepression of \textit{sdc-2} and subsequent inappropriate down-regulation of the single \textit{X} chromosome.

\textit{xol-1} is also required for the specification of male somatic sexual development through repression of \textit{sdc} activity. Low \textit{sdc} activity allows the proper expression of \textit{her-1}, and a male sexual fate. \textit{xol-1} loss of function mutations result in inappropriate expression of \textit{sdc-2} and feminization of dying \textit{XO} animals, due to repression of \textit{her-1}. Ectopic expression of \textit{xol-1} in \textit{XX} animals results in \textit{XX} specific lethality due to repression of \textit{sdc} genes and dosage compensation and the correlating overexpression of \textit{X}-linked transcripts.

In addition to its role as a male specifying gene, \textit{xol-1} also plays a secondary paradoxical role in sex determination. In \textit{XX} animals, sex determination mutations in hermaphrodite specifying genes such as \textit{sdc-1}, \textit{tra-1} (weak), \textit{tra-2}, \textit{tra-3}, and \textit{her-1} (gf) result in partial masculinization of the animals. \textit{tra-2}; \textit{xol-1} double mutants are completely masculinized and are fully viable \textit{XX} males. Therefore wild type \textit{xol-1} must have a weak feminizing role in \textit{XX} animals (Miller et al., 1988); (Rhind et al., 1995).

\textit{xol-1} has been cloned and it encodes three transcripts (2.5kb, 2.2kb, and 1.5kb) that differ at their 3' ends due to alternative splicing. Northern hybridization experiments showed that expression of the 2.2 kb transcript is significantly higher in \textit{XO} animals and peaks in young embryos (Rhind et al., 1995).
In order to identify the functional roles of the three different transcripts of \textit{xol-1}, Rhind et al. made a set of constructs expressing two wild type transcripts, and one truncated transcript. These constructs were tested for their ability to rescue either the \textit{XO} specific lethality, or to perform the feminizing role in a \textit{tra-2} background. Only the 2.2 kb transcript was necessary to rescue the \textit{XO} lethality of \textit{xol-1}. Interestingly the 2.2 kb transcript was also necessary for the feminizing role of \textit{xol-1}; the truncated version of the 2.2 kb transcript resulted in enhanced masculinization. Further, use of the 2.2 kb transcript alone resulted in rescue of both \textit{XX} and \textit{XO xol-1} phenotypes. These experiments showed that the 2.2 kb transcript was both necessary and sufficient for both known \textit{xol-1} activities.

Further mutational analysis showed that the highly acidic carboxyl terminus was necessary for the \textit{XX} specific feminization role of \textit{xol-1}, but not for the \textit{XO} male specific functions of \textit{xol-1}. Another region in the third common exon appears to fulfill the \textit{XO} specific role of \textit{xol-1} (Rhind et al., 1995).

\textit{sdc-1} was the first gene identified that linked the developmental processes of sex determination and dosage compensation in the worm (Villeneuve and Meyer, 1987). Mutations in the X-linked \textit{sdc-1} result in variable sexual transformation (Tra) of \textit{XX} animals. \textit{XX} animals are partially masculinized and have egg laying defects (Egl). Epistasis analysis of \textit{XX} animals doubly mutant for the sex determining gene \textit{her-1} and \textit{sdc-1} showed that \textit{her-1} was necessary for the masculinizing ability of \textit{sdc-1} mutants and thus placed \textit{sdc-1} upstream of \textit{her-1}.
In addition to sexual fate transformation, \textit{sdc-1} mutant \textit{XX} animals also show dosage compensation defects such as the dumpy phenotype (Dpy) associated with elevated levels of \textit{X}-linked transcripts. The first indication that \textit{sdc-1} affects an additional pathway was the observation that in \textit{XX} animals that are \textit{sdc-1}, suppression of the sex determination phenotypes can be achieved by mutations in \textit{her-1}, yet these double mutants still exhibit dosage compensation defects (dumpy phenotype).

The \textit{sdc-1} dosage compensation defects are karyotype specific rather than sex specific. \textit{XO} animals that have been transformed to phenotypic hermaphrodites by \textit{her-1} loss of function mutations can also be made mutant for \textit{sdc-1} without phenotypic consequences. \textit{XX} hermaphrodites with the same mutations in \textit{sdc-1} are Egl and short (dumpy) indicating that the \textit{sdc-1} Dpy and Egl phenotypes are dependent on the number of \textit{X} chromosomes (Villeneuve and Meyer, 1987). Northern analysis confirmed that \textit{sdc-1} mutations result in increased \textit{X}-linked expression, indicating dosage compensation of the two \textit{X} chromosomes had been disrupted. These data established a role for \textit{sdc-1} in dosage compensation (Villeneuve and Meyer, 1987).

\textit{sdc-1} was cloned and found to produce a single 3.8 kb transcript that encodes a 1203 amino acid protein containing seven zinc finger motifs, five of which resemble the consensus \textit{C}_{2}\textit{H}_{2} TFIIIA motif. The expression of \textit{sdc-1} peaks during embryonic development and then transcript levels drop through the larval stages until they rise again in adulthood (Nonet and Meyer, 1991)

Although \textit{sdc-1} has a necessary role for the proper sexual development and dosage compensation of \textit{C. elegans} hermaphrodites, the \textit{sdc-1} null phenotype is not as severe or penetrant as that seen in \textit{sdc-2} or \textit{sdc-3} mutants. There is no significant \textit{XX}
lethality despite the increase in X-linked expression, and the incomplete transformation of sexual phenotype argue that sdc-1 is not the sole gene responsible for hermaphrodite fate specification. Double mutants carrying weak alleles of sdc-2 with sdc-1 null (neither of which have strong phenotypes) result in complete XX specific lethality and a corresponding increase in her-1 transcripts indicating that sdc-1 is likely acting synergistically with sdc-2 to promote the hermaphrodite fate (Nonet and Meyer, 1991); (Trent et al., 1991).

sdc-2 is the master control gene that is responsible for initiating all aspects of hermaphrodite development in C. elegans. sdc-2 regulates the hermaphrodite mode of somatic sexual specification as well as activating X chromosome dosage compensation. Mutations in sdc-2 result in extensive lethality of XX animals, while XO animals are completely unaffected, indicative of dosage compensation defects causing overexpression of X-linked transcripts. In addition, sdc-2 mutations result in the near-complete sexual transformation of hermaphrodites to strongly masculinized pseudo-males, similar to mutations in tra-1, the terminal regulator of somatic sex determination (Nusbaum and Meyer, 1989).

Alleles of sdc-2 were isolated in a screen for X-linked hermaphrodite-specific lethal mutations, as well as in screens for extragenic suppressors of xol-1 lethality. Other genes involved in the dosage compensation process also cause XX specific lethality. Mutations in dpy-21, dpy-26, dpy-27, dpy-28, and sdc-1 (see above) result in disruption of the dosage compensation process and subsequent overexpression of X-linked transcripts in XX animals. (Meyer and Casson, 1986); (Villeneuve and Meyer, 1987); (Meneely and
Wood, 1987); (Plenefisch et al., 1989). Nusbaum and Meyer (1989) showed that \textit{sdc-2} mutations are specific to the \textit{XX} karyotype of an animal and not to the sexual phenotype by comparing \textit{sdc-2 (y55); her-1 XO} animals which are phenotypic hermaphrodites (as \textit{her-1} mutations can suppress the sexual transformation phenotype of \textit{sdc-2} mutants), with \textit{sdc-2(y55); her-1 XX} animals which are also phenotypic hermaphrodites but are \textit{XX}. The mutant \textit{XX} animals had extensive dosage compensation defects such as Dpy and Egl phenotypes, while the \textit{XO} animals displayed no such defects (Nusbaum and Meyer, 1989). Further evidence that \textit{sdc-2} mutations disrupt dosage compensation came from Northern hybridization experiments indicating that X-linked transcript levels are substantially higher in \textit{sdc-2} mutant \textit{XX} animals (Nusbaum and Meyer, 1989).

As mentioned above, loss of function mutations in the male-specifying gene \textit{her-1} can suppress the masculinization phenotype of \textit{sdc-2} mutations and thus \textit{sdc-2} was placed upstream of \textit{her-1} in the regulatory hierarchy of sex determination. In addition to the fact that \textit{sdc-2} mutants are dependent on the presence of \textit{her-1} for their masculinizing activity, it became clear that the dose of \textit{her-1} was also important. \textit{sdc-2 (y55) XX} animals that were homozygous for wild type \textit{her-1} were masculinized, yet \textit{sdc-2 (y55) XX} animals heterozygous for \textit{her-1} had the masculinization activity of \textit{sdc-2 (y55)} suppressed. The fact that \textit{XO} animals heterozygous for \textit{her-1} were completely masculinized or wild type, while the \textit{sdc-2 (y55)} mutant \textit{XX} animals heterozygous for \textit{her-1} had their masculinization suppressed showed that even the residual amount of \textit{sdc-2} activity of the \textit{y55} mutant was able to partially repress \textit{her-1} activity. This was one of the first indications that \textit{sdc-2} may be a direct regulator of \textit{her-1} activity (Nusbaum and Meyer, 1989)
sdc-2 has been cloned and it produces a 9.5 kb transcript that encodes a highly charged 2962 amino acid protein. sdc-2 has 19 exons and the detection of a 4.8 kb deletion that is associated with the y74 mutation confirmed the identity of the transcript as sdc-2 (Dawes et al., 1999).

The role of sdc-2 as the key regulator of hermaphrodite mode of dosage compensation is supported by three molecular observations.

First, immunolocalization experiments showed that sdc-2 was expressed exclusively in XX embryos whereas other components of the dosage compensation complex are expressed throughout the nuclei in both sexes in very early embryos and only later localize to the hermaphrodite X chromosomes. This result implies that SDC-2 is the only dosage compensation protein exclusively expressed in XX animals.

Second, the initial expression of sdc-2 at the 40-cell developmental stage of the embryos coincides with the first evidence of assembly of the dosage compensation machinery on the X chromosomes.

Third, from the onset of its expression, SDC-2 is localized to the X chromosome, and this localization is necessary for the localization of other dosage compensation proteins to the X chromosomes of XX animals (Dawes et al., 1999); (Chuang et al., 1994); (Lieb et al., 1996); (Lieb et al., 1998)

Consistent with a role for sdc-2 as the hermaphrodite-specific switch gene, ectopic expression of sdc-2 results in extensive XO specific lethality. This lethality can be suppressed by corresponding mutations in the dosage compensation dumpy genes, indicating that sdc-2 is capable of initiating dosage compensation (Dawes et al., 1999).
Although the lethality of XO animals ectopically expressing $sdc$-2 is extensive, $xol$-1 loss of function mutations are more severe and result in complete XO lethality. This result implies that there are still other factors that contribute to the implementation of dosage compensation. $sdc$-2 ectopic expression causes 83% of XO animals to die and overexpression of $sdc$-3 causes only 2% lethality of XOs. Ectopic expression of both $sdc$-2 and $sdc$-3 results in 99% XO lethality (Davis and Meyer, 1997). This suggests a synergistic facilitative role for $sdc$-3 in $sdc$-2 mediated dosage compensation. (see below for further discussion of $sdc$-3).

Immunolocalization experiments showed that ectopic expression of $sdc$-2 in XO animals resulted in X chromosome localization of SDC-3. Wildtype XO embryos (lacking SDC-2) have weak expression of SDC-3, but is not localized to the single X chromosome, showing that $sdc$-2 expression is necessary and sufficient for the recruitment of SDC-3 to the X chromosome (Dawes et al., 1999); (Davis and Meyer, 1997).

The protein machine that is involved in globally repressing transcription of the two X chromosomes in C. elegans by half is known as the dosage compensation complex. The dosage compensation complex is comprised of many factors, some of which were initially identified in screens for XX-specific lethality. A large group of proteins involved in such diverse processes as meiosis (DPY-26, DPY-28), mitosis (MIX-1), dosage compensation (DPY-27), and sex determination and dosage compensation (SDC-2 and SDC-3) form a complex that is localized to the two X chromosomes of XX nematodes (Hodgkin, 1983); (Lieb et al., 1996); (Chuang et al., 1996); (Lieb et al., 1998); (Dawes et al., 1999).
Members of this complex have been observed to localize to the \( X \) chromosomes of \( XX \) hermaphrodites at around the forty cell stage of embryonic development coincident with the hermaphrodite specific expression of \( sdc-2 \) (Lieb et al., 1996); (Chuang et al., 1994).

It has been observed that mutations in any one of these proteins prevents the other members from associating with the \( X \) chromosomes. DPY-26, DPY-27, MIX-1, and SDC-3 do not associate with the \( X \) chromosomes in animals mutant for other dosage compensation genes (\( sdc-2, sdc-3, \) and \( dpy-30 \)). SDC-3 is necessary for the localization of all other dosage compensation proteins to \( X \) except SDC-2, and SDC-3 requires SDC-2 for its localization to \( X \). (Lieb et al., 1996); (Chuang et al., 1996); (Lieb et al., 1998); (Dawes et al., 1999).

SDC-2 however appears to have \( X \) chromosome localization regardless of mutations in other dosage compensation genes (\( dpy-26, dpy-27, dpy-28, dpy-30, sdc-3, \) as well as \( sdc-1 \) and \( dpy-21 \)) (Dawes et al., 1999).

This evidence coupled with the sufficiency of \( sdc-2 \) expression for temporal and spatial \( X \)-localization of SDC-3 argues that \( sdc-2 \) is the hermaphrodite-specific initiator of dosage compensation. The current molecular model of dosage compensation is that SDC-2 and SDC-3 bind to sequences on the \( X \)-chromosomes of \( XX \) hermaphrodites and recruit DPY-26, DPY-27, DPY-28, MIX-1, and possibly other factors to \( X \), in order to repress transcription of \( X \)-linked genes by half.

In addition to its role as activator of dosage compensation, there is significant evidence to support a role for \( sdc-2 \) as the key initiator of hermaphrodite sexual development.
The male specifying gene *her-l* is the most upstream regulator of sexual fate. In *XO* animals *her-l* activity is high and male sexual development is implemented. In order to ensure the hermaphrodite sexual fate, *XX* animals need to repress *her-l* activity by a factor of 20 and in so doing ultimately activate *tra-1*, the terminal regulator of somatic sexual fate.

The first evidence that *sdc-2* is the key regulator promoting the hermaphrodite sexual fate, is that loss of function mutations in *sdc-2* result in sexual transformation or variable masculinization of *XX* hermaphrodites. Nusbaum and Meyer found that the rare *sdc-2* mutant *XX* animals that were able to escape lethal overexpression of *X*-linked transcripts were dumpy and masculinized. These *XX* animals homozygous for strong *sdc-2* mutations had male tails and male gonads containing sperm. Almost all of the complex male tail structures were intact and there was no presence of yolk which is an indicator of incomplete masculinization (Nusbaum and Meyer, 1989).

RNA blot analysis also showed that *her-l* transcript levels were elevated to abnormally high levels in *sdc-2* loss of function *XX* animals (Trent et al., 1991). Ectopic expression of *sdc-2* in *XO* animals should result in feminization, but the inappropriate implementation of dosage compensation results in *XO* lethality similar to that seen in *xol-1* mutants due to underexpression of *X*-linked transcripts. By ectopically expressing *sdc-2* in a *dpy-27* null mutant background, it is possible to observe the sexual phenotype while the *XO* lethality is suppressed. Dawes et al. observed that approximately 30% of these *XO* animals were sexually transformed to the point that they were fertile hermaphrodites (Dawes et al., 1999).
Just as sdc-2 is not the sole activator of dosage compensation, it is clear that other factors are involved in activating the hermaphrodite mode of sex determination at the same genetic level as sdc-2. While it is known that sdc-1 mutations causes weak sexual transformation, it appears that sdc-3 (+) plays a fairly important role not only in dosage compensation, but also in sex determination.

sdc-3 is unique in the sex determination and dosage compensation group of genes in that its two functions (activation of dosage compensation and repression of her-1) are separately mutable. Mutant alleles have been isolated that specifically disrupt the sex determination role of sdc-3 and variably masculinize XX animals, yet exhibit no dosage compensation defects. There are also alleles that disrupt the dosage compensation role of sdc-3 and result in XX specific lethality and dumpiness, yet exhibit no sexual transformation whatsoever (DeLong et al., 1993).

sdc-3 has been cloned and it encodes a 250 kD protein. Molecular analysis of the various sdc-3 alleles revealed that there are two independent domains that are responsible for the two developmental processes. The sex determination activity maps to a region which has limited homology to the ATP-binding domain of myosin, while the dosage compensation activity of sdc-3 mapped to a domain containing two TFIIIA like zinc-finger motifs (Klein et al., 1993). Analysis of sdc-3 alleles showed that dosage compensation mutations in sdc-3 eliminated the zinc finger motifs and further site-directed mutagenesis of critical cysteines of the zinc fingers abolished their dosage compensation role. Furthermore, mutations that delete or disrupt the zinc finger motifs abolished X-localization of SDC-3 in vivo (Davis and Meyer, 1997). These zinc finger
mutants were however able to rescue the sex determination phenotype of sdc-3 (Tra) alleles. Further deletion analysis identified another region that is necessary for the dosage compensation activity; removal of amino acids 443-978 prevented the protein from rescuing D.C. defects (Klein and Meyer, 1993).

In addition to sex determination alleles and dosage compensation alleles, there is a third class of sdc-3 null alleles that result in a paradoxical phenotype. These null alleles disrupt both domains of SDC-3 and result in extensive XX lethality, but the rare escapees of X-linked overexpression do not show any obvious signs of masculinization. This result is unexpected because not only is the sex determination domain disrupted, but this same null class also fails to complement the sdc-3 (Tra) mutant alleles, indicating that it has no feminizing activity. A clue as to how the null mutations fail to result in masculinization came from observations of other dosage compensation mutants, in particular the dpy genes. It appears that increases in X-linked transcripts associated with mutations in dpy-26, dpy-27, and dpy-28 can suppress the sex determination defects in both sdc-3 (null) and sdc-3 (Tra) alleles, presumably through a feedback mechanism that reduces her-1 transcript levels (DeLong et al., 1993).

Immunolocalization experiments show that SDC-3 associates with the X chromosomes of XX animals in vivo at around the forty cell stage of embryonic development and persists in somatic cells throughout adulthood. As expected, mutations that disrupt the zinc fingers of SDC-3 prevent its localization to X chromosomes of XX animals, while mutations in the sex determination domain do not. SDC-3 also localizes to the single X chromosomes of dying xol-1 mutant XO animals. The xol-1 mutation results in the derepression of the sdc genes, and subsequent inappropriate activation of dosage
compensation consistent with a role for SDC-3 in participating in the assembly of the
dosage compensation complex on X chromosomes (Davis and Meyer, 1997).

Ectopic expression of SDC-3 in XO animals does not result in X chromosome
localization of the protein indicating the necessity of other hermaphrodite-specific
factor(s) for localization of SDC-3 to the X chromosome. As discussed above sdc-2 is the
likely candidate to regulate all hermaphrodite development since it is zygotically
expressed while all other dosage compensation and sex determination genes are
maternally provided. Also SDC-2 accumulates only in hermaphrodites and is expressed
coincident with the XX-specific assembly of the dosage compensation complex. SDC-2
is also the likely candidate to recruit or facilitate SDC-3 X localization. Analysis of sdc-2
mutant embryos showed that besides the transient (short-lived) maternally contributed
SDC-3, there is no accumulation of SDC-3 in sdc-2 XX >100 cell embryos. Also, SDC-3
does not associate with X chromosomes in sdc-2 mutants that overexpress SDC-3.
Together, these observations indicate that SDC-2 is necessary for SDC-3 accumulation
and X-localization (Davis and Meyer, 1997).

Transgenic experiments that I describe in greater detail later, show that SDC-2
localizes in vivo to the X chromosomes of XX animals and it also colocalizes with large
fragments of her-1 genomic DNA. Interestingly, it was also observed that SDC-3 is
necessary for SDC-2 localization to these large genomic her-1 regulatory regions and that
sdc-3 (Tra) mutations abolished SDC-2 localization to these her-1 arrays (Dawes et al.,
1999).

Additional experiments, revealed that dpy-30 is required for either the expression,
production, or stability of SDC-3, because mutations in dpy-30 eliminate SDC-3 protein
from XX animals. Interestingly \textit{dpy-30} is the only dosage compensation dumpy gene that is not regulated by the \textit{sdc} genes (Hsu et al., 1995). Mutations in \textit{dpy-26, dpy-27,} and \textit{dpy-28} greatly reduce SDC-3 levels indicating its stability may be dependent upon the formation of a complex with DPY proteins and \textit{dpy-27} and \textit{dpy-28} mutations prevent \textit{X}-localization of SDC-3 (Davis and Meyer, 1997).

As noted earlier, mutations in dosage compensation genes including \textit{sdc-3} (null) can suppress the masculinization of sex determination mutations of \textit{sdc-3}. Molecular experiments indicated that this feedback mechanism occurred at, or upstream of \textit{her-1} because \textit{her-1} transcript levels were reduced. This result implicated the presence of another negative regulator of \textit{her-1} other than SDC-3; clearly SDC-2 is a valid candidate due to its important role in the regulation of hermaphrodite sexual development. Indeed overexpression of SDC-2 results in complete suppression of the \textit{sdc-3} (Tra) masculinization (Davis and Meyer, 1997). Dosage compensation mutations may increase levels of SDC-2 available to negatively regulate \textit{her-1}, whether it be through increasing \textit{X}-linked \textit{sdc-2} expression from the derepressed (uncompensated) \textit{X} chromosomes, or by liberating SDC-2 from the disrupted dosage compensation complex, and thus increasing the amount of free protein available to repress \textit{her-1}.

\textbf{1.3.3 Overview of Genes Involved in Dosage Compensation}

\textit{XX}-specific activation of the \textit{sdc} genes results in the subsequent activation of a group of genes that cooperatively act to halve the expression of \textit{X}-linked genes in \textit{XX}
animals. Many of the genes responsible for dosage compensation of \(X\) chromosomes in \textit{C. elegans} were identified in screens for \(XX\)-specific lethal phenotypes associated with overexpression of \(X\)-linked genes.

In addition to \textit{sdc-1}, \textit{sdc-2}, and \textit{sdc-3} genes, another group of genes that are required for dosage compensation has been identified. Mutations in \textit{dpy-21}, \textit{dpy-26}, \textit{dpy-27}, \textit{dpy-28}, and \textit{dpy-30} cause \(XX\)-specific lethality and dosage compensation dumpy phenotypes and identify these genes as being involved in \textit{C. elegans} dosage compensation (Hodgkin, 1983); (Meyer and Casson, 1986); (Plenefisch et al., 1989); (Hsu and Meyer, 1994). Reverse genetics identified a new protein \textit{MIX-1} that is also involved in dosage compensation (Lieb et al., 1998).

\textit{dpy-27} encodes a protein that is similar to members of the evolutionarily conserved structural maintenance of chromosomes (SMC) family of proteins. SMC proteins are involved in the processes of chromosome condensation, chromosome segregation, sister chromatid cohesion, and mitotic recombination repair (Chuang et al., 1994; Chuang et al., 1996); (Lieb et al., 1998). Although DPY-27 is present in both \(XX\) and \(XO\) animals, it is only localized to the \(X\) chromosomes of \(XX\) animals after the 30 cell stage of embryogenesis and is not involved in mitosis or other functions (Chuang et al., 1994).

Immunolocalization experiments revealed that \textit{sdc-2}, \textit{sdc-3}, and \textit{dpy-30} are required for the localization of DPY-27 to \(X\) chromosomes. Similar experiments also revealed that \textit{dpy-26} and \textit{dpy-28} mutations result in complete loss of DPY-27 detection, but not \textit{dpy-27} transcripts, indicating that either \textit{dpy-27} translation or the stability of the protein are dependent on \textit{dpy-26} and \textit{dpy-28}. \textit{In vivo} immunofluorescence experiments
and immunoprecipitation experiments using nuclear extract further demonstrated that DPY-27 is likely stabilized in a complex that contains DPY-26 and DPY-28, as well as one other protein (Chuang et al., 1996).

The *dpy-26* gene encodes a 1263 amino acid protein with two small motifs which share homology to two mitotic proteins; the *X. laevis* condensin complex member XCAP-H, and the *Drosophila* mitotic chromosome segregation protein Barren. In addition to its role in dosage compensation, *dpy-26* is also involved in meiotic chromosome segregation, but does not function in mitosis (Lieb et al., 1998); (Lieb et al., 1996). Mutations in *dpy-26* animals result in 4% male self-progeny as opposed to the expected 0.2% indicating a defect in meiotic chromosome segregation (Lieb et al., 1996). DPY-26 localizes to *X* chromosomes in *XX*, as well as *xol-1 XO* animals, but not wild type *XO* animals. In addition to this, DPY-26 colocalizes with DPY-27 on the *X*. Prior to the implementation of dosage compensation (< 30 cell stage) DPY-26 associates with all chromosomes undergoing mitosis, but after the 60 cell stage when dosage compensation has been implemented DPY-26 staining is specific to *X* chromosomes in *XX* animals (Lieb et al., 1996).

Like DPY-26, DPY-28, another component of the dosage compensation complex associates not only with the *X* chromosomes, but also with meiotic chromosomes in both sexes (Albrecht, 1998).

**DPY-28** has homology to a component of the *Xenopus* 13S-condensin complex, XCAP-D2 and is localized between homologous chromosomes as they pair during pachytene (Albrecht, 1998); (Meyer, 2000).
The other known component of the dosage compensation complex, **MIX-1** (mitosis and X-associated protein) was only recently identified biochemically, by co-immunoprecipitation with DPY-27, and through its homology to the SMC family subclass protein XCAP-E. MIX-1 is an essential component of a complex necessary for mitotic chromosome segregation in both sexes. MIX-1 specifically localizes to the hermaphrodite X chromosomes to down-regulate gene expression presumably with the assistance of hermaphrodite-specific factors. In fact, DPY-27, sdc-2, sdc-3, and dpy-30 are necessary for the hermaphrodite-specific X localization of MIX-1, but not for the chromosome segregation function. In addition, *dpy-26*, and *dpy-28* are necessary for the stabilization of MIX-1 enabling it to fulfill its role in dosage compensation. *dpy-26* and *dpy-28* are not necessary for *mix-1* chromosome segregation function (Lieb et al., 1998).

**mix-1** was also identified as the essential gene *let-29* and immunolocalization analysis of *mix-1* mutants revealed improper localization of dosage compensation proteins and failure of a complex to form. In addition, heterozygous mutants of *mix-1* suppress the previously described masculinizing effects of the *sdc-3* (Tra) mutations, further confirming a role for *mix-1* in repressing transcription of X-linked transcripts through dosage compensation (Lieb et al., 1998).

There are numerous homologies between the dosage compensation components and components of the frog 13S condensin complex involved in mitotic chromosome condensation (DPY-26 \(\equiv\) XCAP-H; DPY-27 \(\equiv\) XCAP-C; DPY-28 \(\equiv\) XCAP-D2 and MIX-1 \(\equiv\) XCAP-E). These homologies, as well as the dual roles of *mix-1* in dosage compensation and mitosis suggest that dosage compensation may have evolved by
recruiting components (MIX-1) from an ancient essential process such as mitosis and used them for a completely new function, in this case gene regulation. This also allows us to speculate as to the mechanism of dosage compensation. The involvement of MIX-1 and other homologous factors in chromosome condensation and segregation suggests that the chromosome-wide repression of gene expression during dosage compensation may result from higher order structural changes in the interphase X chromosomes.
Figure 3: Molecular Models for Dosage Compensation and
her-l Repression

A: In XX animals a complex of proteins is recruited to decorate the X chromosomes to globally repress transcription by half. SDC-2 is responsible for the initiation of all aspects of hermaphrodite development including orchestrating the formation of the dosage compensation complex on X. In addition to the dosage compensation specific DPY-27, this complex is composed of proteins which are also required for sex determination (SDC-2, SDC-3), proteins involved in meiosis (DPY-26 and DPY-28) as well as a protein essential for mitosis (MIX-1).

B: Repression of her-l transcription is necessary to implement the hermaphrodite program for somatic development. her-l transcription is reduced 20-fold in XX animals and both sdc-3 and particularly sdc-2 gene activity are required for this repression. It has recently been shown that SDC-2 protein co-localizes with large fragments of her-l genomic DNA, this localization is dependent on the sex determination domain of SDC-3, consistent with a direct repression of her-l mediated by SDC-2 and SDC-3. The site of SDC-mediated repression has not yet been established, nor have additional factors involved in direct her-l repression been identified. Although sdc-l has a role in sex determination, it is not known whether it, or other dosage compensation components directly interact with her-l to facilitate this strong repression.
Figure 3: Molecular Models for Dosage Compensation and \textit{her-l} Repression

A: Dosage Compensation

\begin{figure}
\centering
\includegraphics[width=\textwidth]{dosage_model}
\caption{Dosage Compensation Model}
\end{figure}

B: \textit{her-l} Repression

\begin{figure}
\centering
\includegraphics[width=\textwidth]{her_l_model}
\caption{\textit{her-l} Repression Model}
\end{figure}
1.3.4 Overview of Genes Controlling Somatic Sex Determination

The hierarchy of genes that regulate somatic sexual fate transmit the signal from the X:A ratio to ultimately set the level of activity of the terminal regulator of sex determination, tra-1. An X:A ratio of 1.0 results in high activity of tra-1 to specify a female somatic cell fate, while an X:A ratio of 0.5 results in low tra-1 activity and somatic cells adopt a male fate. Seven genes form a hierarchy of negative interactions that was elucidated through extensive genetic analyses (Hodgkin, 1980); (Hodgkin, 1986) (figure 2).

All of the genes involved in somatic sex determination have been cloned and sequenced and appear to form a novel signal transduction pathway incorporating cell non-autonomous secreted proteins, transmembrane receptor proteins, numerous cytoplasmic proteins including proteases, phosphatases, and zinc-finger transcription factors (figure 6A,6B).

All of the genes involved are named according to their loss of function phenotypes.

(her = hermaphroditization [XO animals are transformed into self-fertile hermaphrodites], fem = feminization [XO animals are feminized], tra = sexual transformer [XY animals masculinized]) and can be grouped into those that promote male development (her-1, fem-1, fem-2, and fem-3) and those required for proper female development (tra-1, tra-2, and tra-3).
Three fem genes (fem-1, fem-2, fem-3) act at the same genetic level to negatively regulate tra-1, while they in turn are negatively regulated by the other two tra genes (tra-2 and tra-3). her-1 promotes the male fate by negatively regulating tra-2.

Below I summarize the somatic sex determination genes and their products, and the known and predicted interactions between them.

her-1 is the most upstream regulator in the sex determination pathway and is required for proper male development. her-1 activity is high in wild type XO animals and low in XX animals. her-1 null mutations result in complete sexual transformation of XO animals into self-fertile hermaphrodites. her-1 is considered a sexual fate switch gene due to the fact that constitutive activation of the gene completely masculinizes animals regardless of their X:A ratio proving its sufficiency for male development.

Initial recessive loss of function hermaphroditization mutations first defined her-1 and it was linked to linkage group V ((Hodgkin, 1980). Two dominant gain of function (gf) alleles (n695) and (y101) were identified as egg-laying defective mutants and were later characterized as general sexual fate determination mutations. These two gf alleles were subsequently shown to be the same single base pair substitution (a G → A transition) resulting in sexual transformation of XX animals into partially masculinized animals (Trent et al., 1983); (Trent et al., 1988); (Perry et al., 1994). The allele n695 resides in the P1 promoter of her-1, 2 nucleotides upstream of the +1 transcriptional start site. This mutation results in inappropriate expression of her-1 in, and subsequent masculinization of XX animals. Sequences closely conserved to the octamer sequence highlighted in yellow that n695 is located in (see figure 4), are found three times in the
her-1 locus. This site is known as the gain of function or gf site and may define a site of negative regulation of her-1 transcription (Perry et al., 1994).

Weak temperature-sensitive, loss of function mutations were identified in the first (P1) promoter that result in feminization of XO animals. n826, n1100, e1561 are located at approximately -40 to -50 upstream of the first transcriptional start site and are indicated by ^ above the sequence. All three mutations are single nucleotide C to T substitutions and the heptamer, containing n1100 and e1561 highlighted in yellow (see figure 4) is found four times in the her-1 locus and has been designated the loss of function site (If site). This If site could define a target for a trans-acting constitutive activator of her-1.
Figure 4: *her-*1 P1 Promoter Region

The top line depicts the *her-*1 locus on linkage group V. The four *her-*1 exons are shown as boxes. White boxes are the 5' and 3' untranslated regions and the yellow boxes are translated. P1 and P2 denote the two XO-specific promoters. The loss of function site and the three additional consensus *If* sites are indicated above the locus as activation sites. The gain of function site and the two additional consensus *gf* sites are indicated below the locus as repression sites. An expanded view of the proximal sequence of the P1 promoter is depicted below and the sites of loss of function alleles *n826*, *n1100*, and *e1561* are indicated at -52, -46 and, -44 respectively by a ^. The wild type consensus *If* site is in a yellow box. The wild type consensus *gf* site containing the *n695* allele is indicated by a blue box and the site of the *n695* mutation is indicated by a ^.
Figure 4: her-1 P1 Promoter Region

Activation sites

GTCTCTT GTCTCTT AAGAGAC GTCTCTT

Repression sites

GCAGGGAT ACAGGGAG GCAGGGAG

5'

^ mutation sites

-80 -60 -40 -20 -10

Pst I

(cap site)

additional CAP sites

EcoRV

GGAGGATGGAAGACGAAGATAAAAGAGTCGCGTAGAACAGGTGGATGTCATCTMTCTCCTCCCTTTCCTTACTGCATTATGAGATATCTCCCMTT

3' 500 bp

(n826, n1100, e1561)

(n695)

MRYLPI
her-1 is regulated at the transcriptional level and has two tandem promoters P1 and P2 (figure 5) that produce two male specific transcripts. her-1 has four exons. The larger, relatively rare 1.2 kb transcript contains all four exons, while the smaller, more abundant 0.8 kb transcript expressed from the P2 promoter is comprised of only the last two exons (Trent et al., 1991); (Perry et al., 1993) (figure 5).

The larger 1.2 kb transcript expressed from the P1 promoter is the functional regulator of male development and it is transcribed exclusively in XO animals. While RNA analysis shows this 1.2 kb transcript is present throughout developmental stages, expression appears to peak in embryos and L1 larvae coinciding with the first signs of sexual dimorphism. The 1.2 kb transcript is predicted to encode a novel 175 amino acid, cysteine-rich, 20 kD protein with an N-terminal secretion signal sequence necessary for her-1 activity. The smaller 0.8 kb transcript is also expressed in XO animals, but low levels of it can also be detected in the embryos and L1 larvae of XX animals as well. There is no known function for the smaller transcript. Ectopic expression of the smaller transcript alone has no masculinizing activity and exclusion of the P2 promoter from constructs carrying the her-1 rescuing sequence does not interfere with its ability to rescue her-1 null phenotypes (Perry et al., 1993). XX animals that carry the dominant her-1 gain of function n695 alleles, or sdc loss of function alleles, have elevated levels of both the 1.2 kb and the smaller 0.8 kb transcripts indicating that her-1 is ectopically expressed in n695 mutants, and that transcription of both transcripts is to some level under control of the P1 promoter. The 1.2 kb transcript is missing in some her-1 null and if recessive
mutant strains correlating with the strongest \textit{her-l} phenotypes (Trent et al., 1991); (Perry et al., 1993); (Perry et al., 1994).
Figure 5: Physical Map of the her-1 Locus

The top line represents the her-1 gene on linkage group V as described in figure 4. *her-1* produces two transcripts from two tandem promoters, P1 and P2. *her-1* has four exons and the P2 promoter lies within the large second intron. Yellow boxes indicate translated sequence and the grey boxes indicate 5' and 3' UTRs. The larger 1.2 kb transcript *her-la* encodes the functional regulator of male development HER-1. *her-la* contains all four exons and is predicted to encode a 20 kD novel secreted protein that acts cell non-autonomously to coordinate masculinization of all cells.
Figure 5: Physical Map of the *her-1* Locus

**Activation sites**
5'

**Repression sites**

Rare mRNA (masculinizing)
~1.2kb (*her-1a*)

Abundant transcript
~.8kb (*her-1b*)
Mosaic analysis performed by Hunter and Wood (Hunter and Wood, 1992) showed that *her-1* acts in a cell non-autonomous manner to ensure that all cells adopt the male sexual fate regardless of their individual genotype (e.g., *her-1* (-) cells in a *her-1* (+) background were able to adopt a male fate). Consistent with this, the findings of Perry et al. show that HER-1 is likely to be a secreted protein that orchestrates the adoption of a male fate (Perry et al., 1993). The likely receptor for this putative signaling molecule is the predicted transmembrane protein and downstream target of *her-1*, TRA-2 (see below).

*her-1* expression is genetically negatively regulated by the *sdc* group of genes, and there is growing evidence that SDC-2 and SDC-3 may be direct physical regulators of *her-1* activity as well. Loss of activity of either *sdc* gene results in the derepression and inappropriate expression of *her-1* indicating that there is constitutive activation of *her-1* transcription with loss of negative regulation. The gain of function mutation, *n695*, described above occurs 2 nucleotides upstream of the first *her-1* transcriptional start site (cap site) in the P1 promoter (Perry et al., 1994). This mutation occurs in an eight nucleotide sequence, RCAG{G→A}GAK, which occurs three times within the *her-1* gene, (and statistically would not be expected to randomly occur as many times) (figure 4). This sequence could define a cis-acting site of negative regulation of *her-1*, possibly by the products of the *sdc* genes.

In addition to the gain of function site, temperature-sensitive single nucleotide substitutions that occur in the P1 promoter could define a cis-acting site of constitutive
activation of her-1 transcription. Three C → T transitions (e1561, n826, n1100) occur in the -50 to -40 region upstream of the transcriptional start site, that cause weak partial feminization of XO animals at the restrictive temperature of 25°C (figure 4) (Perry et al., 1994). n1100 and e1561 are located in a heptad sequence GTCTCTT that occurs once more at +30 downstream of the her-1 transcriptional start site and twice more in the P2 promoter. This same heptad repeat has been observed near the start of many C. elegans genes, suggesting that it may be bound by a general transcriptional activator (Perry et al., 1993) (Perry et al., 1994). RNA blot analysis showed reduced expression of the large her-1 transcript in the e1561ts, indicating that the promoter alleles are likely affecting the transcriptional activity of her-1. Because both n826 and n1100 were isolated as suppressors of the n695 gain of function mutation, we know that this positive activating cis-element is necessary regardless of the absence of negative regulatory cis- elements. We also know that this element is involved in the constitutive activation of her-1 because all that is necessary for activation of the gene is loss of negative regulation (Perry et al., 1994). Genetic analysis of the sex determination pathway also indicated that her-1 is regulated by repression and that activation of her-1 is constitutive. It is therefore unlikely that the IF site is regulated by the sex determination pathway.

Recently published work (Li et al., 1999), as well as my observations described in chapter III, show that transgenic XX animals carrying various fragments of her-1 genomic DNA in extrachromosomnal arrays exhibit varying degrees of masculinization as well as varying degrees of dumpy morphology, similar to the phenotypes observed in sdc loss of function mutations. XO animals are apparently unaffected by the presence of the her-1
fragments. This sdc loss of function mimicry or “phenocopy” could result from \textit{in vivo} sequestration or titration of trans-acting factors necessary for the proper implementation of dosage compensation and hermaphrodite sexual specification, such as the sdc gene products.

Further evidence to support a role for SDC-2 and likely SDC-3 as direct physical regulators of \textit{her-l} transcription was provided in a recent publication by Dawes et al. (Dawes et al., 1999). In a clever immunocolocalization experiment, Dawes et al. made transgenic strains that carried extrachromosomonal arrays containing either a large 3.9 kb fragment of \textit{her-l} genomic DNA (including 1253 bp of the P1 promoter, the first two exons of \textit{her-l} and 2408 bp of the P2 promoter) or a control fragment of DNA. In addition, the arrays carried \textit{lacO} repeats as well as a transgene encoding a LacI::GFP fusion protein. This allowed for autofluorescence detection of the array carrying the \textit{her-l} DNA. Anti-SDC-2 antibodies were then used to immunolocalize SDC-2 \textit{in vivo}. In addition to observing that SDC-2 localized to the X chromosomes as would be expected, Dawes et al. found that a very high percentage of embryos carrying the \textit{her-l} arrays showed colocalization of SDC-2 with the arrays, while the negative control arrays didn’t show colocalization, implicating a direct role for SDC-2 as a \textit{her-l} transcriptional repressor (Dawes et al., 1999).

\textit{tra-2} is the downstream target of \textit{her-l}. In order to ensure that all cells in an organism adopt the same fate, many multicellular organisms have implemented cell-cell signaling in order to coordinate the development of groups of cells. For the process of sexual fate determination, it appears that \textit{C. elegans} utilizes a secreted ligand (HER-1)
and a transmembrane receptor (TRA-2) to mediate cell communication and ensure all somatic cells adopt the same sexual fate.

The *tra-2* gene promotes female somatic development in *XX* animals. Loss of function mutations in *tra-2* result in incomplete masculinization of *XX* animals (Hodgkin and Brenner, 1977). More recently identified *tra-2* “enhanced gain of function” (eg) alleles have been identified that cause *XO* animals to develop as self-fertile hermaphrodites (Hodgkin and Albertson, 1995); (Kuwabara, 1996a).

*tra-2* has been cloned and molecular analysis of the *tra-2* locus showed that it expresses three transcripts with sizes of 4.7 kb, 1.9 kb, and 1.8 kb. Only the 4.7 kb mRNA encoding TRA-2A with 23 exons contains both the 5' and 3' end of the gene, while both of the other transcripts contain only the 3' end encoding the carboxy terminus of the protein. Mutational analysis, as well as temporal and spatial expression patterns revealed that only the 4.7kb transcript fulfilled the primary somatic feminization role of *tra-2* (Okkema and Kimble, 1991); (Hodgkin and Brenner, 1977); (Klass et al., 1976). Although this 4.7 kb transcript is normally present in both *XX* and *XO* animals, the transcript is 15 times more abundant in wild type *XX* animals, and is actually regulated in response to the phenotypic sex as opposed to the karyotype of the animal, indicating a transcriptional regulation feedback loop originating at or downstream of *tra-1* (Okkema and Kimble, 1991). The mRNA is predicted to encode a 1475 amino acid protein, TRA-2A, with a secretion signal sequence. Hydrophobicity plots predict that there are likely either 9 or 11 membrane spanning domains, indicating that it is an integral membrane protein (Kuwabara et al., 1992) and it is predicted to be the receptor for the HER-1 protein. In *XO* animals high levels of HER-1 likely bind to TRA-2 and inactivate it thus allowing the
fem genes to negatively regulate tra-1 to promote a male somatic fate. Alternatively in XX animals, her-1 is transcriptionally repressed allowing TRA-2 to have high activity to repress the fem gene products and allow high activity of tra-1 to promote the hermaphrodite fate (Kuwabara and Kimble, 1992).

In addition to having a consensus secretion signal sequence and numerous hydrophobic helices, TRA-2A is predicted to have N-linked glycosylation sites that would be extracellular, as well as PEST sequences which are sometimes associated with proteolysis targeting, and potential serine/threonine phosphorylation sites in the intracellular carboxy-terminus (Kuwabara et al., 1992).

Transgenic studies showed that TRA-2A expressed from a heat shock promoter could cause feminization of the soma of tra-2 loss of function mutant XX animals (Kuwabara and Kimble, 1995). In addition to rescuing tra-2 mutants, this same construct when ectopically expressed in XO animals was able to completely sexually transform them to fertile hermaphrodites, indicating that tra-2 is a developmental switch gene, and that the relative ratio of HER-1 to TRA-2A is important for the sexual fate decision.

Molecular analysis of the tra-2 (eg) alleles have shown that a conservative missense mutation in the predicted extracellular N-terminal region may be disrupting the negative regulation of tra-2 through loss of interaction between HER-1 and TRA-2 (Kuwabara, 1996b).

Further transgenic studies showed the predicted intracellular C-terminal TRA-2 domain (TRA-2B) is capable of partially feminizing both XO and XX (tra-2) animals presumably through repressive interactions with the fem gene products (Kuwabara and Kimble, 1995). Further to this, Mehra et al., (Mehra et al., 1999) demonstrated a direct
physical interaction between the intracellular TRA-2B and FEM-3, one of the downstream targets of \textit{tra-2}. In this way TRA-2 may sequester an essential \textit{fem} product, preventing the negative regulation of \textit{tra-1}. This interaction could be a mechanism by which \textit{tra-2} is able to negatively regulate downstream targets and promote the hermaphrodite fate.

In addition to its role in the soma, \textit{tra-2} has an added level of complexity in the germline, where it appears that the 1.8 kb transcript or the cytoplasmic domain of TRA-2A may have a role in germline sex determination (Kuwabara et al., 1998).

The \textit{tra-3} gene also promotes female development in \textit{XX} hermaphrodites, and loss of function \textit{tra-3} mutations result in the transformation of \textit{XX} animals into pseudomales (Hodgkin, 1986). \textit{tra-3} encodes an atypical calpain protease that lacks the typical calcium-binding EF hands. TRA-3 does however have a calcium-dependent proteolytic activity that is necessary for its feminizing activity. TRA-3 proteolytic activity was demonstrated by its ability to autolyse in the presence of calcium. Potential target proteins for TRA-3 proteolysis included TRA-2, and the FEM proteins. Because TRA-3 was previously suggested to be a cofactor for TRA-2 and calpain proteases are often found to regulate their substrates by localization to the membrane, TRA-2 became a good candidate for a substrate of TRA-3 (Sokol and Kuwabara, 2000); (Suzuki and Sorimachi, 1998); (Hodgkin, 1986). Also, as mentioned earlier, TRA-2 has PEST sequences which are commonly associated with targeting a protein for proteolysis. Co-expression of TRA-2A with TRA-3 in Sf9 cells results in two bands that reacted with anti-TRA-2 (C-term) antibodies; one corresponds to full length TRA-2, and a 55kD band likely represents the C-terminal fragment containing the FEM-3 binding. Expression of TRA-2A alone
resulted in only the larger band being present establishing TRA-2A as a substrate for TRA-3. Genetic evidence suggests that TRA-2 proteolysis has biological relevance in C. elegans. The ability of ectopically expressed TRA-3 to feminize XO animals is dependent on the presence of TRA-2A, also possibly placing tra-2 epistatic to tra-3 in the genetic pathway. TRA-3 is likely to act as a potentiator of TRA-2 activity by cleaving off (releasing) the intracellular feminizing domain of TRA-2A. (Sokol and Kuwabara, 2000).

The fem genes are the downstream targets of tra-2 and tra-3, and act to promote male development. The fem genes act by negatively regulating the terminal regulator of sex determination tra-1. Activity of all three fem genes is necessary for proper development; loss of function mutations in any fem gene results in the feminization of XO animals as well as XX animals, meaning that the XX are fertile, yet spermless (Doniach and Hodgkin, 1984); (Hodgkin, 1986); (Kimble et al., 1984)

The fem-1 gene produces a single 2.1 kb transcript which is expressed in both sexes. This transcript encodes a 656 amino acid protein containing seven ankyrin repeats (Gaudet et al., 1996); (Spence et al., 1990). Ankyrin motifs are known to mediate protein-protein interactions in many proteins and in some fem-1 alleles the ankyrin-motif-encoding sequences are mutated, giving support for a biologically significant role (Chin-Sang and Spence, 1996). However, FEM-1-interacting proteins remain to be identified.

fem-2 is also expressed in both sexes and encodes a 449 amino acid protein that is part of the type II C Ser/Thr protein phosphatase family (PP2C) (Pilgrim et al., 1995). FEM-2 has phosphatase activity that is necessary for proper male development. FEM-2 interacts with FEM-3 in vitro, but the biological significance of this interaction is not clear (Chin-Sang and Spence, 1996). The existence of a phosphatase as a key component
of the sex determination pathway suggests that phosphorylation of components of the pathway is likely regulated by FEM-2. One possible candidate for a substrate of FEM-2 is TRA-1. FEM-2 and TRA-1 may have a direct physical interaction, and TRA-1 has different phosphoisoforms in vivo (D. Lum, pers. comm.).

*fem-3* encodes a 388 amino acid protein with no homology to any proteins in the databases. As mentioned earlier, FEM-3 interacts with the intracellular C-terminal region of TRA-2 and this interaction has been shown to be important for *fem* activity (Mehra et al., 1999). Gain of function mutations in the 3' UTR of *fem-3* result in derepression of *fem-3* translation resulting in masculinization of *XX* animal germlines (Ahringer et al., 1992); (Barton et al., 1987). Ectopic expression of *fem-3* in *XX* animals results in somatic masculinization transforming *XX* animals into pseudomales (Mehra et al., 1999).

Of all the *fem* genes, the feminizing phenotype seems to be most sensitive to the levels of *fem-3* activity. *fem-3* exhibits haploinsufficiency in the germline resulting in 5-10% of heterozygous animals having female germ lines. Heterozygous progeny of homozygous mothers exhibit some feminization in both the germline and the soma, indicating that levels of *fem-3* activity are crucial to male development (Hodgkin, 1986).

As noted earlier, FEM-3 interacts with FEM-2 but little is known about the nature of this interaction (Chin-Sang and Spence, 1996).

*tra-1* is the terminal regulator of somatic sexual determination. Null alleles of *tra-1* result in the complete masculinization of the somatic tissue of *XX* animals, regardless of the state of activity of any of the other genes in the sex determination pathway. Dominant *gf* alleles that result in feminization, regardless of genotype or state of other sex
determination genes, designate \textit{tra-1} as a switch gene that is both necessary and sufficient for hermaphrodite sexual specification (Hodgkin, 1987a); (de Bono et al., 1995)

\textit{tra-1} produces a 5 kb transcript and a 1.5 kb transcript differing at their 3' ends. The 5 kb transcript encodes TRA-1A which is a 1110 amino acid protein, while the 1.5 kb transcript encodes TRA-1B a 288 amino acid peptide (Zarkower and Hodgkin, 1992).

The functional \textit{tra-1} activity is due to the larger TRA-1A protein which appears to be a transcription factor. TRA-1A contains five tandem \textit{Kruppel}-like zinc-finger motifs resembling those found in the human glioblastoma tumor oncogenes of the GLI family, as well as the \textit{Drosophila cubitus interruptus} protein (Zarkower and Hodgkin, 1992).

TRA-1A is capable of binding to a consensus nonameric sequence \textit{in vitro} and appears to be nuclear localized (Zarkower and Hodgkin, 1993); (Lum et al., 2000). It is believed that TRA-1A acts by binding DNA and transcriptionally regulating downstream targets. There is no known function for TRA-1B.

Mutations in a small region (gf) in the amino-terminus of TRA-1A protein result in a dominant gain of function phenotype, presumably by releasing TRA-1A from post-translational negative regulation by the \textit{fem} gene products (de Bono et al., 1995)

Regions of TRA-1A have recently been identified that are capable of interacting with the upstream regulator of feminizing activity TRA-2. It is believed that this interaction enhances \textit{tra-1}-dependent feminizing activity in the soma. Mutations in the previously described \textit{mx} region of TRA-2 (Kuwabara et al., 1998) which masculinize the soma, yet feminize the germline disrupt this TRA-2/TRA-1A interaction, indicating that in the soma this complex functions to promote female development while in the germline
it promotes masculinization, or production of sperm necessary for hermaphrodite self-fertility (Lum et al., 2000).

Two potential downstream targets of \textit{tra-1} have been identified, \textit{mab-3} and \textit{egl-1}. \textit{mab-3} mutants have a male abnormal phenotype wherein they lack some of the male tail structures and have production of yolk proteins indicative of weak feminization. \textit{mab-3} is expressed in males and interestingly it contains the TRA-1A consensus binding site. This is consistent with a role for TRA-1A as a transcriptional repressor of a male-specific gene (Conradt and Horvitz, 1999); (Raymond et al., 1998).

\textit{egl-1} is also a putative target of TRA-1A regulation. \textit{egl-1} activity is required for the programmed cell death of the hermaphrodite specific neurons (HSNs) that are necessary for egg laying. \textit{egl-1} also has a TRA-1A consensus binding site and is expressed in males. TRA-1A regulation of \textit{egl-1} is mediated through a TRA-1A consensus binding site in the 3’ end of the gene in order to directly repress \textit{egl-1} (Conradt and Horvitz, 1999).

I have summarized the known and predicted interactions of the components of the sex determination pathway in two molecular models representing the states of activity of the two sexual fates in figures 6A and 6B.
Figures 6A and 6B: A Molecular Model for Sex Determination in *Caenorhabditis elegans*

Recent molecular and biochemical analyses have given insight into putative molecular mechanisms for the genetically defined sex determination pathway. This has allowed the building of a speculative molecular model.

Fig. 6A: In XX animals, SEX-1, the nuclear hormone receptor homologue, and region 1 are synergistically responsible for repressing *xol-1* transcription, while FOX-1, the putative RNA binding protein and region 2 work together to repress *xol-1* activity post-transcriptionally. This allows for the derepression of *sdc-2* transcription, allowing the SDC proteins (SDC-2 and SDC-3) to repress *her-1* transcription. Because *her-1* is not active, TRA-2 the trans-membrane receptor is free to inhibit the FEM proteins, likely through its interaction with FEM-3. This allows high *tra-1* activity and TRA-1A is able to act on its downstream transcription targets to specify the hermaphrodite sexual fate. The activity of TRA-3, the Calpain protease may facilitate the somatic feminizing activity of TRA-1 by cleaving the intracellular domain of TRA-2, enabling the interaction between TRA-1 and TRA-2, thereby enhancing feminization.

Fig. 6B: In XO animals, the low dose of X-signal elements allows *xol-1* transcription and translation, resulting in XOL-1 repressing *sdc-2* transcription. *her-1* is therefore derepressed, translated and secreted from the XO cell allowing it to possibly bind to the extracellular domain of the TRA-2 receptor. The intracellular FEM proteins are then free to act to repress the feminizing activity of TRA-1 and allow specification of
a male fate.
Molecular Model for Sex Determination in XX Animals
Molecular Model for Sex Determination in XO Animals
The primary focus of my thesis work was to identify cis-elements and trans-acting factors involved in the regulation of the *C. elegans* male-specifying gene her-*l*. I performed transgenic experiments utilizing a novel titration effect as a phenotypic indicator to test the significance of various promoter regions. I also tested the model that fragments of her-*l* genomic DNA are able to titrate important regulatory factors by doing genetic crosses in an attempt to suppress the XO lethal mutation xol-*l*. These experiments indicated that numerous regions in the two her-*l* promoters are involved in the regulation of her-*l* and that they may work in concert to mediate the regulation of her-*l*. These experiments also confirmed prior genetic observations that factors involved in her-*l* regulation are also likely involved in the essential process of dosage compensation and are titrated by her-*l* promoter fragments.

In order to study factors involved in her-*l* regulation, I used biochemical techniques that have rarely been used in *C. elegans*. Electrophoretic mobility shift assays indicated that factors present in embryonic nuclear extracts were able to bind specifically to the genetically defined *gf* and *lf* sites. These factors could represent constitutive activators and negative regulators of her-*l*. Supershifts did not verify whether SDC-2, the putative direct negative regulator of her-*l*, binds to the *gf* site.

Finally, using DNase I footprinting of the P1 promoter region, I identified many new regions that are occupied by factors in *C. elegans* embryonic nuclear extract which may be involved with the regulation of her-*l*.
Chapter II: Materials and Methods

II.1 Nematode Strains and Culture Methods

General methods for culturing *C. elegans* were as described by Brenner (Brenner, 1974). 

*C. elegans* Bristol strain N2 is the wild type progenitor of all strains used. Genetic nomenclature follows Horvitz et al. (Horvitz et al., 1979). Mutant nematode strains used were:

- CB1490 *him-5(e1490)* V
- NT47 *him-5(e1490) unc-76(e911)* V
- TY469 *unc-32(e189) III; lon-2(e678) xol-1(y70)* X

Abbreviations are as follows: *dpy* (dumpy), *egl* (egg laying defective), *fem* (feminization), *her* (hermaphroditization), *him* (high incidence of males), *lon* (long), *sdc* (sex determination and dosage compensation), *tra* (sexual transformer), *unc* (uncoordinated), and *xol* (XO lethal).

All strains were grown on petri dishes containing MYOB {0.55g Tris-HCl, 0.24g Tris (base), 4.6g Bacto-Tryptone, 2g NaCl, 8 mg cholesterol, 17g agar per litre; (Church et al., 1995)} and spotted with OP50 *E. coli*. All incubations were at 19°C unless otherwise indicated. Large scale growth in liquid culture is described in section II.6.
II.2 Bacterial Strains and Culture Methods

The *E. coli* strains used were DH5α, OP50, and BL21 (λDE3, pLysS).

*E. coli* strain DH5α (supE44 ΔlacU169 (φ80 lacZAΔM15) hsdR17 recA1 gyrA96 thi-1 relA1) was used for routine molecular biology preparations of plasmid DNA (Sambrook et al., 1989). *E. coli* strain OP50 is a uracil auxotrophic mutant used to “seed” MYOB plates as food for *C. elegans*. *E. coli* strain BL21 (λDE3, pLysS) [hsdS gal (λcI857 indI Sam7 nin5 lacUV5-T7 geneI)] was used for stable expression of recombinant proteins from the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible T7 promoter (Studier et al., 1990).

The media used for the culture of *E. coli* were YT and 2 x YT, (16 g Bacto-tryptone, 10 g Bacto-yeast, 5 g NaCl/litre) (Sambrook et al., 1989), and Super (Terrific) broth (4 ml glycerol, 24 g Bacto-yeast extract, 12 g Bacto-tryptone, 17 mM KH₂PO₄, 72 mM K₂HPO₄/litre) (Tartof and Hobbs, 1987) and incubations were at 37°C. The concentrations of antibiotics used were: ampicillin 100μg/ml; chloramphenicol 25μg/ml; kanamycin 25μg/ml.
II.3 Construction and List of Plasmids

The numbering system used for the *her-l* sequence refers to the *her-l* genomic sequence beginning at the *DpnI* site from file Gbwt.Seq.

**pNR12** contains the ~3.4 kb *her-l* genomic *BamHI* - artificial *BamHI* (linker formerly *HinfI* site) fragment constituting nt 2662 to 6084 ligated into the *BamHI* site of the GFP fusion vector pPD95.77 (Fire et al., 1990).

**pNR13** contains the ~2.5 kb *HindIII* - *PstI* *her-l* genomic fragment of the P1 promoter in *PstI* / *HindIII* MCS sites of the *lacZ* fusion vector pPD95.03 (Fire et al., 1990).

**pMG2** contains the ~2.89 kb *SphI* - *BamHI* *her-l* genomic fragment constituting nucleotides 3196 - 6084. Created by deletion of the 0.52 kb *SphI*-*SphI* fragment from pNR12.

**pMG3** contains the ~2.4 kb *BamHI* - *BglII* *her-l* genomic fragment constituting nt. 2662 to 5069. (pNR12 Δ *BglII* *SmaI*).

**pMG4** contains the ~1.88 kb *SphI* - *BglII* *her-l* genomic fragment constituting nt. 3196 to 5069. (pMG3 Δ *SphI*).

**pMG33** contains the 534 bp *BamHI* - *SphI* *her-l* genomic fragment constituting nt. 2662 - 3196 ligated into pT7/T3α-18 (Gibco BRL).

**pMG36** contains the 367 bp *AatII* - *EcoRV* *her-l* genomic fragment constituting nt. 2082 - 2449 ligated into the *HincII* site of pT7/T3α-18 (Gibco BRL).
pTLV4 contains the ~1.77 kb *HindIII - HindIII her-1* genomic fragment constituting nt. 3838 - 5609 ligated into pBluescript II SK+ (Stratagene).

pTLV5 contains the 855 bp *HincII - BamHI her-1* genomic fragment constituting nt. 1807 - 2662 ligated into pBluescript II SK+ (Stratagene).

pTLV8 contains the 1.53 kb *PstI - HindIII* fragment of *her-1* genomic DNA constituting nt. 2310 - 3838 ligated into pBluescript II SK+ (Stratagene).

pDB35 contains the PCR amplified fragment containing nt. 27-1391 of the *sdc-2* sequence subcloned into the multiple cloning site of one of the pRSET™ expression vectors (Invitrogen) and encodes 455 aa. of the N-terminal region of SDC-2 (provided by Heather Dawes and Barbara Meyer).

pUNC76 contains the genomic fragment of *unc-76* capable of rescue of the Unc phenotype (Bloom and Horvitz, 1997).

Figure 10 shows a graphic representation of the fragments of *her-1* genomic DNA contained in some of these plasmids.
II.4 Production of Transgenic Worm Strains

Healthy L4 NT47 larvae were transferred to separate seeded plates one day prior to injections to provide a source of minimally fertilized young adults for injections. The worms were immobilized on dried agarose pads on glass cover slips with a drop of hydrocarbon oil covering the worm to prevent dessication. Microinjection needles were prepared by "pulling" capillary tubes under high heat. The injection mixture generally consisted of a total of 100-200 ng/µl of high quality plasmid DNA (Qiaprep or Prep-a-gene), composed of variable concentrations of test DNA (fragments described in results section), and carrier DNA (pBSII SK+), while the marker DNA (pUNC76) was generally more than half of the mixture. The DNA mixtures were injected using a microinjection apparatus into the hermaphrodite distal syncitial gonad where the immature germ-line nuclei share syncitial cytoplasm and plasmid DNA can be incorporated into newly forming oocytes. Injected animals were recovered in a small drop of M9 buffer and transferred to new plates. Progeny of the injected hermaphrodites were transferred to separate plates and monitored for the marker plasmid to establish clonal lines of each transgenic strain.
II.5 Large Scale Growth of *C. elegans* strain N2 and Preparation of Gram Quantities of Embryos

Worm populations of Bristol N2 wild type strain were initially grown on approximately 10 (150mm. x 15mm.) petri dishes containing MYOB and spread with OP50 *E. coli* paste. Growth was at 19°C for approximately seven days until the plates were confluent. Worms were harvested by washing the large plates with S basal media (Sulston and Brenner, 1974) and the worms were subsequently rinsed with S basal media over two layers of Miracloth (Calbiochem) through a Buchner funnel. These worms were used to inoculate 3 x 500 ml liquid cultures of complete S media (Sulston and Brenner, 1974) supplemented with 40 - 50 ml each of a 50% paste of OP50 in 2800 ml unbaffled Fernbach flasks. Liquid cultures were incubated at 20°C and shaken at 160 rpm for 4 to 5 days. The cultures were harvested initially by sedimentation at 4°C and aspiration of the media and subsequent filtration through two layers of Miracloth and numerous washes with S basal media. Larva which were able to migrate through the Miracloth into the filtrate were used to inoculate a second overnight culture that was grown and harvested in the same fashion. The total yield of worms was approximately 70 ml of wet pellet.

Embryos were prepared by bleaching adults in an alkaline hypochlorite solution of 30 ml fresh bleach (4-6%Na HCO₃), 15 ml 5M KOH, 105 ml ddH₂O and monitoring the breakage of adult carcasses to free the eggs. Two rounds of successive bleachings and centrifugation in 50 ml conical tubes @ 2900 rpm in a tabletop clinical centrifuge were
carried out for a total time in bleach of approximately 20-25 minutes. The worm embryos were then washed extensively in distilled water and collected by centrifugation. A typical yield of embryos was ~ 6-8 grams.

II.6 Nuclear Extract Preparation

Embryos were resuspended in 3 ml homogenization buffer (15 mM Hepes, pH 7.6, 10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM EGTA, 44 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 mM sodium metabisulfite) per starting gram of embryos and homogenized in a precooled Wheaton stainless steel tissue homogenizer (Lichtsteiner and Tjian, 1995). This homogenate was filtered through one layer of Miracloth and the cellular debris was pelleted at 900 rpm, 4°C for 5 min. in a Sorvall SS34 rotor.

Nuclei were pelleted from the supernatant at 8000 rpm., 4°C for 15 min., resuspended in 5 ml of HB/gram starting embryos and dispersed in an all glass dounce homogenizer fitted with the 'B' pestle, and pelleted as before. Nuclei were resuspended in 1 ml nuclei buffer (25 mM Hepes, pH 7.6, 100 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 mM sodium metabisulfite) and dispersed with 5 strokes of pestle 'B'. Nuclei were transferred to 10 ml Oakridge polycarbonate tubes and 0.1x vol. of 4 M (NH₄)₂SO₄, pH 8 was added to lyse the nuclei and strip soluble proteins off of the chromatin. Tubes were inverted and incubated on ice for 30 min.
Chromatin was pelleted by ultracentrifugation at 36,500 rpm/4°C for 1 hr. in a Ti75 rotor. The supernatant was transferred to a fresh 10 ml Oakridge tube. 0.3g of ground (NH₄)₂SO₄/ml of supernatant were added and the solution was dissolved at 4.0°C, and subsequently incubated on ice overnight to precipitate the nuclear protein. The nuclear protein was collected by centrifugation at 36,500 rpm/4°C for 15 min. The pellet was resuspended in 0.1ml nuclear dialysis buffer (NDB)(25mM Hepes pH 7.6, 20mM KCl, 0.1mM EDTA, 10% glycerol, 1mM DTT) /g starting eggs using a pipette and gentle rocking for 3 hrs. at 4°C.

Nuclear extract was injected into Pierce Slide-A-Lyzer cassettes (10,000 MW cut off) and dialyzed against 100 vol. nuclear dialysis buffer for two hrs. at 4°C, and then overnight with 100 vol. fresh NDB. Protein concentration was determined by Bio-Rad protein assay. Aliquots were quick frozen in liquid N₂ and stored at -80°C.

II.7 Oligonucleotide Probes used for EMSA with Embryonic Nuclear Extract

All oligonucleotides synthesized by Genosys Corp.

MP111      GGCCTACCCGTACGACGTCCCAGATTACGC

MP112      ATGGGCATGCTGCAGGGTCTAP-TGCGCCGG
MP130  TTGAGTATCTAAGTCTCTTCAGGTTCTATAT
MP131  CTCATAGATTTCAGAGAGTTCCAAGATATAGG
MP132  TTGAGTATCTAAGTCTTTTCAGGTTCTATAT
MP133  CTCATAGATTTCAGAAAGTTCCAAGATATAGG
MP134  AACCAAAATGACACTGCAGGGATGCCTATCT
MP135  GGTTTACTGTGACGTCCCTACGGATAGAGG
MP136  AACCAAAATGACACTGCAGAGATGCCTATCT
MP137  GGTTTACTGTGACGTCTCTACGGATAGAGG
JA1    CTAGCATCTAAGTCTCTTCAGGTTCTA
JA2    GTAGATTCAGAGAAGTCCAAGATGATC
JA3    CTAGCATTTAAGTTTTTTCAGGTTCTA
JA4    GTAAATTCAAAAAAGTCCAAGATGATC
II.8 Electrophoretic Mobility Shift Assay

$^{32}$P- labeled double stranded probes were created in the following manner.

Oligonucleotides (section II.8) were synthesized by Genosys corp. 10 pmol of one strand of each complementary pair was labeled with 3μl 6000 Ci/mmol (γ-$^{32}$P) ATP (10μCi/μl.) using 20 units T4 Polynucleotide Kinase (New England Biolabs Inc) in a 20 μl reaction (37°C, 1.5 hrs.). Enzymes were inactivated at 100°C and 100 pmol of the unlabelled complementary strand were added and allowed to anneal slowly to room temperature. The 10x excess of “cold” strand prevents the presence of labelled single-stranded oligonucleotides which can bind single-stranded DNA binding proteins in the nuclear extract and cause spurious shifts (Stroeher et al., 1994). The reaction was phenol extracted, and unincorporated nucleotides were removed by Sephadex G-25 micro-spin column chromatography (Pharmacia Biotech).

Although parameters varied between experiments, typical gel shifts were 20 μl reactions which contained 0.1-0.4 pmol of labeled oligonucleotide probe, 12-18 μg of crude embryonic nuclear protein, 100ng poly (dI-dC) competitor DNA, in a buffer resulting in final concentration of 10 mM Heps, (pH 7.9), 50 mM KCl, 0-5 mM EDTA, 1 mM DTT, 0 - 0.2μM ZnSO₄, 10% glycerol. Reagents were added in this order: ddH₂O, buffer, poly (dI-dC), nuclear extract, +/- cold competitor oligos, or +/- antibodies, labelled probe.
Reactions were incubated at either room temperature or as indicated for particular shifts for 1 hr. and loaded on pre-run 4-6% non-denaturing polyacrylamide gels. Running buffer was 0.5x TBE (0.045 M Tris base, 0.001M EDTA), except for reactions that contained ZnSO₄, which used 0.5x TB (-EDTA) supplemented with .2µM ZnSO₄. Gels were also supplemented with 0.2µM ZnSO₄, and used 0.5X TB instead of 0.5X TBE when binding reactions used zinc. Wells were preloaded with binding buffer and gels were run at 160 volts until the bromphenol blue dye ran approximately 2/3 the length of the gel. Gels were dried and exposed to Kodak Biomax film.

II.9 Purification of SDC-2 Antigen

The plasmid pDB35 was kindly supplied by Heather Dawes and Barbara Meyer (University of California at Berkeley). pDB35 contains nucleotides 27-1391 of the sdc-2 sequence subcloned into the multiple cloning site of one of the pRSET™ vectors (Invitrogen) and encodes 455 aa. of the N-terminal region of SDC-2 (Berlin, 1995).

pDB35 was transformed into the E.coli strain BL21(λ DE3)pLysS and plated on TY plates supplemented with 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. Plates were incubated overnight at 37°C. A single colony was picked and used to inoculate a 1.5 ml starter culture which was subsequently inoculated into a 1 litre liquid culture of 2x TY/25 µg/ml chloramphenicol and 100 µg/ml ampicillin. The culture was grown at 37°C until the O.D.₆₀₀ = 0.9. T7 RNA polymerase was induced by the addition of 0.4ml of 1M IPTG to give a final concentration of 0.4 mM IPTG. The culture was induced for three hours at 37°C until the O.D.₆₀₀ =1.37. Cells were harvested by centrifugation, and
resuspended in 10 ml of buffer A (6M guanidine hydrochloride, 0.1M NaH₂PO₄, 0.01M Tris pH8.0) supplemented with 100.0 μg/ml PMSF, 2.0 μg/ml aprotinin, 1.0 μg/ml pepstatin, and 2.0 μg/ml leupeptin and frozen at -80°C. Cells were thawed (and lysed), refrozen in liquid N₂, thawed again and spun at 10,000 rpm to pellet cellular debris. The 6x-his-tagged fusion protein in the supernatant was bound to 4.0 ml of Ni²⁺NTA resin pre-equilibrated in buffer A by nutation at room temperature. The resin was washed in buffer A, and buffer C (8M urea, 0.1 M NaH₂PO₄, 0.01M Tris, pH adjusted to 6.3), and bound protein was then eluted with 250 mM imidazole in buffer C. Aliquots of all fractions were run on two 10.0% SDS polyacrylamide gels. One gel was fixed in MeOH-HOAc and stained with Coomassie Brilliant Blue, and the other was electroblotted to a nitrocellulose filter using a semi-dry transfer apparatus at 375mA for 30 min. After transfer, the filter was stained with Ponceau S for 5 min. and lanes and MW markers were marked with a lead pencil. The filter was then destained and incubated in BLOTTO (Tris-buffered saline, 0.2% Tween, 5% non-fat milk powder) and probed with r1497 crude anti-SDC-2 antisera (figures 7 and 8).
Figure 7 & 8; Purification of pDB35 SDC-2 Antigen

Figure 7: Coomassie stained 10% SDS-PAGE gel

Lane 1: Molecular weight markers
Lane 2: Pre-induction lysate
Lane 3: Post-induction precleared lysate
Lane 4: Post-induction cleared lysate
Lane 5: Unbound (flowthru) supernatant
Lane 6: Buffer C wash#1
Lane 7: Buffer C wash#2
Lane 8: Elution 1, 1.5 ml of 250 mM imidizole, 8M urea in buffer C
Lanes 9-14: Elutions 2-7, 1.5 ml of 250 mM imidizole, 8M urea in buffer C.

The strong band indicated as full length pDB35 corresponds to the 75-80 kD band previously observed by Heather Dawes (personal communication).

Figure 8: Western Blot of pDB35 Purification

All lanes are the same as above. Primary incubation; 1/10,000 r1497 crude rabbit sera in TBST & 5% milk. 3 washes in TBST & 5% milk, and secondary incubation 1/10,000 anti-rabbit POD. Developed by ECL chemiluminescent detection system. The band corresponding to full-length pDB35 antigen running at ~ 75 kD reacts specifically with the crude anti-SDC-2 sera as do degradation products visualized in lanes 9 and 10.
Figures 7 & 8: Purification of pDB35 SDC-2 Antigen

Figure 7

Figure 8
II.10 Affinity Purification of Anti-SDC-2 Polyclonal Antisera

Pooled elution fractions E₂ to E₅ of the purified SDC-2 antigen were dialyzed against 20 mM MES dialysis buffer pH 6.0 with successively decreasing concentrations (4M - < 2M) of urea until a precipitate formed. Attempts to resolubilize the protein by adding 8M GuHCl and 10% NP40 to final concentrations of 200mM GuHCl and 0.125% NP40 were unsuccessful. Precipitate which contained high concentration of the expected peptide was resuspended in protein sample buffer, boiled and run on a 10 % SDS polyacrylamide gel in a wide well. The gel was transferred to nitrocellulose filter at 320 mA for 45 minutes and the protein was easily visualized by Ponceau S staining. The band corresponding to the expected full size protein moiety was cut out and blocked overnight against BLOTTO. The blot was rinsed in distilled water.

Crude rabbit sera raised against the same N-terminal fragment of SDC-2 (r1497) was kindly supplied by Heather Dawes and Barbara Meyer from the University of California at Berkeley. 1.0 ml of 1/10 dilution of the sera in TBST (Tris-buffered saline, 0.2% Tween) was incubated with the nitrocellulose blot in a 15.0 ml conical tube while nutating for over two hours. The affinity-purified antibodies were eluted by rinsing the filter with three 300 μl aliquots of 100 mM Tris-glycine pH 2.3 and neutralized immediately with 1/10 vol. of 1M Na₂HPO₄. The eluates were pooled and stored in 0.1% BSA, 0.02% Na azide (Smith and Fisher, 1984).

Samples of the antigen purification elutions, as well as nuclear extract, and BL21 (λDE3)pLysS bacterial lysate as a negative control were run on a 7.5% SDS polyacrylamide gel and transferred to a nitrocellulose filter, lanes were marked and
transfer was confirmed after visualization with Ponceau S staining. Purified antibodies were diluted 1/500 in TBST and milk and incubated with the nitrocellulose filter overnight at room temperature while nutating. The blot was washed 3 times in TBST with milk and incubated with 1/10,000 dilution of anti-rabbit IgG-POD (POD = horseradish peroxidase) (Boehringer Mannheim GmbH) for 1 hour at room temperature, the blot was washed 3 times and developed by ECL western blot detection reagents (Amersham Pharmacia Biotech)(figure 9).
Figure 9: Affinity Purification of Anti-SDC-2 Antibodies

Western Blot of a 7.5% SDS-Page gel using 1: 500 dilution of affinity purified anti-SDC-2 antibody, and 1: 10,000 dilution of secondary anti-rabbit-POD. Immunoreactivity was detected by ECL chemiluminescent detection system.

Lane 1: molecular weight markers

Lane 2: BL21(λDE3)pLysS bacterial lysate

Lane 3: elution aliquot of pDB35 (antigen purification # 3)

Lane 4: elution aliquot of pDB35 (antigen purification # 4)

Lane 5: 20 μl (~ 60 μg) C. elegans N2 embryonic nuclear extract.

Purified anti-SDC-2 Ab reacts with the expected molecular weight band in the 2 antigen lanes (3 and 4), and there is virtually no background in the bacterial lysate lane. There is no observable reaction with nuclear extract from mixed sexual population (99.8% hermaphrodites) from the anti-SDC-2 antibody. This could be due to levels of SDC-2 in the nuclear extract below the threshold for detection.
Figure 9: Anti-SDC-2 Antibody Purification

<table>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>83</td>
<td>62</td>
<td>47.5</td>
<td>Full length</td>
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</table>
II.11 Preparation of Probes for DNase I Footprinting

Linear restriction fragment probes were prepared as described by Spiro and McMurray (Spiro and McMurray, 1995). The 368 bp Aat II to Eco RV fragment (2081-2449) of the her-1 P1 promoter was blunted with the Klenow fragment of E.coli DNA Polymerase I (New England Biolabs), gel purified (MoBio Ultraclean 15) and subcloned into the shrimp alkaline phosphatase (Amersham Life Sciences) HincII site of the MCS of pT7/T3α-18 to create pMG36. pMG36 was cleaved at two outer restriction sites in the MCS (Hind III and Eco RI) to release a ~420bp fragment containing numerous convenient inner restriction sites in the MCS. This fragment was dephosphorylated with shrimp alkaline phosphatase and gel purified. The purified fragment was S’-end labelled with $^{32}$P γ-ATP using T4 polynucleotide kinase in the buffer supplied (NEB). The reactions were incubated at 37°C for 1 hour and heat-killed at 70°C for 10 minutes. The reactions were phenol/chloroform extracted and run on a Sephadex G-25 microspin column (Pharmacia Biotech) to remove unincorporated nucleotides. 1.0 μl of the probe in 5.0 ml of Aquasol L.S.C. cocktail (New England Nuclear) was counted in a Pharmacia Liquid Scintillation Beta Counter. Typical incorporation resulted in ~ $5 \times 10^5$ cpm /μl.

Prior to DMS modification or DNase I footprinting, the linear probe would be split into two aliquots and digested with either Sph I or Bam HI leaving two sets of single end-labelled probes at opposite ends.
II.12 DMS Modification and Cleavage of Footprinting Probe

Single end-labelled probe was diluted 1/10 in DMS modification buffer (50 mM Na-cacodylate, pH 8.0, 150 mM KCl, 1 mM EDTA) to a total vol. of 200 μl. 5μl of freshly diluted 1M DMS/EtOH was added and incubated for 1 min. at R.T.. The reaction was stopped by the addition of 200 μl of DMS quench (2 M NH₄OAc, 1M β-mercaptoethanol, 0.136 mg/ml sonicated salmon sperm DNA) and ethanol precipitated in a dry ice ethanol bath for 15 minutes. The DNA was pelleted and dried completely and resuspended in 20 μl KHPO₄/EDTA. The DNA was denatured at 90°C for 10 min. and put on ice and centrifuged at 14 K rpm for 6 min. The DNA was cleaved by the addition of 2.0 μl 1M NaOH and incubated at 90°C for 5 min. 200 μl of cleavage stop solution (1.8 ml TE pH 8.0, 0.2 ml 3M NaOAc, 4 μl tRNA (10mg/ml)) was added and the DNA was ethanol precipitated and washed in 70% ethanol, dried and resuspended in sequencing buffer.

II.13 DNase I Footprinting

Linear restriction fragment probes for footprinting were prepared as described above and in (Spiro and McMurray, 1995). Appropriate concentrations of DNaseI were determined by prior titration. RQ1 RNase-Free DNaseI (Promega) was diluted into 20mM Hepes-KOH, 20% glycerol, 0.2mM EDTA, 0.1M KCl, 0.1μg/μl BSA, 28mM β-mercaptoethanol, 40mM CaCl₂. The concentrations of DNaseI necessary to cleave the
probes incubated with nuclear extract were considerably higher than for those without nuclear extract and were determined empirically by titration. A typical 8x volume reaction cocktail would include 30µl of DNA probe labeled on one strand (~8x10^4 cpm/µl), 0.8µl poly (dI-dC) (0.1mg/ml), 8µl BSA (0.5mg/ml), 1µl β-mercaptoethanol (14M), 39.5µl buffer D (20mM Hepes-KOH, 20% glycerol, 0.2mM EDTA, 0.1M KCl), 0.1µl NP40. This cocktail was aliquoted into 7 silanized eppendorf tubes (10µl each) and appropriate amounts of nuclear extract or buffer D were added to bring the volume to 40µl. These binding reactions were incubated at room temperature for 1-1.5 hrs. and occasionally overnight at 4°C.

A time course was initiated by the addition of 20µl of Mg²⁺/Ca²⁺ supplement (50mM MgCl₂, 10mM CaCl₂, 10mM Tris-HCl pH 7.5) (essential Mg²⁺/Ca²⁺ were left out of the binding incubation to prevent endogenous nuclear extract nuclease activity prior to initiation of time course) to the tube and immediate incubation at 30°C for 7 min. prior to the addition of 5µl of the appropriate concentration of DNasel. Digestion was for 2 min. at 30°C and the reaction was stopped by the addition of 65µl of stop solution (0.02M EDTA, 4M NH₄OAC, 40µg/ml sonicated salmon sperm DNA, 0.2% SDS, 0.1% proteinase K) and the tube was immediately put on ice. Proteinase K was then activated by incubation at 55°C for 15 min.

2 x phenol/chloroform extractions were performed and the DNA was ethanol precipitated, washed in 80% ethanol, dried and resuspended in formamide loading buffer. Reactions were run on a 6% polyacrylamide, 8M urea sequencing gel using DMS modified and NaOH cleaved probes as a reference G-ladder. Gels were dried and exposed to Kodak
Biomax film.
Chapter III: Results

III.1 Fragments of her-l Genomic DNA Cause Sdc-Like Phenotypes in Transgenic Worms

In order to identify regions of the her-l locus that may harbor cis-elements important to the regulation the her-l, I used transgenic studies of individual her-l genomic fragments as an assay. As previously noted, transgenic animals carrying extrachromosomal arrays containing numerous copies of her-l genomic DNA often exhibit phenotypes similar to those seen in animals with sdc loss of function mutations. Transgenic XX animals carrying these arrays exhibit variable masculinization, including shortening of the female whip tail, abnormal female gonad development, egg laying defects, abnormal vulval development, and variable male tail structure development. In addition to showing sex defects, these XX animals also have dosage compensation defects, such as the dumpy phenotype (short, stumpy, and generally unhealthy) and XX-specific lethality due to overexpression of X-linked genes (M.D.Perry, pers.comm.; Villeneuve and Meyer, 1987; Nusbaum and Meyer, 1989). No abnormal phenotypes are observed in XO animals carrying the same arrays.

It has been suggested that non-coding regions of her-l may be titrating away one or more factors that are necessary for the implementation of dosage compensation, resulting in the disruption of the dosage compensation complex and subsequent
overexpression of X-linked transcripts, leading to lethality (M.D.Perry, pers.comm.; Li et al., 1999) (Figure 11). Masculinization of XX animals indicates that the genomic copies of *her-l* on linkage group V have been derepressed, implying that factors necessary for *her-l* repression may also have been sequestered allowing their inappropriate expression. I used this "Sdc phenocopy" phenotype as an assay to identify regions of the *her-l* promoters that may contain *cis*-elements important for the regulation of *her-l*.

Plasmids containing the fragments shown in figure 10 were injected along with the *unc-76* marker plasmid and pBS carrier DNA into the strain NT47 *him-5 unc-76*. This strain allows detection of transgenic animals by rescue of the uncoordinated phenotype and increases the percentage of *XO* progeny due to the *him-5* mutation (see Materials and Methods). The progeny (F1, F2, and F3) of injected *XX* hermaphrodites were scored, and the results are shown in Figure 10. Those fragments that caused masculinization and dosage compensation defects of transgenic *XX* animals are indicated in figure 10. Due to the possibility that some constructs could result in extensive *XX* lethality, any transgenic non-Unc *XO* male progeny were crossed with NT47 hermaphrodites to propagate the lines.

Consistent with recently published results to be discussed later (Li et al., 1999), I found that the large 3.4kb *her-l* intron 2, containing the P2 promoter, appeared capable of titrating factors necessary for feminization of animals. In other words, pNR12 caused *XX*-specific masculinization and dosage compensation defects. The three subfragments of pNR12 that I tested (in plasmids; pMG33, pMG2, and pTLV4) also caused SDC phenocopy in the F1, F2, and F3 generations. I observed consistent extensive masculinization and dosage compensation defects by the construct pMG33 in numerous
transgenic lines and in hundreds of progeny (F1, F2, and F3). pMG2 and pTLV4 also exhibited similar phenotypes.

The construct pTLV5 contains the proximal P1 promoter region and behaved consistently as a strong masculinizing construct in transgenic animals. pTLV5 XX animals exhibited herniated vulvae, masculinized tails and intersexual gonads, as well as extensive dosage compensation dumpy defects, including XX-specific lethality. This result suggests that the fragment of her-l genomic DNA carried in pTLV5 was capable of titrating factors required for sex determination and dosage compensation.

As had previously been observed in our laboratory, XX animals carrying pNR13 exhibited both sexual transformation, as well as dosage compensation dumpy phenotypes. The two constructs, pMPN17-1 and pCT111, had previously been tested and defined the external limits of her-l DNA capable of SDC phenocopy (Marc Perry, pers. comm.). I did not test these two constructs. As appropriate negative controls, injections of either the marker plasmid (pUNC-76), or injections of the pBSII SK+ vector resulted in no sexual transformation or dumpy phenotype of transgenic animals. My experiments with transgenic animals carrying her-l genomic DNA suggest that regions of both the P1 and P2 promoters are involved in the cis-regulation of her-l activity.
Figure 10: *her-l* DNA Fragments Assayed for Sdc Phenocopy

Fragments of *her-l* genomic DNA diagrammed were subcloned into vectors and injected into NT47 *him-5 unc76 V* as described in Materials and Methods. Transgenic lines containing constructs carrying fragments with a" + " exhibited *XX*-specific variable masculinization and/or dosage compensation defects such as dumpiness (short, fat and generally unhealthy) or *XX*-specific lethality (described in results). *XO* animals were unaffected.
Figure 10: *her-1* DNA Fragments Assayed for Sdc Phenocopy

Diagram showing DNA fragments with restriction sites and vectors labeled: P1, P2, pNR13+, pNR12+, pMPN17-1, pTLV5+, pMG2+, pTLV4+, pMG33+, pCT111.

1 kb
Figure 11: Titration Model of Sdc Phenocopy in \textit{XX} Animals

A model that explains how fragments of \textit{her-l} genomic DNA could cause phenotypes similar to those observed for \textit{sdc} loss of function mutations in \textit{XX} animals.

In \textit{XX} animals \textit{sdc} gene activity is high in order to repress \textit{X}-linked transcription and \textit{her-l} expression. The ability of some \textit{her-l} fragments to cause Sdc phenocopy may be due to their ability to titrate factors necessary for the implementation of dosage compensation, and repression of \textit{her-l} transcription. Numerous copies of \textit{her-l} promoter fragments may sequester a limiting factor for dosage compensation thereby resulting in the dissociation or disruption of the dosage compensation complex. The resultant \textit{X}-linked overexpression causes dosage compensation dumpy defects and lethality specifically in \textit{XX} animals. In addition to this, these same animals are often variably masculinized. This masculinization is due to inappropriate expression of \textit{her-l} in \textit{XX} animals. This is likely due to the titration of limiting factors necessary for \textit{her-l} repression.
Figure 11: Titration Model of Sdc Phenocopy in XX Animals

**her-1** promoter fragments

**X chromosomes**

**D.C. complexes**

**her-1 gene on chromosome V**
III.2 One Large Genomic Fragment of \textit{her-l} DNA is able to Suppress \textit{xol-l} Mutations in \textit{XO} Animals

In order to test our titration model proposed to explain the Sdc-phenocopy observed in transgenic \textit{XX} animals carrying \textit{her-l} genomic fragments, I carried out genetic crosses utilizing the \textit{XO} lethal mutation, \textit{xol-l}. If \textit{her-l} promoter elements indeed titrate factors essential for dosage compensation, then these same \textit{cis}-elements may suppress the \textit{XO} lethality of \textit{xol-l} mutations by relieving the inappropriate repression of the single \textit{X} chromosome (Figure 12). This \textit{xol-l} suppression assay provides a more stringent test of the role of a \textit{cis}-element in dosage compensation.

After establishing stable transgenic lines and assaying their phenotypes, males from the lines carrying the extrachromosomal arrays described in Figure 10 were crossed to hermaphrodites homozygous for the \textit{XO} lethal mutation \textit{xol-l}. \textit{xol-l} is on the \textit{X}-linkage group and \textit{X} chromosomes carrying the \textit{xol-l} (\textit{y70}) mutation were marked with the recessive \textit{X}-linked \textit{lon-2} mutation, which results in a Lon (long) phenotype. These hermaphrodites were homozygous for the recessive \textit{unc-32} mutation (uncoordinated phenotype) on the third linkage group to facilitate the identification of all cross progeny (non-Unc).

A control cross was carried out to see if any of the cross progeny non-Unc animals were also Lon which would indicate that they had only one \textit{X} chromosome and that it had come from the hermaphrodite, (i.e. any Lon, non-Unc progeny would be
escapees of $XO$-specific lethality). No Lon, non-Unc progeny were observed. The $him-5$ mutation results in increased incidence of meiotic non-disjunction and a correlating high incidence of males. $e1490$ is a strong $him-5$ allele that results in 33% $XO$ zygotes at 20°C. This mutation was used to increase the number of $XO$ zygotes for analysis. The control and test crosses are described in detail below.

1.) Negative control cross

$XO$: CB1490 $him-5(e1490)$ $V$

$X$

$XX$: TY469 $unc-32(e189)$ $III; lon-2(e678)$ $xol-1(y70)$ $X$

Any cross progeny should be non-Unc (wild type) with respect to coordination. Any $XX$ cross-progeny should be $lon-2$ / + and therefore non-Lon. Any $XO$ cross-progeny should be $lon-2$ $xol-1$ / $O$ and therefore Lon, but also Xol and therefore should die as embryos. From numerous successful crosses (observation of non-Lon, non-Unc cross progeny $\{XX\}$, $n = 63$), no Lon, non-Unc progeny were ever observed, indicating that without extrachromosomal arrays the cross would not yield live $XO$ animals.

2.) Test Cross

$XO$: NT47 $him-5(e1490)$ $unc-76(e911)$ $V; ucEx$ (test plasmid), $pUNC76$

$X$

$XX$: TY469 $unc-32(e189)$ $III; lon-2(e678)$ $xol-1(y70)$ $X$
Figure 12: Titration Model for Suppression of xol-1 Mutations In XO animals

In wild type XO animals xol-1 activity is high and represses the sdc gene activity. Because sdc activity is low, dosage compensation is not implemented and there is full expression of X-linked transcripts from the single X chromosome. In xol-1 mutant XO animals, sdc activity is not repressed and the inappropriately expressed SDC-2 orchestrates the implementation of dosage compensation on the single X chromosome. The resulting underexpression of X-linked transcripts causes embryonic lethality of all XO animals. These dead embryos are often feminized, due to inappropriate repression of her-1. XO animals that are mutant for xol-1 and have a second loss of function mutation in either sdc-1, sdc-2, or sdc-3 are rescued.

If fragments of her-1 genomic DNA are also able to titrate factors necessary for dosage compensation in XO animals, then they may be able to disrupt the dosage compensation process similar to the sdc If mutations, and allow sufficient X-linked transcription from the single X chromosome to suppress the lethality.
Figure 12: Titration Model for Suppression of \textit{xol-1} Mutations in \textit{XO} Animals

Inappropriately expressed dosage compensation proteins

\textit{her-1} promoter fragments in extrachromosomal arrays

Single \textit{X} chromosome

\textit{her-1} gene on chromosome \textit{V}
The *her-l* genomic fragments in the extrachromosomal arrays carried by the transgenic *XO* males are diagrammed in figure 13. Of all the fragments tested, only the relatively large fragment pNR12 (which had previously been shown to suppress *xol-l* mutations), resulted in Lon, non-Unc progeny. These results indicate that only very large fragments containing most of the P2 promoter region are capable of *xol-l* rescue, and that the Sdc-like phenotypes of the transgenic *XX* animals bearing fragments of *her-l* promoter are due to titration of factors necessary for dosage compensation and sex determination.
Figure 13: *her-l* DNA Fragments Assayed for Suppression of *xol-l* in *XO* Animals

Transgenic male animals from lines carrying the diagrammed fragments were crossed with TY469 hermaphrodites (descriptions above and in Materials and Methods). Both *XX* and *XO* cross-progeny from the matings are heterozygous for *unc-32* III, and are therefore non-Unc. *XX* receive one *X* chromosome from each parent, and therefore are heterozygous for *lon-2* as well as *xol-l*, and have non-Lon phenotype and are unaffected by *xol-l*. *XO* animals are hemizygous for *lon-2* and *xol-l* and are therefore Lon, but also Xol and hence die as young embryos. If fragments of *her-l* are able to titrate factors necessary for dosage compensation, they may be able to suppress *xol-l* mutations in *XO* animals. Such animals would be Lon and non-Unc. Transgenic lines containing constructs that gave Lon non-Unc cross progeny are indicated by a "+".
Figure 13: *her-1* DNA Fragments Assayed for Suppression of *xol-1* in *XO* animals
III.3 Factors in *C. elegans* Embryonic Nuclear Extracts Specifically Bind

*her-1* Promoter Regions *in vitro*

Previous genetic studies defined sites in the *her-1* P1 promoter that may represent target sites for *trans*-acting regulators of *her-1* activity. As described in the introduction, the gain of function site, defined by the mutation *n695*, could represent a target site for negative regulators of *her-1* transcription. SDC-2 is a candidate protein to fulfill the role of direct negative regulator of *her-1*. The loss of function site may define a target for constitutive activation of *her-1* transcription (Figure 14). In order to identify whether these sites were actual targets for regulatory nuclear factors, I carried out a series of electrophoretic mobility shift assays.

**III.3.1 Loss of Function Site Gel Shifts**

To assay whether factors in nuclear extracts bind genetically defined *her-1* regulatory regions, I incubated ³²P-labeled synthetic oligonucleotides (MP130/131 Figure 14), spanning the *her-1* loss of function site, with *C. elegans* embryonic nuclear extracts. Several complexes were detected, indicating that the crude nuclear extracts contain factors able to bind and retard the migration of these oligonucleotides through native PAGE gels (Figure 15). To test binding specificity, I assayed the ability of a 6 x molar excess of the input unlabeled oligonucleotide or a mutated oligonucleotide to compete for formation of the various complexes. I incubated the binding reactions with a 6 x molar excess of either the wild type oligo or an oligo containing all three temperature sensitive,
loss of function mutations (JA3/JA4); (see Figure 14 and Figure 15). Binding was performed at 25°C, the non-permissive temperature for the mutations. The protein-DNA complex represented by the top band labeled "A" was not significantly competed by a 6-fold molar excess of either the wild type or mutant oligo (Figure 15, lanes 2-4). In contrast, complexes labeled "C" and "D" were specifically competed by a 6x molar excess of both wild type and mutant oligo. The mutant labeled probe was also able to bind the factors represented by the C complex, and the formation of this complex was partially competed by an 8x molar excess of unlabelled mutant oligo. In order to test the specificity of the binding of complex "A", I also used a 10-fold and 40-fold molar excess of unlabeled oligo as cold competitor in binding reactions in my gel shifts (compared to a 6-fold molar excess in Figure 15, lane 3). This higher concentration of cold oligo to labeled oligo is also a greater total quantity of oligos able to sequester a larger proportion of factors present in the constant volume of nuclear extract. At this concentration of cold competitor, both the wild type and the mutant her-1 loss of function site unlabeled oligos competed for formation of all complexes (Figure 16, lanes 2-5). Interestingly, in experiments with labeled mutant probe, the intensity of complex "A" was much reduced relative to the labeled wild type probe (Figure 15, lanes 2-4 vs. lane 5). This result suggests that the mutations in JA3/JA4 disrupt the binding of some of the factors to the loss of function site probe. However, there are some inconsistencies between these two experiments and they need to be repeated including controls that attempt competition for the complexes with an unrelated unlabeled oligo. Other experiments that titrate complexes formed on wild type probes with both unlabeled mutant and wild type oligos
Figure 14: *her-l* Promoter Region and Synthetic Oligonucleotides for Electrophoretic Mobility Shift Assays

The top black line represents the *her-l* locus as previously described in figures 4 and 5. The sequence below is the expanded view of the P1 promoter proximal region. The +1 transcriptional start site of the P1 promoter is indicated above the sequence and all other numbering is in reference to it.

Synthetic oligonucleotides were produced and $^{32}$P-labeled as described in Materials and Methods. The loss of function site is highlighted in yellow and the gain of function site is highlighted in blue.

MP130/131 contains the wild type sequence of the loss of function site from base pairs -58 to -30 upstream of the *her-l* P1 transcriptional start site.

JA3/JA4 contains the sequence from -54 to -33 upstream of the *her-l* P1 transcriptional start site including the three temperature sensitive, loss of function mutations n826, n1100, and e1561.

MP134/135 contains the wild type sequence of the gain of function site from base pairs -18 to +10 relative to the P1 transcriptional start site.

MP136/137 contains the *n695* mutation in the same sequence as MP 134/135.
Figure 14: her-1 P1 Promoter Region and Synthetic Oligonucleotides for Electrophoretic Mobility Shift Assays

- P1 and P2 regions with promoter sequences and mutation sites.
- GCAGGGAT and ACAGGGAG regions.
- CAP sites and additional CAP sites.
- Wild-type oligos for gel-shifts and mutant oligos for gel-shifts.

Sequences:

- GTCTCTT GTCTCTT
- AAGAGAC GTCTCTT
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
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- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
- GCAGGGAT
- ACAGGGAG
- GCAGGGGAG
Figure 15: Electrophoretic Mobility Shift Assay of *her-1* Loss of Function Site Oligos using Embryonic Nuclear Extract

Lane 1: 0.1 pmol MP130/131 ~5.6 x 10^5 cpm,

Lane 2: 0.1 pmol MP130/131, ~ 12 μg nuclear extract protein, 100 ng poly(dI-dC)

Lane 3: 0.1 pmol MP130/131, ~ 12 μg nuclear extract protein, 100 ng poly(dI-dC), 0.60 pmol unlabelled MP130/131

Lane 4: 0.1 pmol. MP130/131, ~ 12 μg nuclear extract protein, 100 ng poly(dI-dC), 0.60 pmol unlabelled JA3/JA4

Lane 5: 0.1 pmol JA3/JA4, ~ 12 μg nuclear extract protein, 100 ng poly(dI-dC)

Lane 6: 0.1 pmol JA3/JA4, ~ 12 μg nuclear extract protein, 100 ng poly(dI-dC), 0.80 pmol unlabelled JA3/JA4

Experimental conditions: binding reactions were set up as per Materials and Methods, except incubations were at 25.0°C for 1 hr., reactions were not supplemented with ZnSO₄, and all binding reactions contained EDTA. These probes were annealed with equimolar amounts of each labelled strand.

The letters A, B, C, D in the left hand margin point to complexes discussed in the text.
Figure 15: Electrophoretic Mobility Shift Assay of her-1 Loss of Function Site Oligos using Embryonic Nuclear Extract

<table>
<thead>
<tr>
<th>Lane</th>
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<td>mut</td>
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<td>+</td>
<td>+</td>
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</table>

Labeled probe: MP130/131, JA3/JA4

free probe
Figure 16: Electrophoretic Mobility Shift Assay using *her-l* Loss of Function Oligonucleotides and Embryonic Nuclear Extract.

Lane 1: 0.2 pmol MP130*/131 \( \sim 3.9 \times 10^5 \) cpm, 100 ng poly(dI-dC)

Lane 2: 0.2 pmol MP130*/131 \( \sim 3.9 \times 10^5 \) cpm, \( \sim 12 \) µg nuclear extract protein, 100 ng poly(dI-dC)

Lane 3: 0.2 pmol MP130*/131, \( \sim 12 \) µg nuclear extract protein, 100 ng poly(dI-dC), 2.0 pmol unlabelled MP130/131

Lane 4: 0.2 pmol MP130*/131, \( \sim 12 \) µg nuclear extract protein, 100 ng poly(dI-dC), 8.0 pmol unlabelled MP130/131

Lane 5: 0.2 pmol MP130*/131, \( \sim 12 \) µg nuclear extract protein, 100 ng poly(dI-dC) 8.0 pmol unlabelled JA3/JA4

Lane 6: 0.2 pmol JA3*/JA4, \( \sim 12 \) µg nuclear extract protein, 100 ng poly(dI-dC)

Experimental conditions: Binding reactions were set up as per Materials and Methods, incubations were at room temperature (~22°C) for 1 hr., ZnSO₄ was not added to binding reactions, and all binding reactions contained EDTA. These probes were annealed using a ten times molar excess of the unlabelled complementary strand in order to prevent spurious results from labelled unannealed single stranded DNA binding ssDNA binding proteins in the nuclear extract.

\* = Labelled strand
Figure 16: Electrophoretic Mobility Shift Assay of *her-1* Loss of Function Site Single Strand Labeled Oligos using Embryonic Nuclear Extract

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<tr>
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<td>-</td>
<td>wt</td>
<td>wt</td>
<td>mut</td>
<td>-</td>
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Labeled probes: MP130/131, JA3/JA4
would compare the relative affinities of the various complexes as well as the different oligos. Together, my competition experiments suggest that complexes "A" through "D" represent binding of factors specifically to the *her-l* loss of function site probes, and that biologically relevant mutations in the sequence disrupt the binding of some of these factors. These results are encouraging because these complexes may represent constitutive activators of *her-l* transcription bound to the loss of function site.

### III.3.2 Gain of Function Site Gel Shifts

To examine whether the gain of function site in the *her-l* P1 promoter is binding putative negative regulators of *her-l*, electrophoretic mobility shift assays were performed with labelled wild type gain of function site oligonucleotides (MP134*/135) incubated with embryonic nuclear extracts. These oligonucleotides were also able to specifically bind factors in the embryonic nuclear extract. A single complex was detected that was competed by a 5x molar excess of the same unlabelled oligo (MP134/135); Figure 17, lanes 2 and 3). By contrast, a 5 x molar excess of a random cold oligo (MP111/112) was unable to compete for complex formation (Figure 17, lane 4). Interestingly, labeled probe appears to be retained specifically in the wells of the binding reactions (Figure 17 lanes 2-4), suggesting that a large protein complex may be binding the probe and causing retention of the complex in the well. Additional competition experiments were performed with single strands of unrelated oligo (MP111) as well as single stranded MP134 revealing that the band was specific to double stranded MP134/135 (results not shown). The specific bands observed in these reactions could
represent binding of *trans*-acting, negative regulators of *her-1* transcription found in *C. elegans* embryonic nuclear extract. Possible candidates for these factors are the SDC proteins, and in particular SDC-2.

**III.3.3 Gain of Function Site anti-SDC2 Supershift**

To ask whether SDC-2 is a component of the complexes observed in the gain of function site gel shifts, I designed an antibody “supershift” experiment. Preincubation of the binding reaction with antibodies directed against a DNA binding protein may result in the formation of a protein-antibody complex. If the protein is still able to bind its DNA site, the added mass of the antibody will result in further retardation of the complex through the gel and the presence of a new slower migrating band. If the antibody sterically hinders the binding of the protein to the DNA, then there may be the loss of a band.

My initial attempts to supershift bands observed in the gain of function gel shifts using anti-SDC-2 crude anti-sera resulted in high background and no observable shift or loss of bands. I subsequently affinity purified the anti-SDC-2 antibodies and attempted to supershift the *gf* gel shift bands. The wild type gain of function probes again showed specific binding of factors in the nuclear extract (Figure 18). As before, bands were specifically competed away by a 15 x molar excess of the same wild type *gf* oligos (Figure 18, lanes 2 and 3). Interestingly, more bands were detected in this experiment than in the assays shown in Figure 17. This could be due to the increased porosity (reduced cross-linking) of the gel used in Figure 18 (80:1 vs. 37.5:1) allowing additional complexes to migrate out of the wells. However I obtained no evidence for either a loss
of a band or the addition of a slower migrating complex "supershift" upon pre-incubation of the nuclear extract with anti-SDC-2 antibodies (Figure 18, lane 4). Therefore this assay is not informative about the presence of SDC-2 in the observed complexes.
Figure 17: Electrophoretic Mobility Shift Assay of her-l Wild Type Gain of Function Site Oligos using Embryonic Nuclear Extracts

Lane 1: 0.4 pmol MP134*/135 ~5.0 x 10^5 cpm
Lane 2: 0.4 pmol MP134*/135, ~12 μg nuclear extract protein, 100 ng poly (dI-dC)
Lane 3: 0.4 pmol MP134*/135, ~12 μg nuclear extract protein, 100 ng poly (dI-dC), 2.0 pmol unlabelled MP134/135
Lane 4: 0.4 pmol MP134*/135, ~12 μg nuclear extract protein, 100 ng poly (dI-dC), 2.0 pmol unlabelled MP111/112

Experimental conditions: Binding reactions were set up as described in Materials and Methods, except ZnSO₄ was added to final concentration of 1μM, and EDTA was not added. Reactions were run on a pre-run 4.0% 30:0.8 cross-linking polyacrylamide gel at 120V at room temp. No EDTA was used in running buffer, or in gel buffer. ZnSO₄ was added to 1μM in buffer and gel.

* = Labelled strand, ns = non-specific competition
Figure 17: Electrophoretic Mobility Shift Assay of
her-1 Wild Type Gain of Function Site Oligos
using Embryonic Nuclear Extracts

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<td>5 x competition</td>
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Specific band

Labeled probe MP134/135

well

free probe
Figure 18: Electrophoretic Mobility Shift Assay of Gain of Function Site Oligos in the Presence of anti-SDC-2 Antibodies

Lane 1: 0.2 pmol MP134*/135 ~5.0 x 10^5 cpm, 150 ng poly (dI-dC)
Lane 2: 0.2 pmol MP134*/135, ~21 µg nuclear extract protein, 150 ng poly (dI-dC)
Lane 3: 0.2 pmol MP134*/135, ~21 µg nuclear extract protein, 150 ng poly (dI-dC), 3.0 pmol unlabelled MP134/135
Lane 4: 0.2 pmol MP134*/135, ~12 µg nuclear extract protein, 100 ng poly (dI-dC), 2.0 µl affinity purified anti-SDC-2 antibodies
Lane 5: 0.2 pmol, MP134*/135, ~12 µg nuclear extract protein, 100 ng poly (dI-dC), 0.5 µl 10 mg/ml BSA

Experimental conditions: Binding reactions as described in Materials and Methods except; nuclear extract was preincubated with anti-SDC-2 at room temperature for 2 hours prior to overnight incubation with probe at 4°C. Binding buffer: same as described except; ZnSO₄ was added to final concentration of 1µM, and no EDTA was added.

Reactions were run at 180V on a pre-run 4.0% 80:1 cross-linking polyacrylamide gel at room temp. No EDTA was used in running buffer or in gel buffer. (TB buffer, ZnSO₄ was added to 1µM in buffer and gel.

*= Labelled strand
Figure 18: Electrophoretic Mobility Shift Assay of Gain of Function Site Oligos in the Presence of Anti-SDC-2 Antibodies

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<tr>
<td>Antibody</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>BSA</td>
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</tbody>
</table>

Labeled probe MP134/135

free probe
III.4 Factors in *C. elegans* N2 Embryonic Nuclear Extract Protect Regions in the *her-l* Promoter from DNase I Digestion *in vitro*

III.4.1 DNase I Footprint of the P1 Promoter Region of *her-l*

Although earlier genetic experiments defined some of the regulatory elements of *her-l* such as the *gf* and *lf* sites previously described, it became clear from my transgenic experiments that there are numerous additional *cis*-elements involved in the regulation of *her-l* that have not yet been finely mapped. In order to identify new elements that may be involved in *her-l* regulation, I used DNase I footprinting to analyze part of the P1 promoter region including the *lf* site, the *gf* site, a consensus *lf* site, the transcriptional start site, and the translational start site. The ~250 bp *Aat II/Eco RV* fragment I used for this experiment is diagrammed in Figure 19. Although the resolution of these experiments allowed analysis of only one strand in each area of the fragment, the primary goal of the experiment was realized. Footprinting of this region identified sites that are occupied by factors present in the nuclear extract. These sites may be targets of *trans*-acting factors involved in the regulation of *her-l* transcription (Figures 20 and 21).

Autoradiographs showing the regions of the *her-l* P1 promoter that are protected from DNase I digestion by nuclear extracts are shown in figures 20 and 21. DNase I hypersensitive sites are also highlighted. I varied the amount of DNase I in the binding reactions to find the optimum concentrations necessary to equalize the amount of DNA
digestion between reactions that contained nuclear extract to those that don't. Higher concentrations of protein (nuclear extract) in the reactions is known to inhibit DNase I digestion and therefore a correspondingly higher concentration of DNase I is necessary to correct for this. Future experiments will use a fixed amount of DNase I for reactions containing nuclear extract and a fixed amount for those that don't contain any protein source. I detected a number of protected regions and hypersensitive sites consistent with the binding of factors to numerous regions throughout the her-1 P1 promoter, including the loss of function site and gain of function site as I had expected (results not shown). My results also agree with the earlier functional transgenic results which suggested the involvement of numerous cis-elements in the regulation of her-1. Interestingly it appears that a repeating 10 bp ladder of protection may be evident. Such patterns of protection are characteristic of regions occupied by positioned nucleosomes (Wang W. et al., 1996) and could also possibly indicate factors binding on only one side of the DNA helix as may occur when the DNA is looped around a complex of proteins and only one face of the helix is left exposed for DNase digestion. These results implicate the possible involvement of a protein complex binding the her-1 P1 promoter region. Additional DNase I footprints were carried out and yielded further information on the state of occupation of the her-1 P1 promoter region, but have not been shown. A schematic diagram compiling all results of the protected regions and hypersensitive sites from the various experiments is shown in figure 22.
Figure 19: *her-l* Genomic DNA Fragment Used in DNase I Footprinting

The top line depicts the *her-l* locus on linkage group V. The four *her-l* exons are shown as boxes. White boxes are the 5' and 3' untranslated regions and the yellow boxes are translated. P1 and P2 denote the two XO-specific promoters. The loss of function site and the three additional consensus *lf* sites are indicated above the locus. The gain of function site and the two additional consensus *gf* sites are indicated below the locus. An expanded view of the 250 bp *Aat II* - *Eco RV* fragment comprising the 3' end of the *her-l* P1 promoter is diagrammed below the top line. This restriction fragment contains the loss of function site, gain of function site, transcriptional start site and the translational start site close to the 3' end, and was prepared for DNase I footprinting as described in the Materials and Methods section.
Figure 19: *her-1* Genomic DNA Fragment
Used in DNase I Footprinting

**Diagram**:
- **P1** and **P2** regions marked.
- **AatII** and **EcoRV** restriction sites indicated.
- **Avall** and other markers with nucleotide positions.
- Genomic DNA sequence shown.
- Translational start site indicated at 2443.

**Sequence**:
```
GTCTCTT GTCTCTT AAGAGAC GTCTCTT
GCAGGGAT ACAGGGAG GCAGGGAG
```

**Annotations**:
- **Avall** site at 2170.
- **if site** at 2270.
- **gf site** at 2343.
- **lf consensus** at 2310.
- **Start site** at 2443.
Figure 20: DNase I Footprint of Bottom Strand of her-1 P1 Promoter

Region

Lane 1: DMS modified G-ladder of Aat II / Eco RV fragment
Lane 2: 5 µl of 256 µg/µl DNase I, 20 µl nuclear extract
Lane 3: 5 µl of 512 µg/µl DNase I, 20 µl nuclear extract
Lane 4: 5 µl of 512 µg/µl DNase I, 40 µl nuclear extract
Lane 5: 5 µl of 64 µg/µl DNase I
Lane 6: 5 µl of 128 µg/µl DNase I
Lane 7: 5 µl of 512 µg/µl DNase I, 20 µl nuclear extract
Lane 8: 5 µl of 32 µg/µl DNase I

Lanes 7 and 8 are from a separate gel and are upstream of all 6 lanes in the left hand gel.

Regions that show protection are indicated.

Regions hypersensitive to DNase I digestion are indicated as HS.

Binding conditions and running conditions are described in the Materials and Methods.
Figure 20: DNase I Footprint of Bottom Strand of her-1 P1 Promoter Region

<table>
<thead>
<tr>
<th>Lanes</th>
<th>1</th>
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<td>+</td>
<td>-</td>
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</tbody>
</table>

2390

HS

2400

2410

HS

2420

2430

Protection From DNaseI Digestion
Figure 21: DNaseI Footprint of P1 Promoter Region

Bottom Strand:
Lane 1: DMS modified G-ladder of Aat II / Eco RV fragment
Lane 2: 0 DNase I, 0 nuclear extract, (probe alone)
Lane 3: 0 DNase I, 10 μl nuclear extract
Lane 4: 5 μl of 512 μg / μl DNase I, 40 μl nuclear extract
Lane 5: 5 μl of 1000 μg / μl DNase I, 60 μl nuclear extract
Lane 6: 5 μl of 64 μg / μl DNase I
Lane 7: 5 μl of 128 μg / μl DNase I.

Top Strand:
Lane 8: DMS modified G-ladder of Aat II / Eco RV fragment
Lane 9: 0 DNase I, 0 nuclear extract, (probe alone)
Lane 10: 0 DNase I, 10 μl nuclear extract
Lane 11: 5 μl of 512 μg / μl DNase I, 40 μl nuclear extract
Lane 12: 5 μl of 1000 μg / μl DNase I, 60 μl nuclear extract
Lane 13: 5 μl of 64 μg / μl DNase I
Lane 14: 5 μl of 128 μg / μl DNase I.

Regions that show protection are indicated by a "P"
Regions hypersensitive to DNase I digestion are indicated as HS.

Binding conditions and running conditions as described in the Materials and Methods.
Data for protected region 2265~2272 is not shown.
Figure 21: DNase I Footprint of P1 Promoter Region
Figure 22: Protection of Genomic her-1 DNA Fragment from DNase I Digestion

This schematic diagram shows regions protected from DNase I digestion, as well as regions hypersensitive to DNase I digestion. All regions are described in figure 19. The yellow line indicates the ~250 bp Aat II / Eco RV fragment that was labelled and subjected to footprinting. The results on the right-hand side indicate protected regions that were observed in the bottom strand, and putative occupying factors are schematically represented on the diagram by ovals residing primarily on the bottom of the strand. The protected regions on the left were observed in the top strand and are diagrammed as such. DNase I hypersensitive bases are indicated by an "HS".
Figure 22: Protection of Genomic *her-1* DNA Fragment from DNase I Digestion

- Protection of 2111-2113
- Protection of 2135-2142
- Protection of 2145-2152
- Protection of 2159-2161
- Protection of 2169-2173

- Protection of 2265-2272
- Protection of 2289-2295
- Protection of 2307-2312
- Protection of 2389-2398
- Protection of 2401-2403
- Protection of 2411-2415
- Protection of 2421-2425
- Protection of 2430-2436
Chapter IV: Discussion

IV.1 Numerous Regions of the *her-1* Locus are Involved in its Regulation

One of the purposes of my thesis work was to identify *cis*-acting regions involved in the regulation of *her-1*. I used *in vivo* and biochemical methods to identify, and more finely map these putative regulatory regions. The results of my transgenic experiments compiled in figure 10 show that many genomic fragments of the *her-1* locus are able to cause masculinization of *XX* animals as well as *XX*-specific dumpyness and lethality. The phenotypes that I observed are similar to those of *sdc* loss of function mutations, and these fragments may be able to mimic Sdc phenotypes by titrating limiting *trans*-acting factors. Masculinization of *XX* animals is due to inappropriate derepression of *her-1*. This implies that the factors binding these regions and hence the regions themselves are involved in the negative regulation of *her-1*. The dumpy phenotype observed in *XX* animals is due to the disruption of dosage compensation and subsequent overexpression of *X*-linked transcripts. This data implies that these fragments are binding factors that are necessary for *her-1* regulation and the essential process of dosage compensation.

Numerous regions in both the P1 and P2 promoters were able to cause an Sdc phenocopy, indicating that negative regulation of *her-1* is not limited to the P1 promoter 5' of the *her-1* transcriptional start site. Many of the same regions I identified as having the potential for SDC-phenocopy were independently confirmed in a similar study by Li et al. (Li et al., 1999). Like Li et al., I found that transgenic lines with fragments contained in the
plasmids pNR12 and pMG2 exhibit phenotypes similar to those seen in sdc (lf) mutants. The fragment contained in pTLV4 is also capable of Sdc phenocopy, but was not assayed by Li et al., although some larger fragments containing it exhibited masculinization, while one fragment they tested (pBG4), which was missing a 0.4 kb fragment of pTLV4 was unable to masculinize.

Interestingly, contrary to my observations, Li et al. (Li et al., 1999), did not observe any masculinizing effects in transgenic animals that carried fragments encompassing the fragment contained in pMG33. Three of their constructs containing this fragment were scored as non-masculinizing (pBG6, pBG7, and pRH4). Also, I saw that the construct pTLV5, which contains the proximal P1 promoter region, behaved consistently as a masculinizing construct in transgenic animals, while Li et al. reported that a larger 2.8 kb P1 promoter fragment (pRH1), containing the her-l sequence from pTLV5, did not show masculinizing activity. This is particularly interesting because this fragment contains the genetically identified gain of function site which is most likely a site of negative regulation of her-l transcription.

There are several differences in experimental design which may account for the conflicting results observed in the two studies. Firstly, experimental parameters in the creation of transgenic lines can vary, such as plasmid copy number due to differences in injection volumes and DNA concentration resulting in variable titrating ability. Secondly, Li et al. tested the ability of constructs to masculinize XX xol-1 mutant animals, while my assay tested the ability of constructs to masculinize and cause dosage compensation defects in him-5 XX animals. Li et al. conducted their experiments in a xol-1 background in order to enhance masculinization phenotypes as has previously been observed in tra-2,
and her-1(gf) mutants (Rhind et al., 1995). However, using a xol-1(ly) background automatically excludes the possibility of recovering any XO animals that may be carrying the extrachromosomal arrays. Because some of these arrays cause XX-specific lethality, similar to strong Sdc phenotypes, there is the possibility that no transgenic animals (XO or XX) would be recovered. These arrays could contain some of the most potent titrating fragments, harboring important cis-acting elements.

The construction of my transgenic strains incorporated the him-5 mutation to specifically optimize for recovery of fragments that cause strong Sdc phenocopy. If the fragment was XX-specific lethal, it would be immediately noticed, due to the recovery of primarily male animals, and the lines could still be maintained by crossing to wild type / him-5 strains. I did not, however, find any fragments that caused such extensive XX-specific lethality as to skew the ratio of XX animals to XO animals so severely as to account for the lack of observation of masculinization by Li et al. in the lines mentioned above. I separately analyzed suppression of xol-1 by crossing these extrachromosomal arrays over to xol-1 hermaphrodites, to look for XO progeny regardless of their sexual phenotype.

As mentioned, many of these fragments caused serious dosage compensation defects in XX animals, yet were unable to suppress xol-1 mutations. This could be due to different lethal thresholds for high and low levels of X-linked expression. For example, a fragment that causes an increase in X-linked expression by 50% would result in an increase from 1x to 1.5x X-linked transcription in XX animals that may cause serious dosage compensation defects yet may not be lethal, but the corresponding increase of .5
to .75 in xol-l XO animals may not result in sufficient X-linked expression to suppress lethality.

My initial goal was to refine the minimum fragments necessary to cause Sdc phenocopy in order to identify important cis-acting elements. However I discovered that many of the fragments seem capable of titrating factors involved in her-l repression and implementation of dosage compensation. My results suggest that the regulation of her-l may be concerted between numerous elements along the gene, and could involve synergism between cis-elements.

IV.2 The Large P2 Promoter Fragment of her-l is able to Suppress xol-l Mutations in XO Animals

I wished to address whether the XX-specific lethality, and apparent sex determination defects observed in transgenic animals carrying her-l promoter fragments were due to a titration of factors involved in sex determination and dosage compensation. The genetic crosses were designed to exploit the fact that mutations in the sdc genes are able to suppress xol-l mutations. If her-l promoter fragments are titrating factors necessary for dosage compensation, they may also be able to suppress xol-l mutations. Assays using the large P2 promoter fragment confirmed that her-l DNA was likely able to titrate factors necessary for dosage compensation and allowed suppression of the XO specific lethality of xol-l mutations. This indicated a direct physical correlation between sex determination and dosage compensation. The fact that no smaller fragments were able to suppress xol-l mutations in XO animals means that these fragments were unable to
solely raise the level of \(X\)-linked transcription to a threshold sufficient for an animal to live. In fact, these results support a model where multiple elements act in \textit{cis}, possibly through the involvement of chromatin, to synergistically or cooperatively coordinate the strong repression of \textit{her-l} transcription, thus regulating sex determination.

**IV.3 \textit{C. elegans} Embryonic Nuclear Extracts Contain Factors Capable of Specifically Binding \textit{her-l} Regulatory Regions \textit{in vitro}**

**IV.3.1 Loss of Function Site**

My inability to use a transgenic approach to identify distinct \textit{her-l} regulatory elements necessitated the development of biochemical assays to search for \textit{her-l} regulators. Electrophoretic mobility shift assays would indicate whether genetically defined regulatory regions are targets for factors found in \textit{C.elegans} embryonic nuclei.

I predicted that if the \textit{lf} site is a target for constitutive activators of \textit{her-l}, then factors that bind the site would probably be present in both \textit{XO} and \textit{XX} animals. I also predicted that constitutive activators of \textit{her-l} would be present in a predominantly hermaphrodite nuclear extract. Gel shift assays using labelled synthetic oligos containing the loss of function wild type consensus sequence demonstrated that factors present in the embryonic nuclear extracts I prepared were capable of specifically binding to the \textit{lf} site.

I observed numerous shifted bands, suggesting that multiple complexes were forming on the oligos. Further support for this argument stems from my observation that each complex exhibited characteristic and unique behaviours when one of the experimental conditions varied. Band A may represent a complex containing an abundant
activator that binds the wild type loss of function site (Figure 15). I was able to ask whether the \( \text{if} \) mutations that disrupt the expression of \( \text{her-1 in vivo} \) also affect the binding of factors to the site \textit{in vitro}. The stability of the complex represented by band \textit{A} shows sensitivity to the three if mutations (Figure 15, lanes 5 and 6). I predicted that if the complex represented by band \textit{A} contains a relatively abundant factor, then a higher amount of unlabeled oligonucleotide may be necessary to compete for the formation of a complex. Figure 16 shows that this is true: A greater concentration of competitor oligonucleotide successfully competes away all of the bands. The fact that the mutant loss of function site probes are still capable of binding factors in the extract is not unexpected. The three loss of function mutations are known to be weak temperature sensitive mutations and may disrupt functional binding of only some factors. Band \textit{C} may represent a complex that contains a less abundant transcription factor binding to the loss of function region that is sensitive to competition, regardless of mutations (Figure 15).

The loss of function site could represent a target for constitutive activation of \( \text{her-1} \). The complexes observed in the gel shifts may contain general transcription factors necessary for \( \text{her-1} \) transcription, but with no functional role in sex-specific regulation. This same loss of function consensus sequence has been observed 5' of other \( \text{C. elegans} \) genes, and it is dispersed throughout the \( \text{C.elegans} \) genome. Considering these observations, some factors binding the loss of function site may be abundant general transcriptional activators.
IV.3.2 Gain of Function Site and Negative Regulatory Sequences

The *her-l* gain of function site has been proposed to be a target for negative regulation of *her-l* transcription (Trent et al., 1991); (Perry et al., 1994). The specific bands observed in the gel shifts may represent factors involved in the negative regulation of *her-l* bound to the *gf* site. One candidate for a *trans*-acting negative regulator of *her-l* transcription is the initiator of hermaphrodite development; SDC-2. Attempts to "supershift" the bands using anti-SDC-2 antibodies did not result in either a retarded shift or discernible loss of bands. These results suggest that factors other than SDC-2 may be binding the *gf* site, and hence be involved in the negative regulation of *her-l*.

Unpublished results from yeast-one-hybrid screens in our lab using both the *lf* site and the *gf* site as bait have lent support to our model of *her-l* regulation. Screens using the loss of function site as bait consistently pulled out a 360 aa TFIIIA-type zinc finger containing protein, consistent with a role as a general transcription factor (N. Rajwans, pers. comm.). Yeast-one-hybrid screens using the gain of function site as bait identified a protein with homology to p66, a protein associated with the large histone deacetylase complex in Xenopus, Mi-2-NuRD (N. Rajwans, pers. comm.) (Ahringer, 2000). Histone deacetylases are generally associated with closed chromatin and repressed gene expression. The presence of complexes specifically retained in the wells in the gain of function site gel shifts suggests that a very large complex may be forming that cannot enter the gel. Vilim et al. have observed that the upper limit of inclusion and separation on a 1.3%-10% gradient polyacrylamide gel is ~2MDa (Vilim and Krajickova, 1991). The Mi-2-NuRD complex is approximately 2MDa in size, and in a complex with the p66
homologue and the double stranded synthetic oligonucleotide may surpass the upper limit for inclusion in the gel. These results suggest that different factors may be involved in *her-1* negative regulation at different locations in the *her-1* locus.

This correlates well with new results from Jason Lieb and Heather Dawes from Barbara Meyer’s Lab in Berkeley. Recent evidence has suggested that SDC-2 exerts its role in *her-1* regulation at a significant distance from the *her-1* P1 promoter and *gf* site. Lieb found that protein members (DPY-26, DPY-27, and MIX-1) of the dosage compensation complex colocalize with two 250 bp fragments of the *her-1* P2 promoter. Identical copies of a fifteen bp sequence occur within each of these two fragments (Lieb, 1999). These sequences occur approximately 1 kb and 2 kb downstream of the P1 promoter in the large second intron containing the P2 promoter. Heather Dawes showed that SDC-2 protein colocalized with a large fragment of *her-1* genomic DNA, as well as directing the assembly of the dosage compensation complex on X (Dawes et al., 1999).

Interestingly, four members of the dosage compensation complex have homology to members of the 13S condensin complex responsible for mitotic chromosome condensation in Xenopus (Meyer, 2000). Knowing that SDC-2 and SDC-3 are required for DPY localization in dosage compensation, the negative regulation of *her-1* may involve SDC-2 and SDC-3 recruiting members of the dosage compensation complex to the *her-1* P2 promoter in order to facilitate transcriptional repression of *her-1*.

Because it appears that numerous regions in both the P2 and P1 promoters are involved in the negative regulation of *her-1*, yet many haven't been identified genetically, a methodical biochemical approach may be necessary to first identify specific regions occupied by factors, and secondly to identify the factors that are binding these regions.
DNase I footprinting of the two 250 bp fragments and the regions surrounding them can identify sequences that are occupied by more abundant factors, while gel mobility shift experiments provide a sensitive assay for testing specific sequences (such as the two fifteen bp sequences) for their ability to interact with regulatory partners. Gel shifts using \textit{in vitro} translated protein of the p66 homologue identified in the yeast-1-hybrid screen can confirm its interaction with the \textit{gf} site and it can be tested for interaction with the other \textit{gf} consensus sequences found in the \textit{her-l} locus.

IV.4 Multiple Regions of the \textit{her-l} P1 Promoter are Occupied by Factors Present in Hermaphrodite Embryonic Nuclear Extract.

In order to further understand the role of the P1 promoter in \textit{her-l} regulation I wanted to see what regions of it were occupied by potential regulators of \textit{her-l}. Results from my footprinting analysis of the P1 promoter region indicate that there are numerous sites occupied by factors present in embryonic nuclear extracts prepared from a primarily hermaphrodite population, presumably containing negative regulatory factors of \textit{her-l}. Occupied regions include the gain of function, and loss of function sites as we would expect, as well as many others, consistent with transgenic experiments implicating the involvement of multiple \textit{cis}-elements in \textit{her-l} regulation. Identification of binding partners of these occupied regions may help to resolve the molecular mechanism of \textit{her-l} regulation in \textit{XX} animals. As mentioned in section III.4.1, an alternative explanation of the footprint observed in the P1 promoter region involves the participation of a positioned nucleosome resulting in a repeating 10 bp ladder in a DNase I footprinting assay.
Although the protocol used to prepare the embryonic nuclear extract is designed to remove chromatin, there is the possibility that some histones and other DNA-associated proteins are still present in the extract and can reassemble to form a nucleosome. The prospect of a nucleosome positioning itself over the her-1 P1 promoter is an attractive model for a mode of repression of her-1 transcription and is consistent with a speculative model where the recently identified p66 homologue binds the gain of function site and facilitates repressive chromatin through its association with a histone deacetylase complex.
Chapter V: Conclusion

The results of my work can be used in conjunction with recent results from other groups to postulate a model for her-l regulation in XX animals as well as XO animals.

It appears from the most up-to-date information that the negative regulation of her-l in XX animals may in part be mediated by the remodelling of the chromatin structure of the her-l locus in order to down-regulate expression of her-l transcription. Many regions of the her-l locus have been implicated as cis-acting elements, including, but not limited to: the gain of function site and possibly additional consensus sequences of it, two fifteen bp sequences in the P2 promoter which may be targets for SDC-2, SDC-3, or possibly other members of the dosage compensation complex, as well as the loss of function site and its consensus sequences. Additional sequences that have not yet been “narrowed” to distinct elements are also likely involved and may reside in regions such as those contained in the plasmid pMG33, considering this fragment's ability to phenocopy Sdc in transgenic animals. Additional evidence that the regulation of her-l is dependent on action at a distance and may be mediated by chromatin effects is shown by the fact that the single bp gf mutation n695 in the P1 promoter is able to activate transcription from the P2 promoter almost 4 kb downstream of it (Perry et al., 1994). Delong et al. showed by Northern blot that her-l mRNA steady state levels in n695 (gf) mutants increase in an sdc-3 background (DeLong et al., 1993). This means that n695 mutant
animals are still sensitive to further derepression of her-l, implying that there are different levels, or modes of regulation involved in her-l regulation.

Our knowledge about trans-acting negative regulators of her-l, initially defined by genetics, has been supplemented by immunolocalization experiments, yeast-one-hybrid screens and EMSA. Although it is not known how her-l is negatively regulated, a model is emerging that may have SDC-2 playing the limiting and critical role of initiating her-l repression. sdc-2 is expressed specifically in XX animals, and expression of sdc-2 at the 40-cell stage of development immediately precedes the onset of localization of dosage compensation dumpy proteins to X. Also, sdc-2 expression precedes the onset of her-l expression in XO animals at approximately the 100-cell stage of embryogenesis allowing for the proper time frame for repression of her-l in XX animals. SDC-2, in cooperation with SDC-3, is likely responsible for recruiting members of a repression complex to various sites along the her-l locus, possibly inducing the subsequent formation of repressive chromatin. My results support a model where many regions and factors may mediate this repression through long distance cis-interactions. Indeed, the discovery that the C.elegans homologue of p66, a histone deacetylase associated protein, binds to the gain of function site implicates the possible involvement of the nucleosome remodelling and histone deacetylation (NuRD) complex in cooperation with an SDC-2 mediated regulatory mode to repress her-l activity (Ahringer, 2000). The involvement of a histone deacetylase complex in addition to an SDC/ DC complex could explain the differences in levels of repression observed between dosage compensation and her-l regulation. In XO animals this repression would be relieved by the absence of SDC-2, preventing the
assembly of a repressive complex, and allowing the constitutive activation of her-1 by
general transcription factors.

These recent results are very exciting in that her-1 regulation may be one of the
first examples in C. elegans of the possible involvement of histone deacetylation and
chromatin effects as a mode of developmental regulation. This proposed model for the
regulation of sex determination certainly invites new avenues of experimentation to
elucidate the true mechanism of her-1 regulation.

Generation of antibodies against the newly discovered p66 homologue would
allow for co-immunoprecipitation experiments to search for interacting partners that may
also be involved in her-1 regulation, and possibly general regulation of chromatin. Yeast-
2-hybrid screens could also identify binding partners of the p66 homologue, and confirm
the involvement of the NuRD/HDAC complex in sex determination. RNA interference
experiments can target the endogenous RNA of the newly described p66 for degradation
and in so doing possibly knockout the wildtype gene function. In vivo chromatin
immunoprecipitation experiments and in vivo footprinting would be informative for
observing the chromatin state of the her-1 locus and identifying the regions involved in
the repression complex in hermaphrodites vs. males (Strahl-Bolsinger et al., 1997).
Further footprinting of the P2 promoter can be used to identify prospective regulatory
regions, which can be further analyzed through gel-shift experiments with nuclear extract
and in vitro translated proteins. Production of DNA affinity columns using defined
regulatory sites can isolate trans-acting factors which can be analyzed by MALDI-TOF
spectroscopy of trypsin digests to identify factors that may be involved in her-1
regulation.
Literature Cited


