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Evidence for Multiple Promoter Elements Orchestrating Male-Specific Regulation of the *her-1* Gene in *Caenorhabditis elegans*

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ABSTRACT

The sex-determining gene *her-1* is required for male development in *Caenorhabditis elegans.* In XO males, two *her-1* mRNAs, her-1a and her-1b, are transcribed from two separate promoters: P1, located in the 5′-flanking region, and P2, located in the large second intron. In XX hermaphrodites, accumulation of both *her-1* transcripts is repressed by the *sdc* genes, which in turn are negatively regulated by the *xol-1* gene. When introduced into a *xol-1(y9)* background, transgenic arrays, including 3.4 kb of *her-1* intron 2 sequence (P2), result in phenotypes that mimic those of *sdc(lf)* mutants, including suppression of XO lethality and masculinization of both XX and XO animals. The masculinization, but not the suppression of XO lethality, is dependent on endogenous *her-1* activity. These effects could therefore result from sequestration (titration) of *sdc* gene products by sequences in the arrays, causing derepression of *her-1* (masculinizing effect) and disruption of the dosage compensation machinery (allowing survival of XO animals). We used these effects as an assay in a deletion analysis of the two *her-1* promoter regions to define potential *cis*-regulatory sites required for the putative titration. Several regions in P2 contributed to these effects. P1 was effective only in combination with certain P2 sequences and only if a particular P1 site previously implicated in *her-1* repression was intact. These results suggest that normal repression of transcription from P1 in XX animals may involve cooperative interaction with sequences in the P2 region. In experiments to test for a possible role of the her-1b transcript in regulation of *sdc* genes, no significant effects could be demonstrated.

THE coupled developmental processes of sex determination and dosage compensation in the nematode *Caenorhabditis elegans* are both under primary control of the ratio of X chromosomes to autosomes (X:A). An XX animal (X:A = 1.0) normally develops into a hermaphrodite and an XO animal (X:A = 0.5) into a male. Dosage compensation is accomplished by globally decreasing X-linked gene expression in XX animals by one-half so that it is equivalent to that in XO animals; therefore, this process normally occurs only in hermaphrodites (Meyer and Casson 1986; Donahue *et al.* 1987). Previous studies have shown that dosage compensation as well as sex determination are both controlled by the *sdc* genes (see Figure 1), which exert their effect on somatic sex determination by repressing the masculinizing gene *her-1* in XX animals (reviewed in Cline and Meyer 1996). It will be interesting to understand how the *sdc* gene products, which globally repress X-linked genes, also specifically repress expression of a particular autosomal gene, *her-1 V*, and how these two types of regulation are coordinated.

Other genes are also involved in control of dosage compensation. The upstream gene *xol-1* acts to repress *sdc* functions and thus affects sex determination as well as dosage compensation. The downstream dosage-compensation Dumpy (*dpy*) genes (DCD genes) function to effect dosage compensation and are not directly involved in sex determination. Loss-of-function (*lf*) mutations in *sdc-1, sdc-2*, *sdc-3*, and the DCD genes cause either XX-specific lethality or "dosage-compensation Dumpiness," both resulting from overexpression of X-linked genes as a consequence of disrupting the dosage-compensation machinery. Recent molecular work has shown that SDC-2 and SDC-3 as well as the DCD gene products DPY-26 and DPY-27 are associated with interphase X chromosomes in XX but not in XO embryos, suggesting that these proteins mediate global X repression by direct association with the X chromosomes (reviewed in Cline and Meyer 1996; Meyer 1997). Additional evidence has shown that these proteins may form complexes on the X chromosome and function cooperatively (Chuang *et al.* 1996; Lieb *et al.* 1996; Davis and Meyer 1997; H. Dawes, D. Lapidus, T. Davis and B. Meyer, personal communication).

Genetic studies had indicated previously that *xol-1(lf)* mutations such as *y9* result in XO-specific lethality because of failure to inhibit *sdc* and DCD gene functions in XO animals, thereby causing insufficient X-linked expression (Miller *et al.* 1988; Rhind *et al.* 1995). This model has been further supported by molecular studies

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showing that in *xol-1(y9)* XO embryos the dosage-compensation complexes are inappropriately assembled onto the X chromosome (Chuang *et al.* 1996).

As a switch gene required for male development, *her-1* behaves genetically as the first gene in the sex-determination branch of the pathways that regulate sex determination and dosage compensation (Figure 1). *Lf* mutations in *her-1* transform XO males into hermaphrodites (Hodgkin 1980), and hypermorphic *gf* mutations cause masculinization of XX animals (Trent *et al.* 1988). Previous studies have suggested that HER-1 functions as a secreted ligand to promote the male fate. Mosaic analysis showed that the *her-1* gene functions nonautonomously, indicating that a *her-1* gene product or a gene under its regulation is involved in cell interactions (Hunter and Wood 1992). Further molecular analysis revealed that *her-1* encodes a small novel protein, HER-1A, with a secretion signal at the N terminus, which appears to be essential for its masculinizing function (Perry *et al.* 1993). The current molecular model for the sex-determination mechanism is that in males, HER-1A binds to and inhibits the TRA-2 receptor, allowing a signal to be sent to the nucleus via the cytosolic *fem* gene products (FEM proteins), which repress the activity of TRA-1, the transcription factor that promotes hermaphrodite-specific gene expression (Hunter and Wood 1992; Kuwabara *et al.* 1992; reviewed in Meyer 1997). In hermaphrodites, where no HER-1A is produced, the TRA-2 receptor inhibits FEM protein activities, allowing TRA-1 to be active.

Consistent with genetic epistasis analysis (Hodgkin 1980, 1983), the *her-1* gene has been shown molecularly to be negatively regulated by the *sdc* genes, probably at the level of transcription. As diagrammed in Figure 2, the *her-1* locus gives rise to two transcripts: the rare 1.2-kb her-1a transcript and the moderately abundant 0.8-kb her-1b transcript (Trent *et al.* 1991; Perry *et al.* 1993). These transcripts are driven from separate promoter sequences, designated as P1 and P2, respectively, and located as shown in the figure. The her-1a transcript encodes the HER-1A protein, which appears to be necessary and sufficient for *her-1* masculinizing function. The her-1b transcript, predicted to encode the C-terminal portion of HER-1A if translated, has no known function. RNA-blot analysis (Trent *et al.* 1991) showed that both her-1a and her-1b transcripts normally accumulate only in males, but are present at elevated levels in either *sdc-1(lf)* or *sdc-2(lf)* mutant XX animals. This result suggested that the male specificity of her-1a and her-1b expression results from negative regulation by SDC-1 and SDC-2. Run-on transcription assays (Schauer and Wood 1990) detected the her-1b transcript only in nuclear extracts from embryo populations containing males, further suggesting that her-1b is regulated male specifically at the level of transcription. Finally, a combination of genetic and molecular studies showed that two independently isolated *her-1(gf)* mutations, both resulting in the same single nucleotide substitution in the P1 basal promoter, cause elevated accumulation of both her-1a and her-1b mRNAs in XX animals (Perry *et al.* 1994). These results implicated transcriptional repression in the male-specific regulation of the *her-1* gene as well as possible coregulation of P1 and P2.

In this article, we show that transgenes, including *her-1* P2 promoter regions, can mimic effects of *sdc(lf)* mutations, and that these transgenes may act by *in vivo* titration of SDC gene products. We have used this effect as an assay to locate elements in the *her-1* promoters that contribute to the putative titration and therefore are likely to contain *cis*-regulatory sites. Finally, we have explored the possibility that her-1b transcripts could play a role in regulation of *sdc* gene functions.

MATERIALS AND METHODS

Strains, culture conditions, and genetic crosses: The *C. elegans* strains listed below were obtained from our collection or the *Caenorhabditis* Genetics Center, or were constructed from these strains and maintained using standard methods (Sulston and Hodgkin 1988). They are, N2 (wild type), TY1807 *xol-1(y9)*X, CB190 *unc-54(e190)*I, CB950 *unc-75(e950)*I, BW1945 *unc-75(e950)*I; *lon-2(e678) xol-1(y9)*X, BW1756 *her-1(y101hv1)*V; *xol-1(y9)*X, PA43 *him-8(e1489)*IV; *her-1(y101hv1)*V, BW1801 *dpy-11(e224) her-1(n695)*V; *lon-2(e678) xol-1(y9)*X, and BW1790 *her-1(n695)*V *unc-42(e2700)*; *lon-2(e678) xol-1(y9)*X.

Constructs: Plasmids were constructed using conventional molecular cloning techniques (Sambrook *et al.* 1989; Maniatis *et al.* 1990). PCR techniques were used to make constructs with either an HA tag or specific site alterations. For simplicity, we have arbitrarily referred to the 3.4 kb of DNA upstream of *her-1* exon 3 as P2 or the P2 region. This interval includes all of intron 2 and 80 bp of exon 2 sequence. We have referred to a region that includes 2.5 kb upstream of exon 1 and 0.3 kb of coding sequence as P1 or the P1 region (see Figure 2); the sequences farther upstream encode another gene product. For consistency, all constructs used for deletion assays were made with the *gfp* vector pPD95.67 (kindly provided by A. Fire, Carnegie Institution of Washington). The construct pF10HA includes P2, exon 3, intron 3, exon 4 with an HA tag sequence fused in-frame behind the last predicted codon of *her-1*, and 0.5 kb of 3′ flanking region. In pRH5, *gf*-site disruption refers to alteration of AGGGA [the central G is mutated to A in *her-1(gf)* mutants] to ATATC.

Germ-line transformation and analysis of transgenic animals: Germ-line transformation (Mello *et al.* 1991) was used to test the *in vivo* effects of DNA constructs as transgenes. For all DNA injections, a plasmid carrying a dominant mutant *rol-6* gene (pRF-4; Mello *et al.* 1991) was coinjected as a transformation marker that causes a Roller (Rol) phenotype. Solutions for coinjection into *xol-1(y9)* contained a *her-1* promoter construct at 150 ng/μl and pRF4 at 100 ng/μl. For *rol-6*-only controls, the solution contained 200 ng/μl pRF4. Although there may have been small differences in molar concentrations of plasmids among the solutions of deletion constructs injected, it is unlikely that these would have affected the results, for two reasons. First, because 60–90% of the DNA in each construct is contributed by the 4.5 kb of vector sequences, molar differences resulting from differences in construct size were less than a factor of two, which is less than the variation in volume of solution injected. Second, when the construct pBG1 was injected at 80 ng/μl and 150 ng/μl, the effects

observed were similar, indicating that for this construct 80 ng/μl is already saturating.

Transmitting lines: After injection of hermaphrodites and production of self-progeny, all F_1 Rol animals were picked to new plates (two worms/plate) and allowed to self-fertilize. Each plate with five or more F_2 Rol transformants was scored as a transmitting line. The major parameter used to analyze the outcome of each injection was whether XX animals were masculinized to the extent of developing male tails, since this is the most obvious phenotype for scoring. We observed that the same construct can masculinize to different extents in different transmitting lines, probably due to the variation of the arrays both in size (copy number) and in organization. Since the percentage of masculinized lines among total lines should reflect the potency of a construct in the putative titration, these percentages were used as one measure in the assays of deletion constructs. A second measure used was how extensively each line was masculinized. To avoid possible variation among generations in this measure, only the F_2 was scored for percentage of masculinized transformants.

Integrated lines: Two integrated arrays were generated from transmitting lines as described in Perry *et al.* (1993). *ctIs1* was derived from a transmitting array made with the *P2::lacZ* construct pWLG1 and a *rol-6* marker plasmid and was mapped to LG I (Perry *et al.* 1993). *ctIs32* was derived from a transmitting array made with the *P2::gfp* construct pBG1, containing the same promoter sequences as *ctIs1*; the integrated array was shown to be autosomal but was not further mapped. The structures of the two constructs are shown in Figure 2. To test these integrated arrays for masculinization and dosage-compensation-suppression effects, males heterozygous for an array were mated to strain BW1945, and surviving XO progeny were scored as described in the footnote to Table 1.

Microscopy: Worms were mounted on 2% agarose or 5% agar pads, immobilized with 10–20 mm sodium azide solution (modified from Sulston and Hodgkin 1988), and photographed using a 20× or 40× objective on a Leitz microscope equipped with differential interference contrast (Nomarski) optics.

Genomic DNA extraction, Southern blotting, and data analysis: *C. elegans* genomic DNA was extracted from lines carrying *ctIs1* and *ctIs32* using a variation of a published procedure (Emmons *et al.* 1979). DNA was digested with *Bam*HI and the fragments were fractionated on a 1% 0.5 × TBE (Tris-borate and EDTA) gel and then transferred by capillary blotting to a nylon filter. DNA probes containing either P2 sequences or the SL1-5s rDNA sequence (obtained from T. Blumenthal), which is present at ~110 copies per haploid genome (Ferguson *et al.* 1996), were labeled by random primer extension (Feinberg and Vogelstein 1983), hybridized, and washed using standard methods. For the Southern blots shown in the figures, band intensities were quantitated by PhosphorImager analysis and normalized using intensities of bands recognized by the control SL1-5s rDNA probe. Estimation of P2 transgene copy numbers in the two integrants *ctIs1* and *ctIs32* was based on Southern blots probed with P2 DNA sequences, which hybridize both to a band corresponding to the endogenous *her-1* sequence as well as to the P2 transgenes (data not shown).

RESULTS

Some background on negative regulation of the *sdc* genes by *xol-1* (Figure 1) may be helpful in understanding our assays for possible titration of *sdc* functions by transgenic *her-1* promoter sequences. In strains carrying the strong *lf* mutation *xol-1(y9)*, the XX animals appear to be normal hermaphrodites, while XO animals are inviable, dying as partially feminized embryos or young larvae due to inappropriately high *sdc* gene activities, which result in activation of dosage compensation, inappropriate reduction of X-linked gene expression, and repression of *her-1* function (Miller *et al.* 1988). When an *sdc(lf)* mutation is introduced into the *xol-1(y9)* background, XO animals become viable and develop as males, while XX animals become either Dumpy (Dpy) or inviable, due to lack of dosage compensation and resulting overexpression of X-linked genes (see Figure 1). A similar result can be obtained by introducing an *lf* mutation in one of the DCD genes into the *xol-1(y9)* background, except that these mutations do not result in masculinization of the XO animals. In fact, DCD gene *lf* mutations exert a feminizing effect (elimination of dashed arrow in Figure 1), presumably by disrupting the formation of the dosage compensation complex and thereby freeing SDC proteins to repress *her-1* (Villeneuve and Meyer 1987, 1990; Nusbaum and Meyer 1989; Plenefisch *et al.* 1989; Davis and Meyer 1997). Therefore, factors that inhibit the synthesis or functions of SDC proteins should decrease the viability of XX

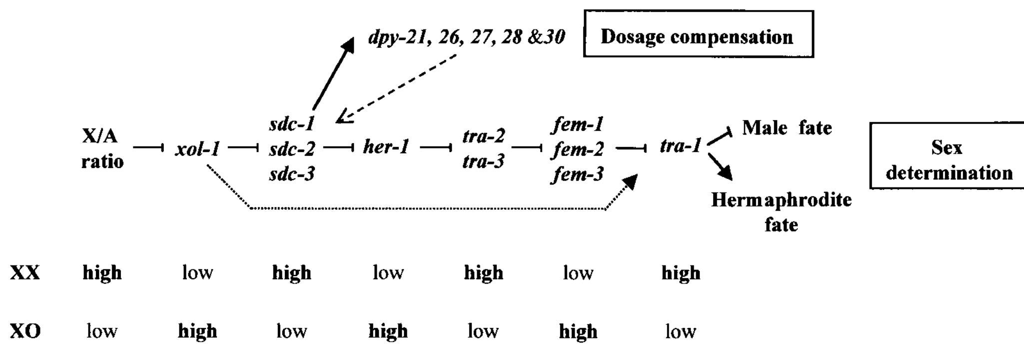

Figure 1.—The proposed regulatory pathway governing sex determination and dosage compensation in *C. elegans* (modified from Perry *et al.* 1993). Genes are named for their *lf* phenotypes: *xol-1* (*XO-lethal*), *sdc* (*sex* and *dosage compensation*), *her-1* (*hermaphroditization*), *tra* (*transformer*), *fem* (*feminization*), and *dpy* (*dumpy*). Arrows and the symbol ⊣ indicate positive and negative regulation, respectively. The arrow with a dotted line refers to an apparent masculinizing role of *xol-1* in XX animals (Rhind *et al.* 1995; see discussion). The arrow with a dashed line refers to the "loop" feedback effect from dosage compensation to sex determination (Davis and Meyer 1997). The terms "high" and "low" refer to the activity status of the sex-determining genes in XX and XO animals.

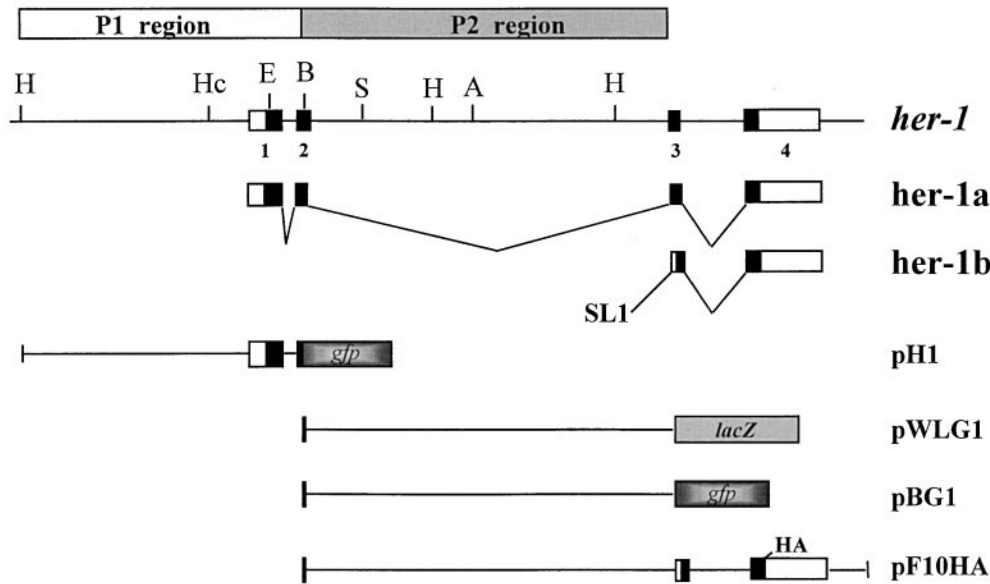

Figure 2.—*her-1* promoter regions, gene structure, transcripts (her-1a and her-1b), and constructs used to make transgenic arrays of promoter regions. In the P1::*gfp* construct pH1, including 2.5 kb of promoter sequences, the *gfp* gene is fused in-frame to exon 2 of *her-1.* pWLG1 and pBG1 are P2 promoter fusions to *lacZ* and *gfp*, respectively; they include no exon 3 sequences. pF10HA is a translational fusion of P2 and the predicted HER-1B coding sequence to two repeats of an HA tag sequence at the C terminus, followed by 0.5 kb of *her-1* 3′ flanking sequence (see materials and methods).

animals, whereas they should suppress the inviability of *xol-1(y9)* XO animals. If a functional *her-1* gene is present, such factors should also masculinize XX animals and suppress the feminization of *xol-1(y9)* XO animals. *xol-1(lf)* mutants provide convenient strains with which to assay such factors. We shall refer to this manner of suppressing *xol-1(lf)* XO inviability as dosage-compensation suppression (DCS).

Multiple copies of *her-1* P2 promoter sequences appear to inhibit *sdc* functions *in vivo*: *Preliminary observations:* We surmised that the P2 region of the *her-1* gene might be able to inhibit *sdc* gene products based on the following observations. First, a construct containing the 3.4-kb P2 region (pF10HA; Figure 2) was observed to cause apparent hermaphrodite-specific lethality when carried by transgenic worms as an extrachromosomal array (data not shown). Second, an integrated array (*ctIs32*) that included a *P2::gfp* construct (pBG1; Figure 2) appeared to cause a DCD-like Dpy phenotype in XX animals, although not apparent lethality.

Tests for DCS and masculinization by P2 arrays in a xol-1 (lf) background: To investigate the possibility that P2 promoter sequences could titrate *sdc* products and thereby disrupt dosage compensation, we first tested whether an integrated array of the P2-containing construct pBG1 (Figure 2) could suppress the inviability of *xol-1(y9)* XO animals. To do so, we crossed individual males heterozygous for the integrated array (*ctIs32/+*) to *unc-75* I; *lon-2 xol-1* X hermaphrodites (Figure 3a). Any non-Unc Lon progeny from this cross would have to be surviving *lon-2 xol-1* XO animals. If all animals of this genotype survived, 50% of the cross (non-Unc) progeny should be Lon, and half of these should carry the array. As shown in Table 1, about 70% of the predicted Lon animals carrying the array survived to adulthood, although they grew slowly and appeared unhealthy. Moreover, all these animals appeared to be complete males, based on gonad (data not shown) and tail morphology (see Figure 4). Although their tails appeared less well developed than those of wild-type males, this imperfection seemed likely to be a result of general unhealthiness rather than incomplete masculinization, since no Lon animals were observed with intersexual phenotypes. Males of this genotype from similar experiments described below were shown to be capable of mating, further supporting the view that these are complete males. These results showed that, like *sdc(lf)* mutations, an integrated array containing P2 sequences can result in DCS and masculinization effects on *xol-1(y9)* XO animals.

To observe the effects of an extrachromosomal array containing P2 sequences, we generated a transmitting line of genotype *xol-1(y9);Ex(pBG1, pRF4)* by coinjecting pBG1 and the *rol-6* marker plasmid pRF4 into *xol-1(y9)* hermaphrodites. Animals of this strain were variably Dpy, consistent with X-chromosome overexpression, and although most if not all should have been XX, they were variably masculinized (phenotypes ranged from apparently complete males, slightly dumpier than wild-type XO males, to animals with masculinized tails but also a hermaphrodite gonad and vulva). To verify karyotype and test their degree of masculinization, several of the most masculinized animals were mated with *unc-75* hermaphrodites. Of the F_1 non-Unc cross-progeny produced, all were hermaphrodites (71/71), indicating that the parental males were capable of mating, one of the strictest criteria for complete masculinization, and were XX in karyotype. The F_1 cross-progeny included many non-Unc animals carrying the array; the observation that none of these were masculinized indicates that the array had no appreciable masculinizing effect on

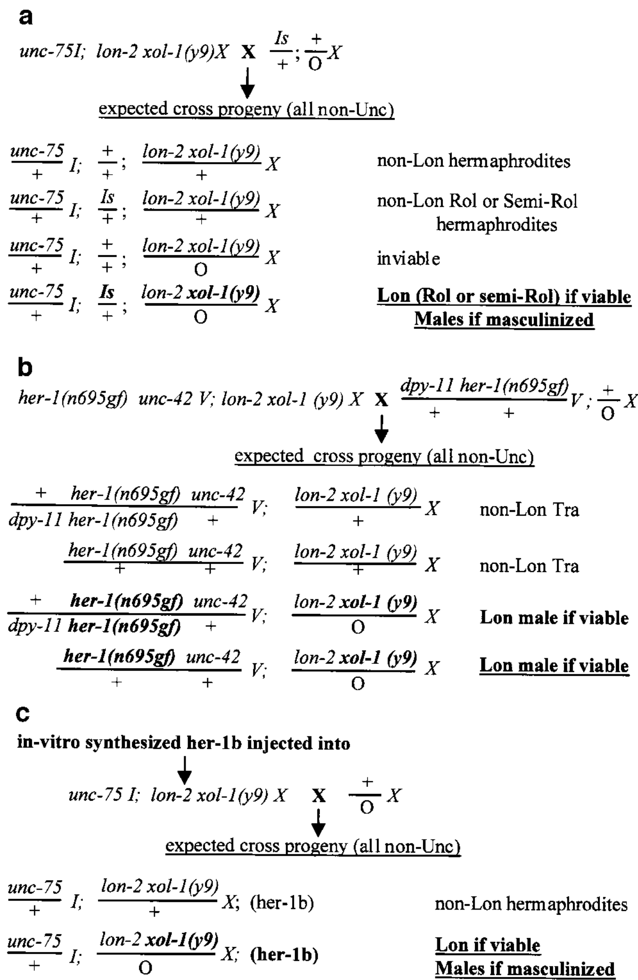

Figure 3.—Diagram of crosses used to test for suppression of *xol-1(y9)* XO lethality and masculinization by transgenic arrays of *her-1* promoter regions or presence of her-1b RNA. For each cross, parental genotypes are shown with the hermaphrodite on the left and the male on the right. Key genotypes and phenotypes are in boldface. The recessive, X-linked *lon-2* marker is used in all crosses for recognizing *xol-1(lf)* homozygous or hemizygous animals, and the *unc* markers for recognizing self-progeny. (a) Can an integrated array of *her-1* P2 promoter sequences rescue and masculinize *xol-1(lf)* XO animals? *Is* stands for either of the integrated arrays *ctIs32* or *ctIs1* (see materials and methods). *ctIs1* is located on LG I, as is *unc-75*; *ctIs32* is autosomal but its linkage group was not determined. The appearance of Lon males in the progeny indicated that the arrays can both rescue the viability and masculinize XO animals, which would otherwise be inviable due to overexpression of *sdc* genes and hermaphrodite due to resulting repression of *her-1* expression. (b) Can ectopic expression of *her-1* transcripts in *xol-1(lf)* XO animals rescue their viability? The semidominant *gf* allele *her-1(n695)* results in expression of both *her-1* transcripts under normally repressing conditions of *sdc* gene expression [*e.g.*, in XX hermaphrodites or *xol-1(y9)* XO animals]. The appearance of a small number of Lon male progeny suggests that her-1b RNA could weakly suppress *sdc* function, occasionally preventing inappropriate dosage compensation to the extent that these animals could survive. (c) Can injected her-1b RNA suppress *sdc* functions to rescue viability and masculinize *xol-1(y9)* XO animals? Sense RNA corresponding to her-1b was synthesized *in vitro* as described in Powell-Coffman *et al.* (1996). This RNA at a concentration of 2 mg/ml was injected into *unc-75(e950)xol-1(y9)lon-2(e679)* hermaphrodites that had been mated to N2 males. Lon non-Unc progeny were scored among the non-Unc cross-progeny as the surviving XO animals. Brood sizes of the injected animals were greatly reduced. Appearance of a small number of Lon hermaphrodites among the progeny suggests that her-1b RNA could suppress *sdc* function in a few *xol-1(y9)* XO animals to the extent that they could survive (but not sufficiently to derepress *her-1* and cause masculinization). See text for further description of results.

XX animals heterozygous for *xol-1(y9).* Likewise, homozygous *xol-1*(+) animals carrying a similar array showed no apparent masculinization. However, the above results show that the P2-containing array substantially masculinizes XX animals in the homozygous *xol-1(y9)* background, which is known to be sensitized for masculinizing effects (see discussion).

To test the ability of this transmitting array to suppress the inviability and feminization of *xol-1(y9)* XO animals, we mated N2 males with the least masculinized Rol animals from the transgenic line above. In addition to the variably masculinized self-progeny, we observed thinner Rol and semi-Rol apparently complete males that were candidates for surviving *xol-1/0* animals. When these were mated to *unc-75* hermaphrodites, about half the non-Unc progeny produced (19/34) were male, confirming the XO karyotype of the parental males. These results show that the array allowed survival of *xol-1(y9)* XO animals (no quantitation of DCS was attempted in this experiment) and efficiently suppressed their feminization, allowing them to develop into complete, functional males. As a control, we performed parallel experiments with two transmitting lines obtained by injection of the *rol-6* marker plasmid alone into *xol-1(y9)* hermaphrodites and observed neither masculinized XX self-progeny nor surviving masculinized XO progeny following mating to N2 males.

Correlation of masculinizing and DCS effects with transgene copy number in two integrated arrays: We observed differences in the effects of transgenic P2 sequences on DCS and masculinization in two integrated lines carrying P2 reporter constructs that were suspected from expression experiments to differ in transgene copy number. In the *ctIs1* line, made with the P2::*lacZ* construct pWLG1, expression of the reporter was male specific, suggesting that the transgenic P2 sequences were appropriately regulated (Perry *et al.* 1993). By contrast, the *ctIs32* line, made with the P2::*gfp* construct pBG1, expressed the reporter in both XX and XO animals, consistent with the possibility that a higher copy number of P2 transgenic sequences had saturated the normal *sdc* regulation of sex-specific P2 expression. When one chromosomal copy of *ctIs32* was introduced into the *xol-1(y9)* background, both inviability and feminization of XO animals were suppressed as described above. By con-

TABLE 1

Comparison of *ctIs1* and *ctIs32* in suppression of XO lethality in the *xol-1(lf)* mutant

Integrated array	No. surviving XO progeny	% males among XO progeny (*n*)	No. XX progeny	Estimated % of surviving XO progeny with array
ctIs1	0	—	172	0
ctIs32	77	100 (77)	222	70

Males heterozygous for an integrated array were mated to *unc-75 I; lon-1 xol-1 X* hermaphrodites. Surviving XO progeny were scored as Non-Unc, Lon animals, which were hemizygous for *xol-1* and heterozygous for the autosomal array. XX progeny were scored as non-Unc non-Lon animals. Estimated percent survival of XO animals carrying the array is based on the number of XX progeny, assuming survival of all XX progeny, equal numbers of XX and XO embryos, and transmission of the array to half the XO embryos.

trast, no suppression of XO inviability was observed in a parallel experiment with *ctIs1* (Table 1). To ask whether these differences corresponded to different copy numbers of P2 sequences in the integrated arrays, we performed Southern blot analysis on genomic DNA extracted from *ctIs32* and *ctIs1* lines, normalizing the P2 signal by comparison to the signal from a control probe for the SL1-5S-rDNA sequence, which is present in a tandem array of ~110 copies per haploid genome. The results, shown in Figure 5, indicate that *ctIs32* contains 6.4 times more P2 sequences than *ctIs1.* In an independent comparison of band intensities from the endogenous *her-1* gene and the P2 transgenes using P2 sequence to probe Southern blots of DNA from the same two strains, we estimated that *ctIs1* and *ctIs32* contain 7–10 and 45–64 copies of P2 sequences per array, respectively (data not shown; see materials and methods). These results are consistent with the possibility that the DCS and masculinization effects of transgenic P2 sequences are correlated with their copy number, as might be expected for an *in vivo* titration effect. Although other explanations are possible (see discussion), we shall use this term for convenience throughout the remainder of the article.

Dependence of masculinization effects on endogenous her-1 function: If the Sdc-like phenotypes described involve derepression of *her-1* by titration of negative regulators, then the observed masculinization of *xol-1(y9)* animals by P2 arrays should be dependent on endogenous *her-1* activity. To test this supposition, we injected pBG1 and the *rol-6* marker plasmid into a *her-1(y101hv1);xol-1(y9)* strain, in which the endogenous *her-1* gene is nonfunctional (Trent *et al.* 1991). In both of the two transmitting lines obtained, we observed no masculinized animals over several generations. However, both lines displayed the variably severe Dpy phenotype characteris-

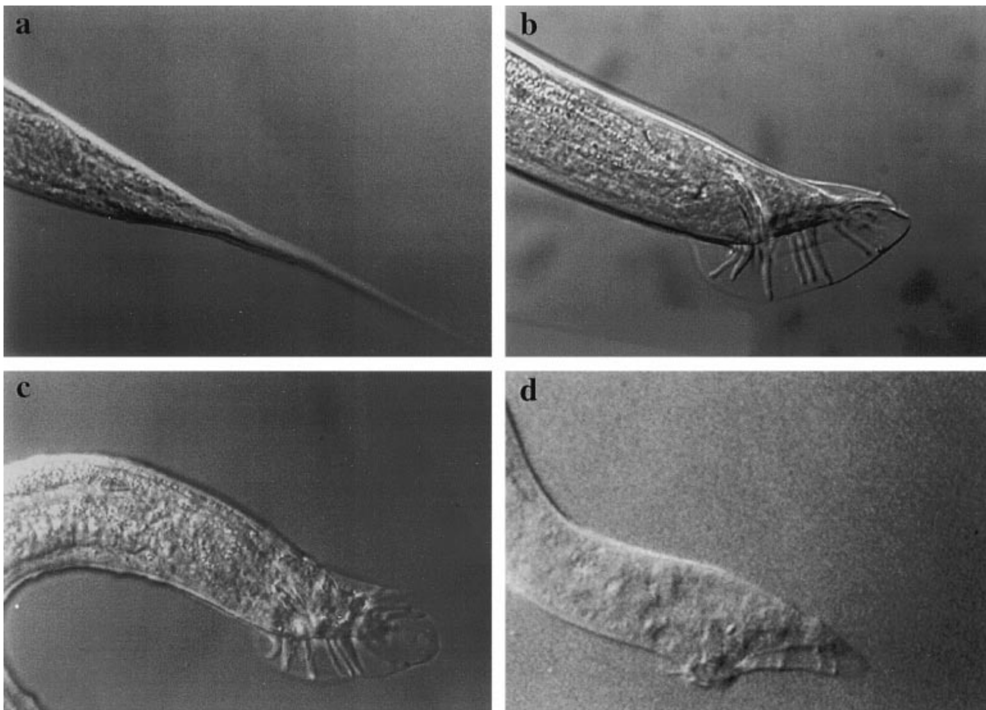

Figure 4.—Masculinized tail phenotypes caused by the apparent titration effects of P2 constructs in *xol-1(lf)* animals. The four parts show the tail regions of (a) a *xol-1(lf)* XX hermaphrodite, which has a wild-type morphology; (b) an N2 male; (c) a *xol-1(lf)* XX animal masculinized by a titration construct (pBG11; see Figure 6); (d) a *xol-1(lf)* XO animal rescued and masculinized by the integrated array *ctIs32.* The tails in these latter animals were not well developed, in contrast to *xol-1(lf)* XO animals rescued by the extrachromosomal array pBG1, which were complete mating males similar to (b) in tail morphology (not shown).

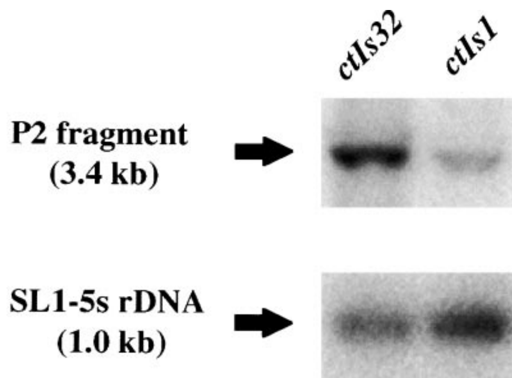

Figure 5.—Southern blot showing that *ctIs32* (left lane) contains a higher copy number of P2 fragments (3.4-kb band) than *ctIs1* (right lane). (Top) Probed with P2 sequences. (Bottom) Probed with SL1-5s rDNA as a loading control. Quantitation by PhosphorImager showed that the *ctIs32* copy number was higher by a factor of 6.4. See text for further explanation.

tic of X-chromosome overexpression resulting from defective dosage compensation. These results confirm that a functional endogenous *her-1* gene is required for masculinization but not for DCS in *xol-1(y9)* XX animals. The transgene effect, therefore, is exerted downstream of *xol-1* and upstream of *her-1* (see Figure 1), strongly implicating as targets one or more *sdc* gene products, which act as negative regulators of *her-1* and positive regulators of dosage compensation as reviewed in the Introduction.

Multiple regions in P2 contribute to the titration effect: The apparent titration effect described above appeared to provide a means of identifying negative *cis*-regulatory elements in the *her-1* promoter regions. Using this effect as a semiquantitative assay, we tested a series of deletion constructs, shown in Figure 6, for ability to masculinize *xol-1(y9)* when present in transmitting arrays with the *rol-6* marker plasmid (see materials and methods). Several to many transmitting lines were scored for each construct. Two measures were used to assess masculinizing activity for each construct: (1) percentage of transmitting lines that produced masculinized animals, and (2) percentage of total masculinized individuals among the F_2 transgenic animals scored. To facilitate description of these experiments, we have labeled three segments of the P2 region as A, B, and C in Figure 6. The results are presented in Table 2 and qualitatively in the right column of Figure 6.

One set of constructs, derived from the starting construct pBG1, included a series of deletions from the distal end of the P2 region that removed increasing amounts of segment A, then segment B, and then segment C (pBG2, 3, 4, and 5). pBG3, which is missing segment A, still exhibited masculinizing activity, though less than that of the entire 3.4-kb P2 region. In contrast, pBG4, which is missing both A and B, did not masculinize. These results suggest that both segment A and segment B include elements involved in titration. The results with pBG6 and pBG7, both missing segment B, confirm that this region is necessary for titration. Furthermore, pBG8, which contains segment B only, caused a low level of masculinization. We conclude that the 0.4-kb segment B is likely to contain one or more element(s) responsible for the titration effects of the P2 region, while segment A may contain additional elements that assist in the titration.

We also tested constructs with deletions from the proximal end of P2 for their masculinizing activity. Since pBG10, including all three segments A, B, and C, exhibited essentially the same masculinizing activity as the parental construct pBG1, it appears that the most proximal 0.5 kb of P2 is not required for titration. When segment C was removed, as in pBG9, the masculinizing activity decreased to a level almost as low as that observed with segment B alone. This suggests that region C, which by itself had no masculinizing activity (see the result with pBG4), can substantially enhance the masculinizing activity of segments A and B (*e.g.*, see pBG1, 2, and 3) and therefore also contains one or more elements that are important for titration by the P2 region.

To investigate whether the observed additivities of titration effects by different regions depend on *cis* interactions in the same construct sequence, we coinjected pBG8 and pBG7, which alone show little or no masculinizing effect, respectively, but together contain all the sequences present in pBG1. In three independent coinjected transmitting lines, no masculinization was seen (Table 2), indicating that when placed *in trans*, segment B does not complement segments A + C in the titration assay. These results suggest that the observed additivities of titration effects with the different segments depend on their normal organization in the P2 region.

P1 sequences that include a functional *gf* site can titrate when combined with some P2 sequences: We also investigated whether P1, defined as the 2.5-kb sequence upstream of the start site for the larger transcript her-1a plus 0.3 kb of the coding sequence, can titrate regulatory proteins *in vivo.* The construct pRH1 (Figure 6), which includes P1 only, did not masculinize *xol-1(lf)* XX animals, indicating that the P1 sequences do not titrate by themselves. However, the P1 sequences can enhance titration by segments A + B from P2, which alone showed only low masculinizing activity (compare pRH2 with pBG9).

Results with pRH3 suggested that promoter-proximal sequences participate in the P1-enhanced titration. Comparison with pRH4, which carries none of the promoter sequence, further directed our attention to the basal promoter region of P1 (failure to detect any titration with this construct, rather than a low level as expected from the pBG9 result, may be due to the low number of transmitting lines assayed; see Table 2). This region includes the genetically identified *gf* site defining a likely target for negative regulation of *her-1* (see Introduction). To test whether this site is important for P1-

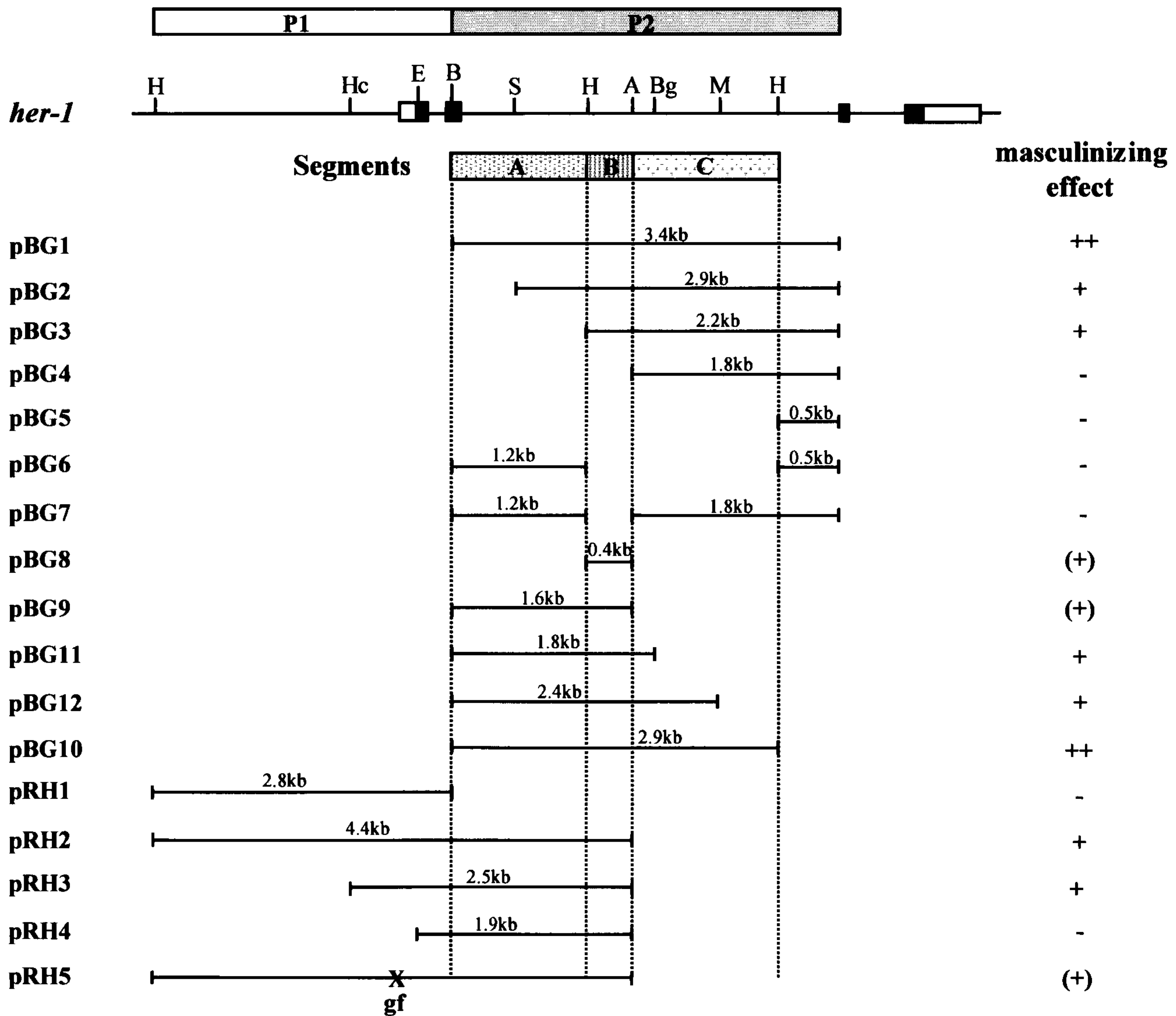

Figure 6.—The deletion constructs of *her-1* used for assaying the apparent titration effect. All constructs carry the *gfp* gene in addition to the indicated promoter sequences (see materials and methods). Segments A, B, and C are shown in shaded boxes. In pRH5, X(gf) is a disruption of the P1 *gf* site (see materials and methods). The titration effect (masculinizing effect; right column) was measured as ability to masculinize XX *xol-1(lf)* animals (see Table 2 and text). ++, strong titration effect; +, some titration effect; (+), titration effect detectable but at a very low level; −, no titration effect. The quantitative results corresponding to these symbols are presented in Table 2. H, *Hin*dIII; Hc, *Hin*cII; E, *Eco*RI; B, *Bam*HI; S, *Sph*I; A, *Avr*II; Bg, *Bgl*II; M, *Msc*I.

enhanced titration, we mutated the *gf* site (see materials and methods) to produce pRH5. This construct titrated significantly less effectively than the parent construct pRH2, suggesting that an intact *gf* site is important for P1-enhanced titration. We conclude that at least one P1 site and multiple P2 sites participate in the apparent titration of SDC proteins and, therefore, may play roles in the sex-specific negative regulation of *her-1* transcription.

Test for a possible regulatory activity of her-1b RNA: As mentioned above under *Preliminary observations*, the construct pF10HA, including both P2 and her-1b coding sequences (Figure 2), causes hermaphrodite-specific lethality in a wild-type background. In contrast, the construct pBG1, which also includes P2 but lacks her-1b coding sequences, does not. Four transmitting lines made by injecting constructs including only the her-1b coding sequences exhibited no apparent lethality (data not shown), indicating that these sequences as DNA are not toxic to XX animals. These results suggested the possibility that overexpression of the her-1b RNA in XX animals might result in DCS and thus cause Sdc-like XX lethality. We took two approaches to investigate this possibility. First, in genetic experiments we exploited the *gf* allele *her-1(n695)*, which is insensitive to *sdc* repression and thus results in constitutive overexpression of

TABLE 2

Masculinization of *xol-1(lf)* XX in transgenic lines carrying various *her-1* promoter-deletion constructs

Construct	% of masculinized lines (n)	% of total masculinized F_2 (n)	Relative severity of masculinization
pBG1	100 (5)	80 (40)	++
pBG2	27.3 (11)	14.7 (136)	+
pBG3	23.1 (13)	1.8 (655)	+
pBG4	0 (61)	0 (1914)	−
pBG5	0 (40)	0 (1930)	−
pBG6	0 (4)	0 (136)	−
pBG7	0 (28)	0 (1216)	−
pBG8	5.0 (20)	0.3 (916)	(+)
pBG9	4.5 (22)	3.8 (649)	(+)
pBG10	100 (7)	56.5 (138)	++
pBG11	62.5 (16)	21.5 (281)	+
pBG12	41.6 (12)	7.4 (376)	+
pRH1	0 (23)	0 (136)	−
pRH2	84.6 (13)	24.6 (240)	+
pRH3	100 (2)	10.2 (59)	+
pRH4	0 (6)	0 (126)	−
pRH5	8.3 (12)	1.6 (256)	(+)
pBG7 + pBG8	0 (3)	0 (95)	−

both her-1a and her-1b. To test whether such overexpression could suppress the inviability of *xol-1(lf)* XO animals, we introduced this allele and an autosomal *unc* marker into a *lon-2 xol-1* background and mated the resulting hermaphrodites to males also carrying the *her-1(gf)* allele, so that surviving XO animals would be identifiable as non-Unc Lon (see Figure 3b). We observed only three viable Lon progeny, all males, in crosses expected to produce ~1000 XO Lon embryos. However, 3/1000 is significantly higher than the background frequency of viable *xol-1(lf)* XO animals, which have never been observed in our laboratory or to our knowledge in others (B. Meyer, L. Miller, personal communications). Second, we injected *in vitro*-transcribed her-1b RNA into *unc-75; lon-2 xol-1* hermaphrodites that had been mated with N2 males (see Figure 3c) to test for suppression of F_1 XO inviability. Two viable Lon animals were recovered among 125 cross-progeny. Both Lon animals were hermaphrodites, which together produced a total of only 26 progeny, 20 hermaphrodites and 6 males, all appearing to be unhealthy. Interpretation of this result is complicated by the small number of Lon survivors and the possibility that both could have been matriclinous XX animals generated by nondisjunction, which might occur at a higher than normal rate following RNA injection. However, the appearance of males among the self-progeny of these animals argues strongly that they were XO. Therefore, both experiments suggest that her-1b RNA may weakly inhibit *sdc* functions, but that if so this effect is unlikely to be significant in normal regulation of sex determination and dosage compensation.

DISCUSSION

Transgenic *her-1* P2 promoter sequences appear to titrate *sdc* gene products *in vivo*: On the basis of earlier observations of competitive titration by transgenic sequences in sea urchin embryos (Franks *et al.* 1990; Livant *et al.* 1991) and a few isolated observations of anomalous effects with *C. elegans* transgenes (reviewed in McGhee and Krause 1997), it was suspected that multiple copies of regulatory sequences in transgenic arrays might result in titration effects on activity of regulatory proteins, but this phenomenon has not been well documented in worms. Some anomalous transgene effects in *C. elegans* are now thought to result from RNA-mediated interference (RNAi; Fire *et al.* 1998; Montgomery *et al.* 1999), but others are probably not. In theory, titration by promoter sequences from a gene that is positively regulated should mimic the gene's *lf* mutant phenotype, whereas promoter sequences from a negatively regulated gene, such as *her-1*, should mimic a *gf* (overexpression) phenotype. As one likely example involving positive regulation, injection of a *fem-2* promoter::reporter construct containing no *fem-2* exon sequences into N2 hermaphrodite gonads can cause feminization of the germ line, which is the *fem-2(lf)* phenotype (D. Pilgrim, personal communication), implying that an activator of *fem-2* transcription is titrated in the syncytial germ line. A possible example involving negative regulation is the apparent titration of SEX-1, a repressor of *xol-1* transcription, by transgenic arrays carrying *xol-1* promoter sequences (Carmi *et al.* 1998). We have shown here that transgenic arrays of the *her-1*

P2 promoter can mimic *her-1(gf)* phenotypes, as if inhibiting a repressor, and several lines of evidence suggest that the observed effects could result from titration of the *sdc* gene products that negatively regulate *her-1* transcript accumulation in normal sex determination. In a sensitized *xol-1(lf)* background, in which masculinizing effects are enhanced (see below), P2 arrays strongly masculinize both XX and feminized XO animals, and this effect depends on presence of a functional endogenous *her-1* gene. Moreover, these arrays cause an apparent Dpy phenotype in XX animals and suppress the inviability of *xol-1* XO animals, which normally die as embryos or L1 larvae because of inappropriate dosage compensation activated by *sdc* gene derepression. Therefore, in many respects, the P2 arrays phenocopy the effects of *sdc(lf)* mutations, namely, masculinization of XX animals due to *her-1* derepression, as well as Dpy phenotypes among XX animals and rescue of *xol-1* XO animals as viable males due to suppression of dosage compensation. The differing DCS activities of two different integrated P2::reporter transgenes correlated with their ability to be sex specifically regulated and their copy number in the genome. Although other explanations such as transgene position effects for the observed activity differences are possible, these results are consistent with a titration model. In general, the transgene effects we have reported cannot be the result of RNAi (Fire *et al.* 1998; Montgomery *et al.* 1999) because many of the active constructs contain no exon sequences, and they cause *her-1(gf)*, rather than *lf*, phenotypes. Instead, our results suggest that the transgenic P2 sequences interact directly with one or more of the SDC proteins, so that they are no longer available to repress *her-1* and activate dosage compensation.

Advantages of titration assays for identifying possible *C. elegans* promoter elements: We utilized the apparent promoter titration effect as an alternative assay to study *her-1* regulation largely because previous approaches using more conventional techniques had been problematic. Due to lack of a reliable *in vitro* transcription system, *C. elegans* researchers have relied on transgenic techniques to study promoter activity (reviewed in McGhee and Krause 1997). However, because copy numbers cannot be well controlled in these experiments, quantitative comparisons of one transgenic construct with another are difficult or unfeasible, and conclusions are reliable only when large qualitative differences are observed. This limitation makes analysis of promoter elements such as those involved in *her-1* regulation, which modulate gene activity under different developmental conditions, very difficult to study using expression from transgenes. In contrast, the titration assay we have employed capitalizes on the high and variable copy number of transgenic sequences, yielding effects in the sensitized background that were dramatic enough to allow collection of sufficient data for comparisons from a reasonable number of transmitting lines for each deletion construct. We sought to maximize the usefulness of these data by using two different criteria in assaying titration effects for each construct: (1) percentage of lines producing masculinized animals and (2) percentage of total masculinized individuals among the F_2 animals scored. Results using these two criteria were in good agreement for most of the constructs tested. Nevertheless, since this assay is clearly only semiquantitative, we have based conclusions only on either qualitative or obvious quantitative differences.

Effects of the *xol-1(lf)*-sensitized background: Although we observed an apparent DCD phenotype from P2 arrays in wild-type XX animals, we found convincing masculinizing effects only when arrays were assayed in a *xol-1(lf)* background. The *xol-1(lf)* mutants would be expected to provide a sensitized genetic background for this assay, based on previous observations that *xol-1 (lf)* mutations enhance masculinization of the XX pseudomales resulting from weak *sdc(tra)*, *her-1(gf)*, or *tra-2 (lf)* mutations (Rhind *et al.* 1995). The still poorly understood feminizing function of *xol-1*(+) in XX animals indicated by these results (represented by the dotted arrow below the pathway in Figure 1) is considered to be separate from its *sdc*-repressing role in XO animals, both temporally and in terms of mechanism (Cline and Meyer 1996). In view of these effects, it is not surprising that *xol-1(lf)* mutations should also enhance the masculinizing effects of P2 transgenes.

One puzzle is that the pBG1 arrays show masculinizing effects almost equivalent to those of either *sdc(lf)* or *her-1(gf)* mutations in the *xol-1(lf)* background and yet, in contrast to these mutations, cause no apparent masculinization in the wild-type background. One possibility is that in wild-type XX animals, titration of the *sdc* gene products acts primarily to disrupt dosage compensation, resulting in suppression of any masculinization by a secondary effect via the "loop" feedback from dosage compensation to sex determination (dashed arrow in Figure 1; see Introduction). It has been well documented that *lf* mutations in DCD genes can suppress the masculinizing effects of a *her-1(gf)* mutation, an *sdc(lf)* mutation, or a 2X:3A karyotype (Cline and Meyer 1996). To explain this suppression, it has been suggested that when the dosage compensation complexes are disrupted, one or more *sdc* gene products are released and available to repress *her-1* (Davis and Meyer 1997). One more difference between P2 titration effects and *sdc(lf)* mutations is that in any such mutant, at least one of the SDC proteins is defective, whereas all are normal in the P2 titration experiments, so that they should be fully functional if they become available at the right place and time due to disruption of a complex.

Another puzzle is how as few as ~50 transgenic copies of the P2 region can compete for SDC proteins with the large number of SDC-binding sites on the X chromosome to the extent that dosage compensation is significantly impaired. A possible explanation could be that

one of the SDC proteins is limiting in the formation of SDC complexes, and that this protein is normally present in XX animals at just above the threshold concentration that allows complexes to form. In this situation a relatively small number of *her-1* promoter sequences—particularly if their affinity for the limiting protein were higher than that of sites on the X—might lower the effective concentration of the free protein to below threshold level so that complex formation would be disrupted.

Evidence for multiple negative regulatory elements in the *her-1* promoter: *The role of P2 sequences:* Use of the titration assay with appropriate deletion constructs allowed us to determine the contributions of three different segments of the 3.4-kb second intron of *her-1* to the titration effect and thereby point to regions likely to include regulatory elements that could interact with SDC proteins. Our results suggest that a small 0.4-kb region near the center of the intron contains the most active titrating sequences, but that its activity can be enhanced by more distal and more proximal sequences, none of which show appreciable titration activity alone.

The role of P1 sequences: It was known that in the *gf* site located 2 bp upstream of the cap site for the her-1a transcript, change of a G to an A causes derepression of *her-1* in XX animals (Perry *et al.* 1994). This result showed that P1 is involved in male-specific regulation. However, we were unable to demonstrate that P1 alone can confer male-specific regulation in studies with transgenic P1 reporter constructs (W. Li and W. B. Wood, unpublished results), and P1 sequences alone failed to show masculinizing activity in the titration assay. Therefore, we hypothesized that sites in the P2 region might confer male specificity on P1. In support of this view, constructs including the P1 promoter sequence, as well as some P2 sequences, did produce a titration effect much stronger than that seen with the P2 sequences alone. When we altered the *gf* site along with four adjacent nucleotides in such a construct, we eliminated the P1-enhanced titration activity, supporting the role of the *gf* site in *her-1* repression and the view that P2 sequences are essential for male-specific regulation of P1.

Why do some regions that are important for titration fail to titrate by themselves? It is possible that such regions affect only the conformation of DNA mediated by regulatory proteins bound at another site, or that by themselves these regions bind regulators too weakly to cause a measurable effect in the assay, or both. We also showed that organization of DNA segments in P2 appears to be important for titration, consistent with the hypothesis that a certain conformation of the *her-1* promoter elements is required for the repression. Thus the titration results together suggest that normal sex-specific repression of *her-1* is achieved by cooperative binding of one or more SDC gene products and perhaps other repressor proteins to multiple regulatory elements distributed within the P1 and P2 regions.

Does her-1b play a role in sex determination or dosage compensation? The functions, if any, of the her-1b transcript and its possible translation product remain a mystery. Studies involving reporter gene expression and immunostaining have suggested that her-1b is not translated (B. Robertson, M. D. Perry, W. Li, A. Streit and W. B. Wood, unpublished results). Previously, her-1b has been shown not to be required for the masculinizing function of the *her-1* gene (Perry *et al.* 1993). We have tested the possibility that her-1b RNA itself could play a regulatory role in feedback inhibition of *sdc* activity in XO animals, serving as a "guardian molecule" in XO animals to inhibit any residual *sdc* activities. However, the marginal effects observed suggest that such a role for her-1b is not likely to be significant, so the function of this RNA is still unclear.

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