DOI: 10.1534/genetics.107.078071

Revisiting the X:A Signal That Specifies *Caenorhabditis elegans* Sexual Fate

John M. Gladden, Behnom Farboud and Barbara J. Meyer[1]

Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3204

Manuscript received June 22, 2007
Accepted for publication September 2, 2007

ABSTRACT

In *Caenorhabditis elegans*, sex is determined by the opposing actions of X-signal elements (XSEs) and autosomal signal elements (ASEs), which communicate the ratio of X chromosomes to sets of autosomes (X:A signal). This study delves more deeply into the mechanism by which XSEs transmit X chromosome dose. We determined the relative contributions of individual XSEs to the X:A signal and showed the order of XSE strength to be *sex-1* > *sex-2* > *fox-1* > *ceh-39* ≥ region 1 XSE. *sex-1* exerts a more potent influence on sex determination and dosage compensation than any other XSE by functioning in two separate capacities in the pathway: *sex-1* acts upstream as an XSE to repress *xol-1* and downstream as an activator of hermaphrodite development and dosage compensation. Furthermore, the process of dosage compensation affects expression of the very XSEs that control it; XSEs become fully dosage compensated once sex is determined. The X:A signal is then equivalent between XO and XX animals, causing sexual differentiation to be controlled by genes downstream of *xol-1* in the sex-determination pathway. Prior to the onset of dosage compensation, the difference in XSE expression between XX and XO embryos appears to be greater than twofold, making X chromosome counting a robust process.

NEARLY 60 years have passed since the initial discovery that the nematode *Caenorhabditis elegans* determines its sex by counting the number of X chromosomes relative to the ploidy, the number of sets of autosomes (Nigon 1951; Madl and Herman 1979). Only recently have molecular genetic approaches revealed the components of this X:A sex-determining signal. A set of X-linked genes called X signal elements (XSEs) relays X chromosome dose, and a set of autosomally linked genes called autosomal signal elements (ASEs) relays the ploidy (Akerib and Meyer 1994; Hodgkin *et al.* 1994; Nicoll *et al.* 1997; Carmi *et al.* 1998; Carmi and Meyer 1999; Powell *et al.* 2005; reviewed in Meyer 2005; Gladden and Meyer 2007, accompanying article in this issue). Both sets of elements communicate chromosome dose by controlling the sex-determining gene *xol-1* (*XO l*ethal), the direct molecular target of the X:A signal. XSEs (repressors) and ASEs (activators) conduct a molecular tug-of-war ending in *xol-1* repression in XX animals and *xol-1* activation in XO animals. Once active, *xol-1* directs male development (Miller *et al.* 1988; Rhind *et al.* 1995). In its absence, hermaphrodite development ensues.

By regulating the activity of *xol-1*, signal elements control not only the choice of sexual fate, but also the rate of X-linked gene expression dictated by the process of dosage compensation (Miller *et al.* 1988; Akerib and Meyer 1994). This process equalizes X chromosome gene products between the sexes by reducing gene expression from both hermaphrodite X chromosomes by half (reviewed in Meyer 2005). In XO embryos, *xol-1* induces the male fate by repressing the activity of the hermaphrodite-specific *sdc* (*s*ex determination and *d*osage *c*ompensation) genes (Miller *et al.* 1988; Rhind *et al.* 1995). In XX embryos, SDC-2 acts with SDC-1 and SDC-3 to repress the male sex determination gene *her-1* and to assemble the dosage compensation protein complex (DCC) onto both X chromosomes (Villeneuve and Meyer 1987, 1990; Nusbaum and Meyer 1989; Nonet and Meyer 1991; Delong *et al.* 1993; Klein and Meyer 1993; Davis and Meyer 1997; Dawes *et al.* 1999; Chu *et al.* 2002; McDonel *et al.* 2006).

To date, XSE activity has been ascribed to four genes defined by mutations and to a 2-MU interval on X (called region 1) defined by chromosomal duplications and deficiencies. SEX-1 (a nuclear hormone receptor), CEH-39 (a ONECUT homeodomain protein), SEX-2, and the region 1 XSE act synergistically to control *xol-1* at the transcript level (Akerib and Meyer 1994; Nicoll *et al.* 1997; Carmi *et al.* 1998; Gladden and Meyer 2007, accompanying article; J. Powell, C. Y. Loh and B. Meyer, unpublished results). FOX-1 (an RNA-binding protein) controls *xol-1* at a post-transcriptional level (Nicoll *et al.* 1997). Both mechanisms of repression function together to ensure the fidelity of the X chromosome counting process. The predominant form of *xol-1* regulation by XSEs appears, however, to be transcriptional (Gladden and Meyer 2007). The autosomal

[1]*Corresponding author:* Department of Molecular and Cell Biology, Howard Hughes Medical Institute, 16 Barker Hall, MC 3204, University of California, Berkeley, CA 94720-3204. E-mail: bjmeyer@berkeley.edu

component of the X:A signal is represented by at least two ASEs. SEA-1 (a T-box transcription factor) and SEA-2 oppose XSEs by activating *xol-1* transcription (Powell *et al.* 2005; P. Nix and B. Meyer, unpublished results).

Recent studies (Gladden and Meyer 2007) have hinted that the earliest aspects of sex determination are controlled through a process more complex than first described (Carmi and Meyer 1999). These studies showed that individual XSEs make unequal contributions to the X:A signal but did not determine their relative contributions or assess whether the majority of XSEs had been defined. Furthermore, they showed that most XSEs appear less potent than SEX-1, but did not define the mechanism by which SEX-1 exerts its stronger influence on sex determination and dosage compensation. Finally, no prior studies have addressed whether the X signal elements used to convey X chromosome dose are themselves repressed through a feedback loop by the very dosage compensation process that they control. Our study provides answers to these questions.

MATERIALS AND METHODS

C. elegans* strains:** All *C. elegans* strains were derived from the Bristol variant N2 and were maintained as described in Brenner (1974). Abbreviations are as follows: *ceh* (C. e**legans* **h**omeobox), *Df* (*de*ficiency), *Dp* (*du*plication), *dpy* (*dumpy*), *egl* (*egg-l*aying defective), *fasn* (*f*atty *a*cid *syn*thase), *fox* (*f*eminizing gene *o*n *X*), *him* (*h*igh *i*ncidence of *m*ales), *nhr* (*n*uclear *h*ormone *r*eceptor), *sdc* (sex determination and *d*osage *c*ompensation), *sea* (*s*ignal *e*lement on *a*utosome), *sex* (*s*ignal *e*lement on *X*), *tra* (sexual *tra*nsformation), *unc* (*unc*oordinated), and *xol* (*XO l*ethal).

The following chromosomal aberrations and mutations were used for this study:

LG III: *dpy-27(y57)* (Plenefisch *et al.* 1989).

LG IV: *him-8(e1489), mIs11, yIs58*[*ceh-39(+),myo-2::gfp*]. *him-8 (e1489)* increases X chromosome nondisjunction, resulting in 37% XO, 57% XX, and 6% Dpy XXX animals (Hodgkin *et al.* 1979). *mIs11* is a multi-construct array carrying *myo-2::gfp, pes-10::gfp,* and *gut::gfp* integrated into LG IV near *dpy-20. yIs58* is an integrated array carrying the wild-type *ceh-39* gene and the co-injection marker *myo-2::gfp.*

LG X: *dpy-3(e27), unc-2(e55), ceh-39(y414), ceh-39(gk296)* (Vancouver group of the *C. elegans* Gene Knockout Consortium), *fox-1 (y303)* (Nicoll *et al.* 1997), *sex-2(y324)* (J. Powell and B. Meyer, unpublished results), *lon-2(e678), xol-1(y9)* (Miller *et al.* 1988), *dpy-6(e14), sex-1(y263)* (Carmi *et al.* 1998), *unc-3 (e151), meDf5 X* (Villeneuve 1994), and *yDf17* and *yDf20* (Akerib and Meyer 1994).

Duplications: *yDp14(X;I), yDp13(X;*f*)* (Akerib and Meyer 1994).

Mutations not referenced are described in this study or in Riddle *et al.* (1997).

Western blot analysis: For Western blot analysis of CEH-39 levels, embryos of different genotypes were prepared by washing gravid hermaphrodites with water, treating for 5 min in a 20% hypochlorite 5% sodium hydroxide solution, and then washing two times with M9. Embryonic extract was generated by boiling embryos in 3 vol of 2× SDS–PAGE loading buffer containing 7 M urea for 10 min. The supernatant was then loaded on a 10% precast polyacrylamide gel (Invitrogen, San Diego). The Western blot was performed with rabbit anti-CEH-39 peptide antibodies, mouse anti-tubulin (DM-1A) antibody (ICN Biochemicals), horseradish-peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Labs), and horseradish-peroxidase-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Labs). For Western blot analysis of SEX-1 levels, wild-type XX and *sex-1(y424)* XX embryo extracts were prepared as previously described (Chu *et al.* 2002), and 1 mg of protein from each extract was precipitated with trichloroacetic acid. Briefly, one-fourth volume of 4 mg/ml deoxycholic acid in trichloroacetic acid was added to an embryo lysate, vortexed, and centrifuged at 4° for 10 min at 13,000 × *g*. The supernatant was then removed, and 3 vol of acetone was added to the pellet, incubated at room temperature for 10 min, and centrifuged at room temperature for 10 min at 13,000 × *g*. After the supernatant was removed, the pellet was resuspended in 1× SDS–PAGE buffer and the equivalent of 2 μg of total protein was loaded per lane on a NuPAGE 4–12% Bis–Tris precast polyacrylamide gel (Invitrogen). After transfer to nitrocellulose, the Western blot was performed with rabbit anti-SEX-1 antibodies (Carmi *et al.* 1998) and horseradish-peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Labs). The Western blot was developed using the ECL Plus reagent (GE Healthcare), and the blot was then stripped and reprobed with anti-α-tubulin antibody, as above.

Isolation of *sex-1* null mutation: A *C. elegans* deletion library was constructed in the Meyer Lab and screened for a *sex-1* deletion following Michael Koelle's *C. elegans* Gene Knockout Protocol (02/09/03 update) retrieved from his Yale University website (http://info.med.yale.edu/mbb/koelle/). *sex-1* primers used were as follows:

Forward outer primer: TGCACACATGTGTAGTAGCGGC;
Reverse outer primer: CCTGGAGATTATTACGCAACCACG;
Forward inner primer: ACATGTGAAGGACTATACTAGG;
Reverse inner primer: TAGCCGCTTGCCTTCACTTTCG;
Forward poison primer: CTCCATACTACAGCCCTTCTGG;
Reverse poison primer: GTGCATACAGAAAGCGGTGTGTCAGG.

RNA interference: RNAi was conducted as described in Gladden and Meyer (2007, accompanying article).

Quantification of transcript levels: Quantitative RT–PCR (qRT–PCR) was performed as described in Gladden and Meyer (2007, accompanying article). In all cases, three independent growths of the stains were used for the measurements. RNA was isolated as described in Gladden and Meyer (2007) except that *sdc-2(y93,* RNAi) was grown on NG–agar plates overlaid with HT115(DE3), and the following strains were grown on NG–agar plates overlaid with HB101 bacteria: *dpy-27(y57), xol-1(y9), sex-1(y424),* and *xol-1(y9) sex-1(y424).* For *dpy-27(y57)* and *sex-1(y424)* animals, plates were monitored, and young males were removed prior to reaching adulthood.

Primers for qRT–PCR are as follows:

ceh-39+, CGAGGTTCGAGGAATTGGTG;
ceh-39–, TGGAACTGGAACTGGTAGTGC;
fasn-1+, GATCCATTTGCAACTGATTCC;
fasn-1–, GCTTGGTAAGGATGGTGGC;
fox-1+, ATGGGACAAACGCAGATTGG;
fox-1–, GGGATATTCGATACGTGAAGTC;
her-1+, ACCAGCCCTTCCATCGACGC;
her-1–, GCAGTATTCTTCGAATTGGAGC;
nhr-64+, TAGAGGAAATGCGACAACGG;
nhr-64–, CCCTCATTTGGTAGCATCAG;
sex-1+, ATGACATGCCGCATTGACGG;
sex-1–, AGGCAACGGAAGTGTTGAGAG;
xol-1+, TGTAATCGCCAAGTTCGAGC;
xol-1–, TTGAAATGCTCCGTTGTCCC.

Immunofluorescence microscopy: Embryos were fixed and stained as described in Gladden and Meyer (2007, accompanying article). Over 1000 embryos were examined for each genotype.

RESULTS

Relative contributions of individual XSEs to the X signal: Prior studies showed that individual XSEs do not contribute equally to the X signal but did not determine their relative contributions (Carmi and Meyer 1999; Gladden and Meyer 2007, accompanying article). This disparity in XSE strength was revealed initially by the wide range in XX lethality (0–30%) caused by the loss of individual XSEs. However, the extent of XX lethality cannot be used as the sole criterion to gauge relative XSE strength, because several XSEs, including *ceh-39*, *fox-1*, and *sex-2*, cause insignificant lethality when disrupted. Therefore, we assessed relative strength using a more sensitive assay that measured the degree to which an XSE mutation enhanced the XX lethality caused by the partial disruption of dosage compensation (Table 1). Incomplete dosage compensation was achieved by RNAi depletion of *sdc-2*, a central dosage compensation gene. No XX animals survive the complete loss of *sdc-2* function achieved by a null mutation, yet 81% of *sdc-2* (RNAi) XX animals survive, affording ample range for mutations to enhance lethality.

Both *ceh-39* alleles enhanced the XX lethality caused by *sdc-2*(RNAi) (Table 1). XX viability was reduced to 54 and 50% for *ceh-39(y414) sdc-2*(RNAi) and *ceh-39(gk296) sdc-2*(RNAi), respectively. *fox-1* appeared to have a more potent effect on the X signal: only 37% of *fox-1(y303) sdc-2*(RNAi) XX animals were viable ($P < 0.01$ for *y303* comparison to *y414* or *gk296*) (Table 1). Both *sex-1(y263)* and *sex-2(y324)* caused complete lethality in combination with *sdc-2*(RNAi), indicating that both *sex-1* and *sex-2* contribute more strongly to the signal than either *ceh-39* or *fox-1* (Table 1). Since *sex-2(y324)* causes only 1% XX lethality, but *sex-1(y263)* causes 30% XX lethality, *sex-1* appears to be a stronger XSE than *sex-2*. All five XSE mutant strains appeared unaffected by administration of control dsRNA, making it unlikely that the differences in viability reflect nonspecific RNAi effects (Table 1). This combined analysis shows that the XSEs defined by point mutations make the following relative contributions to the X signal: *sex-1* > *sex-2* > *fox-1* > *ceh-39*.

A more refined distinction in XSE strength between *fox-1* and *ceh-39* was made by comparing the extent to which *fox-1*(RNAi) or *ceh-39*(RNAi) suppressed the complete XO-specific lethality caused by two copies of the X duplication *yDp14* (Figure 1; Table 2). The XO lethality caused by *yDp14* is due to the increased dose of *fox-1*, *ceh-39*, and other potential XSEs, which repress *xol-1* and thereby activate dosage compensation. *fox-1*(RNAi) permitted 69% of *him-8; yDp14/yDp14* XO animals to live, and *ceh-39*(RNAi) permitted only 4% of XO animals to live. In contrast, no XO *Dp* animals were rescued by control dsRNA made from an RNAi vector lacking a cloned gene. Thus, *fox-1* appears to be a stronger XSE than *ceh-39*.

TABLE 1

The contributions of individual XSEs to the X signal are of different strengths

Genotype[a]	Hermaphrodite viability (%)[b]	*n*[c]
ceh-39(y414)[d]	101	1008
ceh-39(y414) + control RNAi[e]	100	900
ceh-39(gk296)[d]	102	1021
ceh-39(gk296) + control RNAi[e]	100	1134
fox-1(y303)[d]	99	1054
fox-1(y303) + control RNAi[e]	101	938
sex-1(y263)[d]	70	884
sex-1(y263) + control RNAi[e]	70	867
sex-2(y324)[d]	99	1032
sex-2(y324) + control RNAi[e]	99	794
sdc-2(RNAi)	81	1044
ceh-39(y414) sdc-2(RNAi)	54	1120
ceh-39(gk296) sdc-2(RNAi)	50	1079
fox-1(y303) sdc-2(RNAi)	37	1258
sex-1(y263) sdc-2(RNAi)	0	1072
sex-2(y324) sdc-2(RNAi)	0	1087
sex-2(y324) xol-1(y9) sdc-2(RNAi)[f]	93	1093
xol-1(y9) sdc-2(RNAi)	98	1211

[a] Animals were fed bacteria that produced dsRNA to *sdc-2*.
[b] Hermaphrodite viability was calculated by the formula: (no. of adult hermaphrodites)/(total no. of embryos) × 100.
[c] *n* is the total number of embryos from at least six independent sets of progeny counts.
[d] Data are from Gladden and Meyer (2007, accompanying article).
[e] Mutants were grown on bacteria carrying the L4440 empty vector. Data were acquired concurrently with the data in footnote *d*.
[f] This strain also has the *unc-76(e911)* and *unc-10(e102)* mutations.

Using a separate assay, Carmi and Meyer (1999) compared the relative contributions of *fox-1* and the region 1 XSE to the X signal. Relative XSE strengths were assessed by determining the extent to which XSE loss rescued the complete XO lethality caused by the single-copy X duplication *yDp13*, which increased the dose of the region 1 XSE, *ceh-39* (region 2), and *fox-1* (region 3) (Figure 1A). Region 1 deficiency *meDf5* permitted 38% of *meDf5; yDp13* XO animals to live. In contrast, 90% of *fox-1(y303); yDp13* XO animals lived, indicating that *fox-1* is more potent than the region 1 XSE. The combination of our data and that of Carmi and Meyer (1999) indicate that *sex-1*, *sex-2*, and *fox-1* make stronger contributions to the X signal than the region 1 XSE and that the overall order of XSE strength is *sex-1* > *sex-2* > *fox-1* > *ceh-39* ≥ region 1 XSE.

The dose of more than four XSEs must be reduced by half to toggle the X signal from the XX to the XO mode: In view of the modest contribution of each XSE

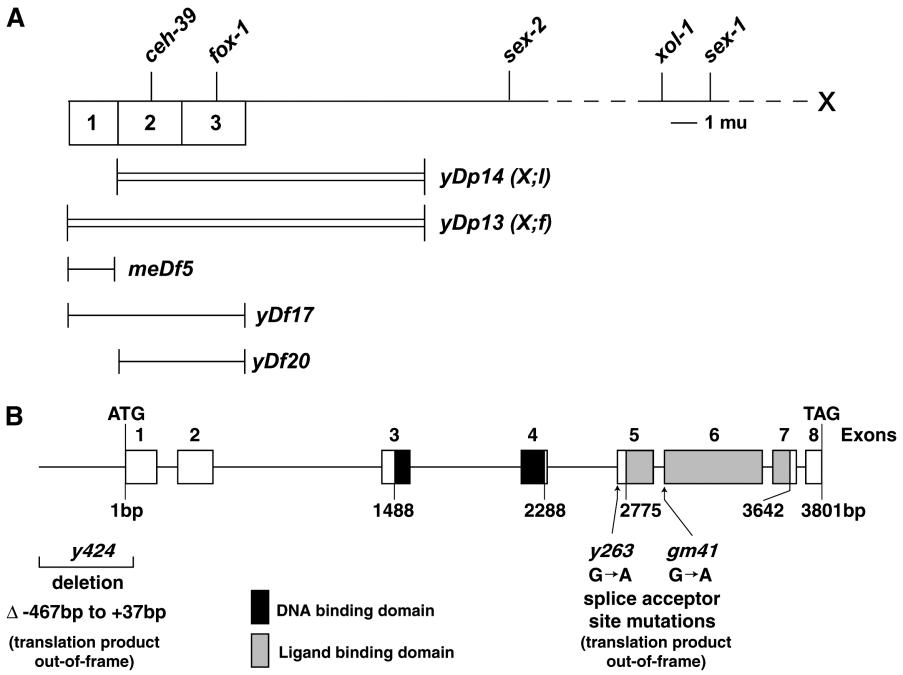

FIGURE 1.—Genetic map of the X chromosome and structure of the *sex-1* gene. (A) The X map shows XSEs and *xol-1* above the line and highlights three regions (numbered boxes) previously shown by duplications (double lines) and deficiencies (single line) to contain X signal elements. The free duplication *yDp13* covers region 1 (XSE not identified), region 2 (*ceh-39*), and region 3 (*fox-1*). The attached duplication *yDp14* covers regions 2 and 3. The deficiency *meDf5* removes region 1, *yDf17* removes regions 1–3, and *yDf20* removes regions 2 and 3. (B) The genomic region spanning *sex-1*. Exons are indicated by boxes and introns by lines. Exon sequences encoding the DNA-binding domain are solid and those encoding the ligand-binding domain are shaded. The *sex-1 (y424)* null allele reported in this study removes 504 bp of 5′ regulatory sequences, including the ATG translation initiation site and at least part of the promoter. The partial loss-of-function mutations *y263* and *gm41* introduce G-to-A transitions that mutate splice acceptor sites, causing translation products to be out of frame.

to the X signal, it was important to address whether the set of genetically defined XSEs constitutes the entire X signal. Since the twofold lower copy number of XSEs in XO embryos *vs.* XX embryos permits high *xol-1* expression and the male fate, one can determine whether the known XSEs constitute the entire X signal by asking whether halving the dose of these XSEs in XX animals activates *xol-1* and induces the male fate. Previous studies using large X deficiencies to reduce XSE dose in XX animals showed that halving the dose of three XSE regions caused mild-to-severe defects in sex determination and dosage compensation and that halving the dose of four regions was sufficient to switch the X:A signal to the XO mode, causing complete masculinization and lethality of XX animals (CARMI and MEYER 1999). To better estimate the minimum number of XSEs that contribute to the X signal, we assessed the effects of heterozygous XSE mutations on XX animals. In contrast to results using deficiencies, our results show that halving the dose of three or four XSEs only partially toggles the X signal toward the XO mode, suggesting that the deficiencies removed several additional, unidentified XSEs and that the X signal includes more XSEs than previously thought.

In our study, *fox-1*+/+ *sex-1* animals were wild type, and all *ceh-39* + +/+ *fox-1 sex-1* animals were viable but exhibited a mild Dpy, Egl phenotype, showing that halving the dose of three XSEs reduces the overall X signal (Table 3). However, the mild Dpy Egl phenotype was much less severe than the phenotype reported previously for *yDf20* +/+ *sex-1* XX hermaphrodites, in which the doses of at least *ceh-39*, *fox-1*, and *sex-1* were halved. Only 29% of the animals were viable, and escapers were Egl and Dpy (Table 3; Figure 1; CARMI and MEYER 1999). The phenotypic discrepancy between the two strains means either that *yDf20* removes other undefined XSEs or that *yDf20* removes other factors essential for the viability of hermaphrodites. Evidence presented in GLADDEN and MEYER (2007) indicated that *ceh-39* and *fox-1* are not likely to be the only XSEs in this region of X, thus making the former possibility the most probable.

fox-1, *ceh-39*, and *sex-1* repress *xol-1* using two different mechanisms, transcriptional (*sex-1* and *ceh-39*; see below) and post-transcriptional (*fox-1*). While halving the dose of both transcriptional (*ceh-39* and *sex-1*) and post-tanscriptional (*fox-1*) repressors of *xol-1* in XX animals caused Dpy and Egl phenotypes, halving the dose of only transcriptional regulators (*sex-1*, *ceh-39*, and *sex-2*) caused no obvious mutant phenotype (Table 3). Since *fox-1* and *sex-2* are of equivalent strength, we conclude that reducing the dose of XSEs that regulate *xol-1* on multiple levels disrupts *xol-1* repression more severely than reducing the dose of XSEs that regulate *xol-1* on the same level. This finding reinforces previous studies (CARMI and MEYER 1999).

As expected, reducing the dose of four XSEs by half using mutant alleles of *ceh-39*, *fox-1*, *sex-2*, and *sex-1* caused more severe phenotypes than halving the dose of only three XSEs. Approximately 60% of the heterozygous quadruple mutant *ceh-39 fox-1* + +/+ + *sex-2 sex-1* animals were viable, and the escapers ranged in phenotype from wild type to Dpy and Egl (Table 3). However,

TABLE 2

fox-1* is a stronger XSE than *ceh-39

yDp14/yDp14; him-8 XO + RNAi of gene[a]	Male viability (%)[b]	*n*[c]
No RNAi	0	929
dsRNA from vector with no gene[d]	0	1055
fox-1(RNAi)[d]	69	1174
ceh-39(RNAi)[d]	4	1030
fox-1(RNAi)[e]	72	1083
ceh-39(RNAi)[e]	4	1060

[a] The relative strength of the XSEs *fox-1* and *ceh-39* was examined by treating hermaphrodites of genotype *yDp14/yDp14* (X;I)*; him-8(e1489)* IV*; unc-2(e55)* X with dsRNA corresponding to the gene listed, and the viability of progeny males was assessed. RNAi-mediated knockdown of an XSE should decrease the male lethality caused by the increase in XSE dose from *yDp14*. *yDp14* is an X duplication attached to LG I that can exist in one copy (*yDp14/+*) or two copies (*yDp14/yDp14*) (Akerib and Meyer 1994). *him-8* XX animals produce 37% XO males, 57% XX hermaphrodites, and 6% Dpy XXX hermaphrodites (Hodgkin *et al.* 1979).

[b] Male viability was calculated by the formula: (no. of adult males)/(expected no. of males) × 100. The number of expected males was (0.37) *n*.

[c] *n* is the total number of embryos from at least six independent sets of progeny counts.

[d] Hermaphrodites were injected with dsRNA corresponding to the indicated gene, and the viability of progeny males was assessed.

[e] Hermaphrodites were fed bacteria producing dsRNA corresponding to the indicated gene, and the viability of progeny males was assessed.

reducing the dose of these four XSEs caused a milder phenotype than was evident for *yDf17 +/+ sex-1* XX animals, all of which were dead despite bearing one wild-type copy of region 1, *ceh-39*, *fox-1*, and *sex-1* (Carmi and Meyer 1999). Since *sex-2* is stronger than the region 1 XSE (Table 1; Akerib and Meyer 1994; Carmi and Meyer 1999), one would logically expect the *ceh-39 fox-1 + +/++ sex-2 sex-1* strain to exhibit a more severe phenotype than the *yDf17 +/+ sex-1* strain. The opposite was found, suggesting that *yDf17* harbors additional XSEs and that the X signal includes more XSEs than the previously estimated number.

The *sex-1* null mutation causes severe disruption of sex determination and dosage compensation: Disruption of *sex-1* causes more extensive XX lethality than disruption of any other XSE, even though the *sex-1(y263)* allele does not appear to be a null allele. That is, *sex-1 (y263)* reduces viability of XX animals to ~70% (Table 4; Carmi *et al.* 1998), but depletion of *sex-1* activity with RNAi into either wild-type animals or *sex-1(y263)* mutants reduces viability to 17% (Table 4). Therefore, to explore the function of *sex-1* further and to determine the cause for its potent effect on sex determination and dosage compensation, we first obtained a *sex-1* deletion allele by screening a *C. elegans* deletion library (materials and methods). *sex-1(y424)* reduces viability of XX animals to 20%, and the viability is not further reduced by treating the mutants with *sex-1* RNAi (Table 4). Both *sex-1(y424)* mutants and *sex-1(y424*, RNAi*)* mutants (19% viable) are comparable in viability to *sex-1*(RNAi) animals (17% viability) and to *sex-1*(*y263*, RNAi) animals (17% viability), suggesting that the *sex-1(y424)* deletion allele removes all gene function. The *y424* deletion removes 504 bp, including the ATG translational start site and part of the *sex-1* promoter region (Figure 1B). Any potential translation products are predicted to be out of frame and in low abundance. Indeed, Western blot analysis detected no protein in extracts from *sex-1(y424)* mutants (Figure 2) even after extended exposure to film. Together, these findings indicate that *sex-1(y424)* is a null allele.

***sex-1* controls sex determination and dosage compensation by regulating *xol-1* and at least one additional target:** The first clue as to how *sex-1* exerts a stronger influence on sex determination and dosage compensation than any other XSE came from correlating *xol-1* transcript levels with XX lethality in mutants lacking a specific XSE. Although the XX lethality caused by *sex-1* mutations is greater than that caused by any other XSE mutation, the *xol-1* transcript levels in *sex-1* mutants are not markedly higher than those in the other XSE mutants (Gladden and Meyer 2007, accompanying article). This finding suggested that *sex-1* might regulate more targets than just *xol-1* and that deregulation of the other targets might be responsible for the increased lethality of *sex-1* mutants. This view was reinforced by our finding that the *xol-1(y9)* null mutation, which deletes the entire gene, cannot suppress all the defects caused by *sex-1*(*y263*, RNAi) or *sex-1(y424)* (Table 5). The *xol-1* null mutation does suppress most of the XX-specific lethality caused by the *sex-1(y263)* partial loss-of-function mutation (from 70 to 87% viability, $P < 0.01$), consistent with *sex-1* functioning as an XSE (Table 5 and Carmi *et al.* 1998), but it only partially suppresses the XX lethality caused by *sex-1*(*y263*, RNAi) (from 17 to 58% viability; Table 5) or by *sex-1(y424)* (from 20 to 56% viability; Table 5). The Dpy, Egl, and Tra phenotypes caused by the disruption of dosage compensation and sex determination were suppressed in all three strains to a significant degree. The incomplete suppression by *xol-1(y9)* of the XX lethality caused by loss of *sex-1* suggests that *sex-1* has a function distinct from its regulation of *xol-1*, possibly in controlling genes elsewhere in the sex-determination and dosage compensation regulatory pathway.

If *sex-1* acts in the pathway downstream of *xol-1* or in an independent pathway, then a *xol-1* mutation should fail to suppress the synergistic lethality caused by partial disruption of both *sex-1* and a downstream dosage compensation gene such as *sdc-2*. Partial disruption of *sdc-2* by RNAi reduced the viability of otherwise wild-type XX animals to 84% and the viability of *sex-1(y263)* XX

TABLE 3

Multiple XSEs must be reduced by half to perturb dosage compensation in XX animals

Genotype[a]	XX phenotype	Hermaphrodite viability (%)[b]	n[c]
fox-1 +/+ *sex-1*[d]	Wild type	100	NA
yDf20 [Δ of *ceh-39, fox-1*]/+[d]	Wild type	99	NA
yDf20 [Δ of *ceh-39, fox-1*] +/+ *sex-1*[d]	Dpy, Egl, Tra	29	NA
yDf17 [Δ of region 1, *ceh-39, fox-1*] +/+ *sex-1*[d]	Dead	0	NA
ceh-39 + +/+ *fox-1 sex-1*[e]	Wild type or mild Dpy, Egl	107[f]	1401
ceh-39 + +/+ *sex-2 sex-1*[g]	Wild type	98[f]	1867
ceh-39 fox-1 + +/+ + *sex-2 sex-1*[h]	Dpy, Egl	60[f]	1081

[a] Alleles used were *sex-1(y263), fox-1(y303), sex-2(y324),* and *ceh-39(y414).*

[b] Hermaphrodite viability was calculated by the formula: [no. of adult hermaphrodites]/[expected no. of hermaphrodites, $(0.5)n$] × 100.

[c] n is the total number of embryos from at least six independent sets of progeny counts.

[d] Data are from Carmi and Meyer (1999).

[e] Crosses between *fox-1 sex-1* XO males and *unc-32; ceh-39* hermaphrodites yielded non-Unc cross progeny that ranged from wild type to mild Dpy Egl. The phenotype of *ceh-39* + +/+ *fox-1 sex-1* animals (Dpy and Egl) is more severe than that of *fox-1*+/+ *sex-1* animals (wild type), showing that changing the dose of *ceh-39* by half reduces the overall X signal.

[f] Hermaphrodite viability was calculated as the [no. of adult hermaphrodites (either non-Unc or non-Dpy-3)]/[$(0.5)(n$ − no. of Unc or Dpy-3 adults)] × 100. Unc or Dpy-3 adults represent self-progeny.

[g] Crosses between *sex-2 sex-1* XO males and *unc-32; ceh-39* hermaphrodites yielded non-Dpy non-Unc cross progeny.

[h] Crosses between *sex-2 sex-1* XO males and *ceh-39 dpy-3(e27) fox-1* hermaphrodites yielded non-Dpy-3 cross progeny that ranged from Dpy to wild type. The *ceh-39* allele *gk296* showed results similar to *y414.*

animals to 0% (Table 5). Consistent with *sex-1* playing a role in dosage compensation beyond its role in regulating *xol-1*, a *xol-1* mutation failed to suppress the synergistic lethality between *sex-1(y263)* and *sdc-2*(RNAi). Although 87% of *xol-1 sex-1(y263)* XX double mutants were viable, only 6% of *xol-1 sex-1(y263) sdc-2*(RNAi) triple mutants were viable (Table 5). Similar results were obtained with the *sex-1* null allele. *sdc-2*(RNAi) reduced the viability of *sex-1(y424)* XX mutants from 20 to 0%. Although 56% of *xol-1 sex-1(y424)* double mutants were viable, 0% of *xol-1 sex-1(y424) sdc-2*(RNAi) triple mutants were viable (Table 5).

Further results demonstrating *sex-1*'s function in dosage compensation beyond *xol-1* regulation were obtained through RNAi disruption of the dosage compensation gene *dpy-28* (Table 5) in a *sex-1* mutant. About 91% of *dpy-28*(RNAi) animals and 89% of *dpy-28*(RNAi); *xol-1* animals were viable, but only 6% of *dpy-28*(RNAi); *sex-1(y263)* double mutants and 4% of *dpy-28*(RNAi); *xol-1 sex-1(y263)* triple mutants were viable, indicating that a

TABLE 4

***sex-1* null allele behaves like *sex-1*(RNAi)**

Genotype[a]	Hermaphrodite viability (%)[b]	n[c]
sex-1(y263)	74	1107
sex-1(RNAi)	17	1090
sex-1(y263, RNAi*)*	17	1304
sex-1(y424)	20	2308
sex-1(y424, RNAi*)*	19	876

[a] Animals were fed bacteria producing dsRNA generated from plasmids encoding the gene listed.

[b] Hermaphrodite viability was calculated by the formula: (no. of adult hermaphrodites)/(total no. of embryos) × 100.

[c] n is the total number of embryos from at least six independent sets of progeny counts.

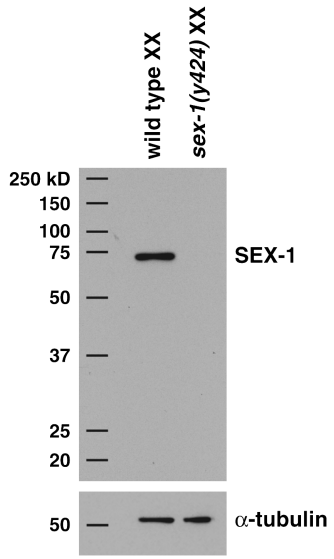

Figure 2.—*sex-1* null mutants have undetectable levels of SEX-1. Western blots of whole-cell protein extracts from wild-type XX and *sex-1(y424)* XX embryos were probed with anti-SEX-1 antibody followed by anti-α-tubulin antibody for extract normalization. Positions of molecular weight markers are indicated on the left.

TABLE 5

***sex-1* functions as an XSE and has an XSE-independent function**

Genotype[a]	Hermaphrodite viability (%)[b]	*n*[c]
sex-1(y263)	70	884
xol-1(y9) sex-1(y263)	87	690
sex-1(y263, RNAi*)*	17	1304
xol-1(y9) sex-1(y263, RNAi*)*	58	891
sex-1(y424)	20	2308
xol-1(y9) sex-1(y424)	56	1701
sdc-2(RNAi)[d]	84	1512
sex-1(y263) sdc-2(RNAi)[d]	0	1072
xol-1(y9) sex-1(y263) sdc-2(RNAi)[d]	6	2018
xol-1(y9) sdc-2(RNAi)[d]	98	1211
sdc-2(RNAi)[d]	86	1244
sex-1(y424) sdc-2(RNAi)[d]	0	912
xol-1(y9) sex-1(y424) sdc-2(RNAi)[d]	0	1256
xol-1(y9) sdc-2(RNAi)[d]	97	1283
dpy-28(RNAi)	91	1439
dpy-28(RNAi); *sex-1(y263)*	6	2273
dpy-28(RNAi); *xol-1(y9) sex-1(y263)*	4	1506
dpy-28(RNAi); *xol-1(y9)*	89	1315
mom-2(RNAi)[e]	24	1064
mom-2(RNAi); *xol-1(y9)*[e]	58	999
unc-22(RNAi)[e]	100% viable, 64% Unc	550
unc-22(RNAi); *xol-1(y9)*[e]	100% viable, 32% Unc	983

[a] Animals were fed bacteria producing dsRNA generated from plasmids encoding the gene listed.

[b] Hermaphrodite viability was calculated by the formula: (no. of adult hermaphrodites)/(total no. of embryos, *n*) × 100.

[c] *n* is the total number of embryos from at least six independent sets of progeny counts.

[d] All RNAi treatments and progeny counts performed simultaneously.

[e] The *xol-1(y9)* mutation appears to reduce the effectiveness of RNAi against some genes. *mom-2* encodes the WNT signaling molecule; loss of *mom-2* function causes embryonic lethality. *unc-22* encodes a muscle protein; loss of *unc-22* function causes a twitching phenotype. *xol-1(y9)* reduces the effectiveness of *mom-2*(RNAi) and *unc-22*(RNAi). This phenomenon probably accounts for why 84% of *sdc-2*(RNAi) animals are viable, but 98% of *xol-1; sdc-2* (RNAi) animals are viable. The *xol-1(y9)* mutation probably reduces the effectiveness of *sdc-2* (RNAi). However, *xol-1(y9)* does not suppress the lethality of *sdc-2* mutants. The ability of *xol-1(y9)* to interfere with RNAi against some genes does not compromise any of our conclusions. First, *xol-1(y9)* suppresses the lethality caused by the *sex-1 (y424)* null mutation (see Table 3) to the same degree that it suppresses the lethality caused by *sex-1*(RNAi) (this table). Second, suppression of XX lethality in *sdc-2*(RNAi) *sex-1 (y263)* or *dpy-28*(RNAi)*; sex-1(y263)* animals by *xol-1(y9)* is very poor, and the ineffectiveness of RNAi in *xol-1(y9)* could cause only the opposite effect: the extent of suppression would be greater than it would otherwise be.

xol-1 mutation cannot suppress the synergistic lethality caused by partial disruption of *sex-1* and a dosage compensation gene. As an important aside, the *xol-1(y9)* mutation reduces the effectiveness of RNAi against some genes (Table 5). In our experiments, any reduction of RNAi by *xol-1(y9)* would only cause us to overestimate the suppression of lethality by *xol-1(y9).* Since suppression is negligible, any abrogation of RNAi by *xol-1(y9)* would not compromise our conclusions. Evidence presented in the next section shows that *sex-1* positively regulates dosage compensation by acting downstream of *xol-1.*

***sex-1* acts downstream of *xol-1* to regulate both dosage compensation and sex determination:** *xol-1* mutations induce all XO animals to activate the XX-specific *sdc* genes and thereby adopt the hermaphrodite mode of sex determination and dosage compensation. These feminized *xol-1* XO mutants die as embryos or L1 larvae due to reduced X-linked gene expression caused by assembly of the dosage compensation complex on the single male X (Miller *et al.* 1988; Chuang *et al.* 1994; Lieb *et al.* 1996, 1998; Dawes *et al.* 1999). One can determine whether a gene functions downstream of *xol-1* in the sex-determination and dosage compensation pathway and, if it does, which branch, by assessing whether mutations in the gene suppress the sex-determination defects, the dosage compensation defects, or both classes of defects in *xol-1* XO mutants. For example, mutations in genes such as *sdc-2*, which control both sex determination and dosage compensation in XX animals, suppress both the death and feminization of *xol-1* XO mutants, causing rescued XO animals to develop as males (Miller *et al.* 1988). In contrast, mutations in dosage compensation genes such as *dpy-28* suppress the death but not the feminization of *xol-1* mutants, causing the rescued XO animals to develop as hermaphrodites (Miller *et al.* 1988).

By themselves, neither *sex-1(y263)* nor *sex-1(y424)* rescues *xol-1* XO mutants to adulthood, but both permit *xol-1* XO mutants to develop beyond the L1 larval stage, indicating that a *sex-1* mutation weakly disrupts dosage compensation even when its impact on the X signal has been abrogated by a *xol-1* mutation (Table 6). Furthermore, a *sex-1* mutation can further suppress the lethality of *xol-1* XO animals whose dosage compensation machinery has been partially disrupted. RNAi of *sdc-2* rescued only 6% of *xol-1* XO males, but a *sex-1* mutation increased the viability of *sdc-2*(RNAi) *xol-1* XO animals to 35% (Table 6, $P < 0.01$). The rescued animals develop as males. These results show that *sex-1* acts downstream of *xol-1* as a positive regulator of dosage compensation (Table 6).

A complementary set of genetic experiments showed that *sex-1* also controls sex determination through its action downstream of *xol-1* (Table 6). *dpy-28*(RNAi) rescued almost all *xol-1* XO animals, and all rescued *dpy-28* (RNAi); *xol-1* XO animals developed as hermaphrodites. In contrast, 33% of the rescued *dpy-28*(RNAi); *xol-1 sex-1 (y263)* XO animals developed as males, indicating that

TABLE 6

***sex-1* acts downstream of *xol-1* to control sex determination and dosage compensation**

Genotype[a]	X chromosomes	% viability	Sexual phenotype	n^b
xol-1(y9)/+[c]	XX	96	♀	1187
xol-1(y9)[c]	XO	0	NA	1187
xol-1(y9) sex-1(y263)/+ +[c]	XX	93	♀	1450
xol-1(y9) sex-1(y263)[c]	XO	0[d]	NA	1450
sdc-2(RNAi); *xol-1(y9)/+*[c]	XX	94	♀	1292
sdc-2(RNAi); *xol-1(y9)*[d]	XO	6[e]	♂	1292
sdc-2(RNAi); *xol-1(y9) sex-1(y263)/+ +*[c]	XX	39	♀	1273
sdc-2(RNAi); *xol-1(y9) sex-1(y263)*[c]	XO	35[e]	♂	1273
dpy-28(RNAi); *xol-1(y9)/+*[c]	XX	93	♀	1119
dpy-28(RNAi); *xol-1(y9)*[c]	XO	97[f]	♀	1119
dpy-28(RNAi); *xol-1(y9) sex-1(y263)/+ +*[c]	XX	60	♀	1854
dpy-28(RNAi); *xol-1(y9) sex-1(y263)*[c]	XO	85	33% ♂[g] 52% ♀	1854

[a] RNAi was applied by feeding hermaphrodites undergoing mating with bacteria producing dsRNA corresponding to the indicated gene.

[b] n is the total number of embryos from at least six independent sets of progeny counts.

[c] These strains are marked with the *unc-3(e151)* mutation. XX and XO animals were generated by crossing males homozygous for *mIs11*, an integrated *gfp*(+) transgene, with *xol-1(y9) unc-3(e151)* or *xol-1(y9) sex-1(y263) unc-3(e151)* hermaphrodites with or without *dpy-28*(RNAi) or *sdc-2*(RNAi). XX cross progeny are of the genotype *mIs11/+; unc-3(e151)/+* and were identified as Gfp non-Unc, while XO cross progeny are of the genotype *mIs11/+; unc-3(e151)* and were identified as Gfp Unc. Male viability was calculated by the following formula: (no. of Gfp Unc animals) (0.5) $n \times 100$. Hermaphrodite viability was calculated by the following formula: (no. of Gfp non-Unc animals) (0.5) $n \times 100$. Since the progeny were generated from a cross, the expected number of XX or XO adults is half the total number of cross-progeny embryos. The crosses appeared to go to completion because all adults were Gfp. Also, at least 98% of scored embryos were Gfp, and the non-Gfp embryos were too young to express *gfp*.

[d] Although all *xol-1(y9) sex-1(y263)* XO animals are dead, they survived to a later developmental stage than *xol-1(y9)* XO animals. A similar result was found for *xol-1(y9) sex-1(y424)* XO animals.

[e] The 6% of *sdc-2*(RNAi); *xol-1(y9)* XO and the 35% of *sdc-2*(RNAi); *xol-1(y9) sex-1(y263)* XO animals scored grew slowly and developed into very sick adult males with partially developed or completely developed male tail structures. Partially developed tails were missing some rays and had malformed spicules and fans. Another 80% of *sdc-2*(RNAi); *xol-1(y9)* or 41% of *sdc-2*(RNAi); *xol-1(y9) sex-1(y263)* XO animals arrested in what appeared to be the L2 larval stage and were not scored as viable. These animals were too sick to determine their sex. Although these XO animals were considered inviable, they survived to a later developmental stage than *xol-1(y9)* XO animals.

[f] *dpy-28*(RNAi) rescues the viability of almost all *xol-1(y9)* XO animals but they were feminized and developed as hermaphrodites.

[g] The presence of males indicated that *sex-1(y263)* rescued the feminization of *dpy-28*(RNAi); *xol-1(y9)* XO animals. The percentage is based on the number of embryos.

sex-1 acts downstream of *xol-1* to promote the hermaphrodite sexual fate.

Independent molecular experiments confirmed the XSE-independent function of *sex-1* and showed that this downstream effect controls sex determination by repressing transcription of the male sex-determination gene *her-1*, either directly or indirectly (Table 7). *her-1* is one of the few genes in the sex-determination pathway that is controlled at the level of transcription and acts downstream of *xol-1*, making it an appropriate gene to monitor *sex-1* function (Trent *et al.* 1991). *her-1* is repressed directly through binding of SDC proteins to the promoter and second intron (Chu *et al.* 2002). The difference in *her-1* transcript levels between XO and XX animals is estimated to be ~20-fold (Trent *et al.* 1991). Using qRT–PCR, we found that normalized *her-1* transcript levels were elevated 6.5-fold in *sex-1* mutants ($P < 0.01$) and 4.6-fold in *xol-1 sex-1* mutants ($P < 0.01$), consistent with the XSE-independent function of *sex-1* acting downstream of *xol-1* and upstream of *her-1* (Table 7). The combined effect of the XSE-independent function of *sex-1* on dosage compensation and sex determination suggests that it acts at the level of the *sdc* genes. Thus, *sex-1* controls not only dosage compensation, but also sex determination, by acting in two separable capacities, as a repressor of *xol-1* and also as a downstream inducer of the hermaphrodite fate, including the activation of dosage compensation.

TABLE 7

***sex-1* regulates *her-1* transcript levels**

Genotype	Transcript measured by qRT–PCR[a]	
	her-1[b]	*fasn-1*[c]
xol-1(y9) XX	1.2 ± 0.2	1.0 ± 0.1
sex-1(y424) XX	6.5 ± 0.4	1.1 ± 0.1
xol-1(y9) sex-1(y424) XX	4.6 ± 0.7	1.0 ± 0.1

[a] The levels of *her-1* transcripts were measured in embryos of different genotypes (listed by genotype) by qRT–PCR and are expressed as the fold change compared to the transcript levels measured in wild-type XX embryos. For example, a value of 2.0 means that twice as many gene-specific transcripts were measured in mutant embryos than in wild-type XX embryos. All transcripts levels were normalized to the levels of the control gene, *nhr-64*, whose expression is not affected by dosage compensation. Fatty acid synthase *fasn-1*, another gene not affected by dosage compensation, was used as a control to gauge the variability and reliability of measurements made using qRT–PCR. See Van Gilst *et al.* (2005) for protocol. Experimental error is expressed as the standard error of the mean. Similar results were obtained for all genotypes in separate qRT–PCR experiments in which transcript levels were normalized to the levels of *fasn-1*.

[b] qRT–PCR primers amplify both the short and long transcripts of *her-1*, which are coordinately regulated in a sex-specific manner despite being produced by two different *her-1* promoters. Measurements thus reflect changes in total *her-1* transcripts.

[c] A critical control was to compare the *nhr-64*-normalized *fasn-1* transcript levels in three independent preparations of wild-type embryos. That comparison showed the *fasn-1* transcript levels to be statistically equivalent among the independent RNA preparations (*fasn-1*, 1.1 ± 0.2).

The XSE-independent function of *sex-1* regulates dosage compensation by affecting the stability and localization of the dosage compensation complex: To determine the step at which the downstream function of *sex-1* affects dosage compensation, the localization and abundance of the DCC component DPY-27 were examined in *xol-1* XX mutants with reduced *sex-1* and *sdc-2* activities using DPY-27 antibodies. Disruption of *sex-1* through RNAi in *xol-1 sex-1(y263)* mutants (58% viable, Table 5) caused only a mild decrease in DPY-27 staining (Figure 3, G and H and g and h). In contrast, the *sex-1(y263)* mutation reduced the DPY-27 staining and disrupted its assembly onto the X chromosomes of *xol-1 sdc-2*(RNAi) XX mutants (Figure 3, E and F and e and f). DPY-27 staining was undetectable in most nuclei, and the residual DPY-27 staining appeared somewhat diffuse in other nuclei. DCC localization in neither control *sdc-2*(RNAi) XX animals nor *xol-1 sdc-2*(RNAi) XX animals appeared significantly compromised (Figure 3, A–D and a–d).

Similarly, SDC-3 staining and localization appeared only partially disrupted in *sex-1*(*y424* null) mutants (20% viable), *xol-1 sex-1*(*y424* null) mutants (56% viable), or *sdc-2*(RNAi) animals, but were severely disrupted in *sex-1* (*y424* null) *sdc-2*(RNAi) mutants (0% viable) or *xol-1 (y9) sex-1*(*y424* null) *sdc-2*(RNAi) mutants (0% viable) Figure 4, A–H and a–h). This result further demonstrates that loss of the XSE-independent function of *sex-1* disrupts the dosage compensation complex.

The differential DCC disruption explains why partial depletion of *sdc-2* activity by RNAi reduces XX viability to a greater extent in *xol-1 sex-1* animals than in wild-type animals or in *xol-1* mutants. These results indicate that the XSE-independent function of *sex-1* promotes the stability and proper localization of the DCC. The dual functions of *sex-1*, as a direct repressor of *xol-1* and as a downstream positive effector of hermaphrodite sexual fate and dosage compensation, account for why *sex-1* mutations confer more severe phenotypes in XX animals than mutations in other XSEs such as *ceh-39*, which appear to relay the X signal strictly through *xol-1*.

After XSEs communicate X chromosome dose to determine sex, the dosage compensation process equalizes expression of XSEs between the sexes: The twofold difference in copy number of X signal elements between XO and XX embryos forms the basis for *C. elegans* sex determination. Once sex is determined, the dosage compensation machinery is activated in XX animals, providing the potential for the dosage compensation process to feed back and repress expression of the very X-linked XSEs that activate the DCC. If so, the DCC can modulate expression of the X signal in a temporal manner and diminish the sex signal in XX animals. To determine whether expression of XSEs is controlled by the DCC, we first determined the expression level of each XSE in XO and XX embryos after sex had been determined using quantitative RT–PCR and then measured the XSE transcript levels in dosage-compensation-defective XX mutants.

We compared *fox-1*, *ceh-39*, and *sex-1* transcript levels in XX and XO embryos of two genotypes, wild-type XX hermaphrodite embryos, and *her-1; xol-1 sdc-2* XO hermaphrodite embryos (Table 8). This latter strain grows as an XO hermaphrodite strain that lacks the dosage compensation machinery. The *xol-1* mutation would kill all XO animals by activating the dosage compensation machinery, but the *sdc-2* mutation rescues the XO animals by preventing dosage compensation proteins from loading onto X. The *her-1* mutation transforms the XO animals into hermaphrodites. The majority of animals in the population are XO, but some XX and nullo-X animals are also present. We found the XSE transcript levels to be equivalent in the XO and XX embryos, suggesting that the XSEs are dosage compensated (Table 8).

A further criterion for a dosage-compensated gene is that the transcript levels not only should be equivalent between the sexes, but also should be elevated in XX mutants defective in dosage compensation. The levels of XSE transcripts were first measured in the hypomorphic *dpy-27(y57)* XX mutant strain, which is 77% viable and therefore only partially defective in dosage com-

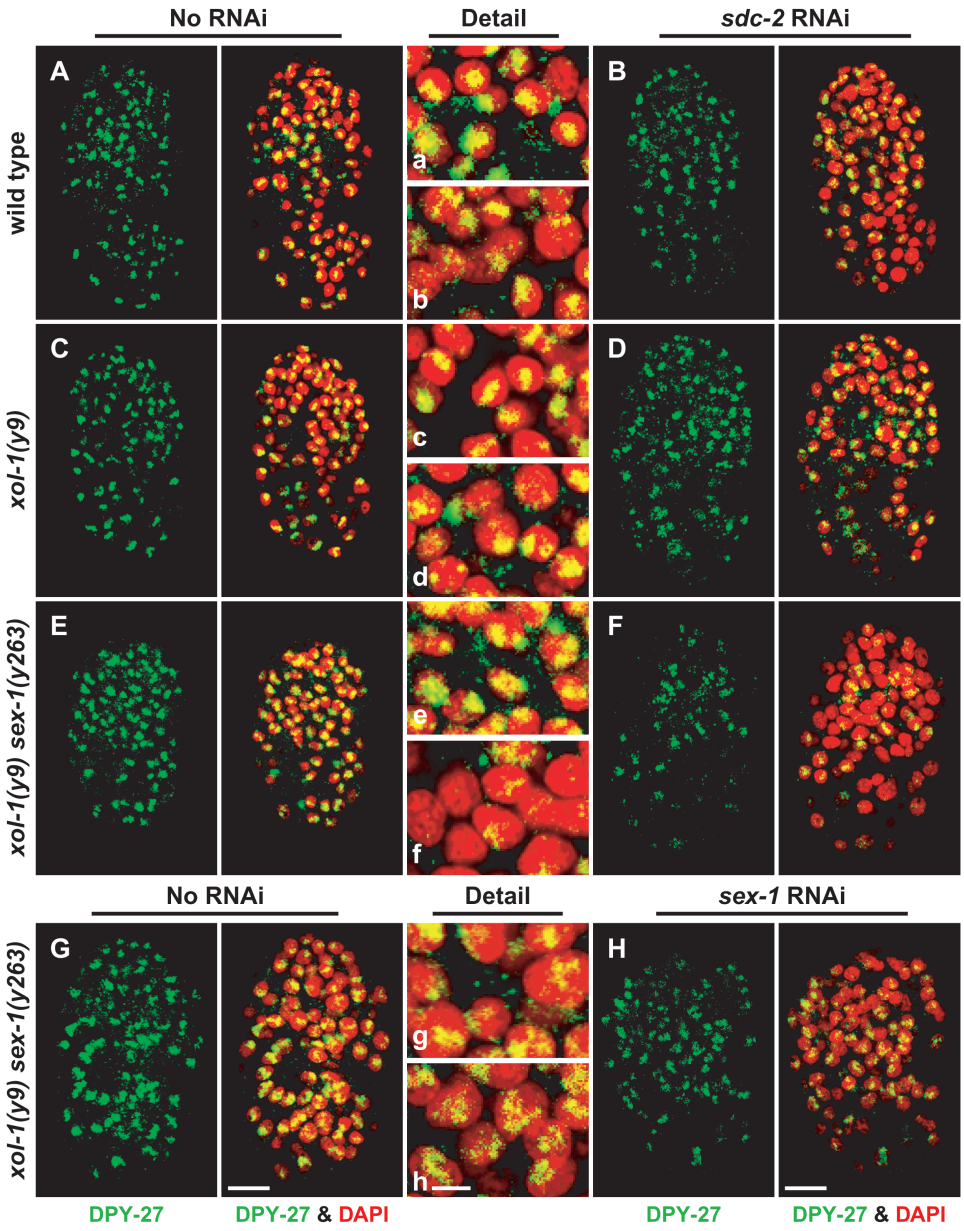

FIGURE 3.—X chromosome localization of the DCC is disrupted by inhibiting the XSE-independent function of *sex-1*. (A–H) Partial projections of false-colored confocal images of wild-type and mutant XX embryos costained with antibodies against the dosage compensation protein DPY-27 (green) and DAPI (red). (a–h) Enlargements of nuclei from A–H, respectively. (A–D) RNAi treatment of *sdc-2* in wild-type and *xol-1* mutant embryos mildly disrupted the X localization of DPY-27, as indicated by reduced DPY-27 levels and diffuse nuclear localization. (E and F) RNAi of *sdc-2* disrupted DPY-27 more severely in *xol-1 sex-1* mutants than in either wild-type or *xol-1* animals, indicating that the XSE-independent function of *sex-1* is important for proper stability and assembly of the DCC on X. (G and H) RNAi of *sex-1* in *xol-1 sex-1* XX embryos disrupts DPY-27 localization, indicating that loss of only the XSE-independent function of *sex-1* impairs dosage compensation. Bars (A–H), 10 μm; (a–h), 3 μm.

pensation (Table 8). Both *sex-1* and *ceh-39* transcript levels were found to be elevated 1.8-fold above the levels in wild-type XX animals ($P < 0.01$ for *sex-1*, $P = 0.017$ for *ceh-39*), but the *fox-1* transcript level was not significantly elevated in the *dpy-27(y57)* strain. The XSE transcript levels were then assessed in XX mutants more severely disrupted in dosage compensation. Fully dosage-compensation-defective XX mutants cannot be propagated as a strain; therefore we devised a means to obtain XX embryos that had died from severe disruption of dosage compensation. A large population of XX larvae carrying the very weak *sdc-2(y93)* mutation (100% viable, NUSBAUM and MEYER 1989) were treated with *sdc-2*(RNAi) through feeding for one generation, and the dead, dosage-compensation-defective progeny embryos (100% inviable) were harvested for RNA isolation. This growth regime permits acquisition of reasonable quantities of severely dosage-compensation-defective animals for transcript analysis. The majority of embryos were at a developmental stage in which their sex should already have been determined. The transcript levels of all three XSEs were elevated by the severe reduction of *sdc-2* activity: *sex-1* by 2.9-fold ($P < 0.01$), *ceh-39* by 5.2-fold ($P < 0.01$), and *fox-1* by 2-fold ($P < 0.01$). Thus, the XSEs meet the second criterion for a dosage-compensated gene.

We also found that disruption of dosage compensation caused an elevation of *xol-1* transcript levels in XX animals, suggesting that *xol-1* is also repressed in response to the activation of dosage compensation. In *sdc-2(y93*, RNAi) XX embryos, the *fasn-1*-normalized *xol-1* transcript level was increased 4.5 ± 0.6-fold compared to that in wild-type XX embryos, and in *dpy-27(y57)* XX embryos, the normalized *xol-1* transcript level was increased 1.9 ± 0.2-fold.

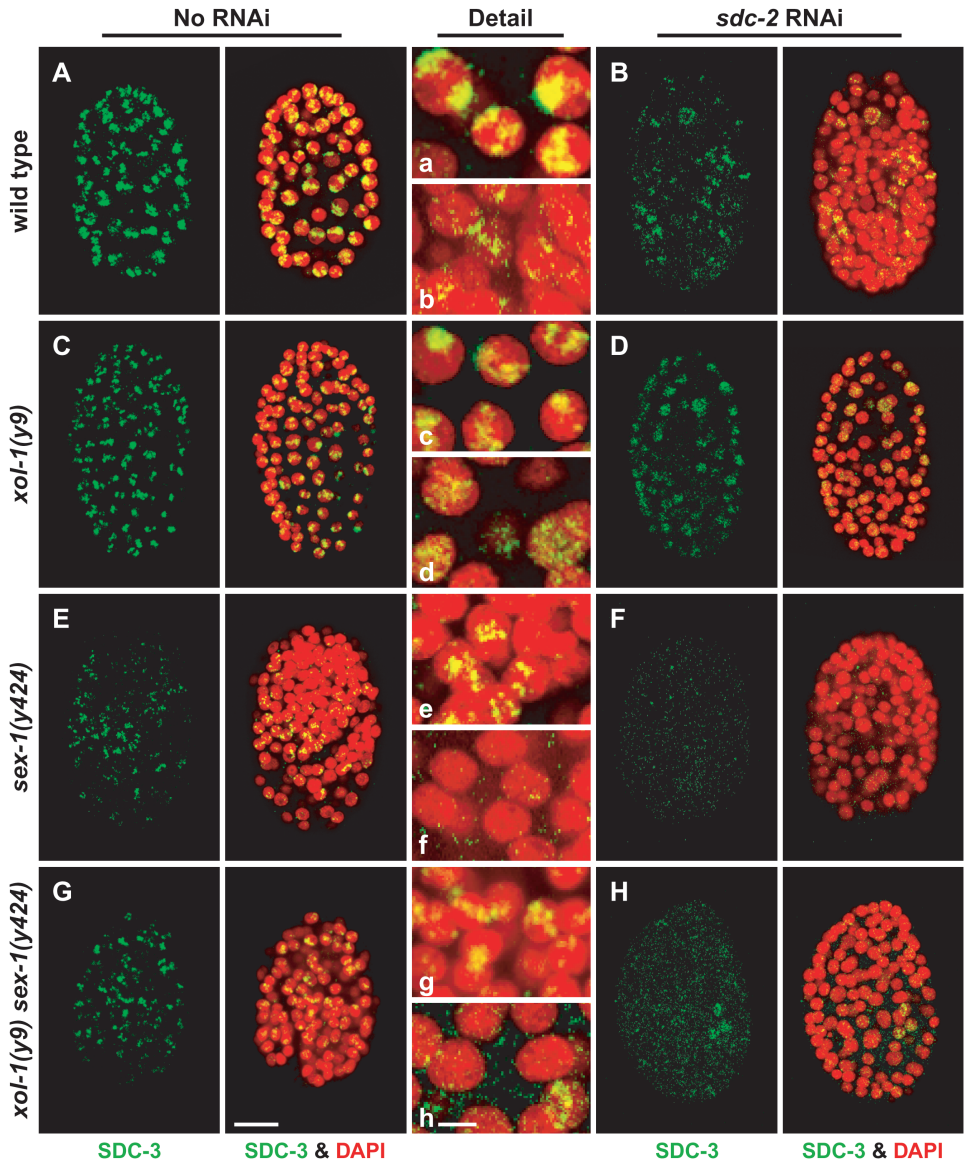

FIGURE 4.—DCC disruption by a *sex-1* null mutation and by the XSE-independent function of *sex-1*. (A–H) Partial projections of false-colored confocal images of wild-type and mutant XX embryos costained with antibodies against the dosage compensation protein SDC-3 (green) and DAPI (red). (a–h) Enlargements of nuclei from A–H, respectively. (A–D) RNAi disruption of *sdc-2* in wild-type and *xol-1* mutant embryos reduced the abundance and partially disrupted the X localization of SDC-3. (E and F) The *sex-1(y424)* null allele partially disrupted dosage compensation, but X-localized SDC-3 was evident in many nuclei. Some nuclei lacked SDC-3 staining. RNAi of *sdc-2* into *sex-1(y424)* completely disrupted the DCC. Virtually no SDC-3 was detectable. (G and H) RNAi of *sdc-2* in *xol-1 sex-1* mutants also disrupted SDC-3 severely, similarly to *xol-1(y9) sex-1(y424) sdc-2*(RNAi) embryos, showing that the XSE-independent function of *sex-1* is important for proper stability and assembly of the DCC on X. Bars: A–H, 10 μm; a–h, 3 μm.

Two important conclusions can be drawn from these experiments. First, the XSEs are dosage compensated, indicating that the very signal that communicates the difference in X chromosome dose between XO and XX animals is diminished once dosage compensation is fully activated in the embryo. Lowering *xol-1* transcript levels in XX animals in response to dosage compensation could partially compensate for the reduction in expression of XSEs, the *xol-1* repressors. Ultimately, however, the sex-determination decision must be maintained and transmitted to newly developing cells by sex-determination and dosage compensation genes that act downstream of *xol-1*. Second, XSE transcripts levels are derepressed by more than twofold when the dosage compensation complex is disrupted, implying that XSEs are repressed more than twofold by dosage compensation, and the difference in XSE expression between XO and XX embryos prior to dosage compensation is potentially greater than twofold.

Disruption of dosage compensation by mutation of one XSE feeds back to enhance expression of other XSEs: XSE transcript levels are also affected by mutations in the XSE genes themselves (Table 8). For example, in a *sex-2* mutant, *ceh-39* transcript levels were elevated 2.6-fold ($P < 0.01$) and *fox-1* transcript levels 2.2-fold ($P < 0.01$) above their levels in wild-type animals. Also, *ceh-39* and *fox-1* transcript levels were elevated 2.8-fold and 2.1-fold ($P < 0.01$ for both), respectively, in *sex-1* mutants compared to wild-type animals. This latter elevation was suppressed in large part by a *xol-1* mutation, which reduced the increase in *ceh-39* transcript levels from 2.8- to 1.8-fold ($P = 0.02$) and the increase in *fox-1* transcript levels from 2.1- to 0.9-fold ($P < 0.01$). The fact that wild-type *xol-1* function is required to elevate the levels of XSE transcripts in XSE mutants indicates that this effect is caused by disruption of dosage compensation and not due to direct regulation among the XSEs themselves.

TABLE 8

XSEs become dosage compensated after sex is determined

Genotype	Transcript measured by qRT–PCR[a]			
	sex-1	*ceh-39*	*fox-1*	*nhr-64*[b]
her-1; xol-1(y9) sdc-2(y74) unc-9 XO[c]	0.8 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
sdc-2(y93, RNAi) XX[d]	2.9 ± 0.2	5.2 ± 0.6	2.0 ± 0.1	1.0 ± 0.1
dpy-27(y57) XX	1.8 ± 0.1	1.8 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
sex-1(y263) XX	0.9 ± 0.1[e]	2.8 ± 0.4	2.1 ± 0.5	1.3 ± 0.1
sex-2(y324) XX	1.4 ± 0.1	2.6 ± 0.3	2.2 ± 0.3	1.2 ± 0.1
ceh-39(y414) XX	1.2 ± 0.1	3.9 ± 0.4	1.8 ± 0.2	1.1 ± 0.1
ceh-39(gk296) XX	1.3 ± 0.1	NA	1.3 ± 0.1	1.1 ± 0.1
fox-1(y303) XX	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
xol-1(y9) sex-1(y263) XX	0.9 ± 0.1	1.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.1
yIs58[*ceh-39(+)*] XX	1.0 ± 0.1	4.1 ± 0.5	1.1 ± 0.1	0.9 ± 0.1
yIs58[*ceh-39(+)*]; *sex-1(y263)* XX	1.0 ± 0.1	26.4 ± 2.8	3.6 ± 0.5	1.2 ± 0.1
yIs58[*ceh-39(+)*]; *xol-1(y9) sex-1(y263)* XX	1.0 ± 0.1	3.1 ± 0.3	2.3 ± 0.2	1.0 ± 0.1

[a] The levels of XSE transcripts in embryos of different genotypes (listed by genotype) were measured by qRT–PCR and are expressed as the fold change compared to the transcript levels measured in wild-type XX embryos. All transcripts levels were normalized to the levels of the control gene, *fatty acid synthase-1* (*fasn-1*), whose expression is constant throughout embryogenesis and is not affected by dosage compensation. See Van Gilst *et al.* (2005) for details and protocol. *nhr-64*, another gene not affected by dosage compensation, was used as a control to gauge the variability and reliability of measurements made using qRT–PCR. Experimental error is expressed as the standard error of the mean. Similar results were obtained for all genotypes in separate qRT–PCR experiments in which transcript levels were normalized to the levels of *nhr-64*.

[b] A critical control was to compare the *fasn-1*-normalized *nhr-64* transcript levels in three independent preparations of wild-type embryos. That comparison showed the *nhr-64* transcript levels to be statistically equivalent among the independent RNA preparations (*nhr-64*, 1.3 ± 0.1).

[c] The full genotype is *her-1(hv1y101); xol-1(y9) sdc-2(y74) unc-9(101)* XO.

[d] XX animals carrying the weak *sdc-2(y93)* mutation were fed *sdc-2*(RNAi), and the dead, dosage-compensation-defective progeny embryos were harvested for RNA isolation.

[e] The *sex-1(y263)* mutation affects *sex-1* mRNA splicing and may destabilize the mutant *sex-1* transcripts, causing a decrease in their overall levels, specifically in *sex-1* mutants.

The most dramatic effect of an XSE mutation on the transcript level of another XSE was evident in *sex-1* mutant XX embryos carrying a transgenic array expressing *ceh-39* (Table 8). *yIs58*[*ceh-39(+)*] animals express *ceh-39* transcripts at a level 4.1-fold higher than that in wild-type embryos, and the level increased by 6.5-fold in *sex-1* mutants to a level 26.4-fold above that in wild-type animals. This increase in *ceh-39* transcript levels was suppressed by a *xol-1* mutation. In *yIs58; xol-1 sex-1* mutants, *ceh-39* transcripts drop back down to a level 3.1-fold higher than in wild-type embryos, further emphasizing that the increase in XSE transcript levels in XSE mutants is not caused by direct regulation of one XSE by another, but rather is a consequence of a dosage compensation disruption.

yIs58 contains multiple copies of the *ceh-39* locus integrated into an autosome, yet it appears to be affected by the X chromosome dosage compensation process. At least two explanations might account for this phenomenon. First, the *ceh-39* locus may contain a DCC recruitment site that attracts the DCC to the *yIs58* integration site and permits *ceh-39* to be repressed. Second, an X-linked activator of *ceh-39* might be dosage compensated, and the *sex-1* mutation causes this activator's expression to increase, thereby indirectly increasing *ceh-39* expression in *yIs58*. The former possibility is unlikely because the DCC did not localize to the *yIs58* locus in XX animals (data not shown). The latter case is potentially true for any X-linked gene and is a caveat that must be considered in assessing whether the process of dosage compensation represses an XSE directly or instead acts indirectly by repressing the XSE's potential regulator.

The changes in *ceh-39* transcript levels measured by qRT–PCR correlated with changes in CEH-39 protein levels assessed by Western blots (Figure 5). CEH-39 levels were elevated 5-fold in *sex-1* mutants compared to levels in wild-type animals and the increase in protein level was suppressed by a *xol-1* mutation, as was the increase in *ceh-39* transcript levels. CEH-39 levels were only 2-fold higher in *xol-1 sex-1* animals. CEH-39 levels made from *yIs58*[*ceh-39(+)*] were also subjected to repression through the dosage compensation process. CEH-39 levels were 8- and 20-fold higher in *yIs58*[*ceh-39(+)*]

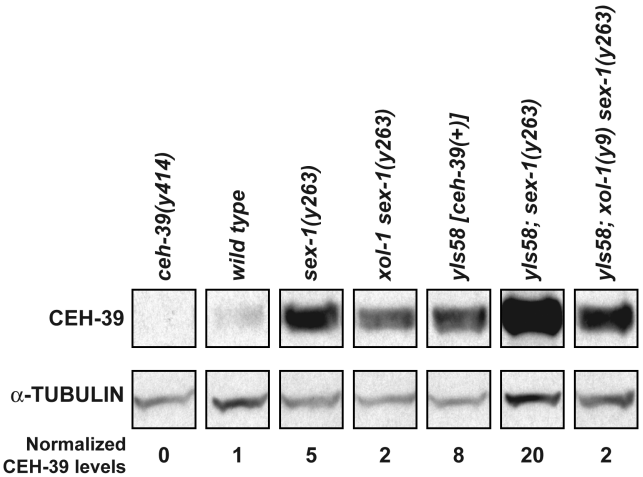

FIGURE 5.—CEH-39 levels are affected by the process of dosage compensation. A Western blot of whole-cell extracts from wild-type and mutant XX embryos shows that CEH-39 levels increase when *sex-1* is mutant. The levels of CEH-39 in all protein extracts were normalized to the levels of α-tubulin. The normalized CEH-39 levels were then compared in mutant and wild-type (N2) extracts. CEH-39 was detected by CEH-39 antibodies in all extracts except those from *ceh-39(y414)* mutant embryos, which lacked the epitope for the antibody. CEH-39 level was increased by 5-fold in *sex-1* mutants compared to wild-type embryos and by 2-fold in *xol-1 sex-1* mutants, indicating that the increase in CEH-39 level was caused by a dosage compensation disruption. The level of CEH-39 in *yIs58* [*ceh-39(+)*] embryos was increased by 8-fold compared to that in wild-type embryos, showing that CEH-39 is indeed overexpressed in this strain. A *sex-1* mutation caused CEH-39 to be expressed at an even higher level from *yIs58* (20-fold); mutation of *xol-1* greatly reduced the CEH-39 level (2-fold).

animals and in *yIs58*[*ceh-39(+)*]; *sex-1* animals, respectively, compared to levels in wild-type animals and were suppressed 2-fold higher in *yIs58*[*ceh-39(+)*]; *xol-1 sex-1* animals compared to levels in wild-type animals (Figure 5). Thus, the dosage compensation disruption caused by loss of *sex-1*'s XSE activity created a feedback loop that elevated *ceh-39* expression.

DISCUSSION

We have shown that the earliest aspects of sex determination are regulated through a more elaborate mechanism than previously understood. The X component of the X:A sex-determination signal includes more elements than first predicted, and each has a more modest effect on sex determination than the nuclear receptor SEX-1. The more potent influence of SEX-1 on sex determination and dosage compensation derives from its dual roles in the pathway: as an XSE to repress *xol-1* and as a downstream activator of hermaphrodite fate. Furthermore, the dosage compensation process reduces expression of the XSEs once the X:A signal has determined sex. After the activation of dosage compensation, expression levels of XSEs are equivalent between XX and XO embryos, indicating that the X:A signal cannot guide sexual fate decisions later in development.

Composition of the X signal: Prior studies using heterozygous X chromosome deletions to remove one copy of XSEs in XX animals suggested that reducing the dose of only four XSEs by half was sufficient to switch the X:A signal from its XX mode of *xol-1* repression to its XO mode of *xol-1* activation (AKERIB and MEYER 1994; CARMI and MEYER 1999). In contrast, the severity of phenotypes that we observed by reducing the dose of XSEs using mutations rather than chromosomal deficiencies was less extreme, suggesting that the deletions also reduced the dose of additional, undefined XSEs and caused an overestimate of the contribution made by an individual XSE to the X:A signal. In our analysis, reducing the dose of four individual XSEs (*sex-1*, *sex-2*, *ceh-39*, and *fox-1*) by half was not sufficient to mimic the XO state. Moreover, the XO state is unlikely to be induced in XX animals only by halving the dose of these four XSEs plus the XSE in region 1, which is comparable in strength to *ceh-39*. Thus, the X component of the X:A signal likely utilizes more than five XSEs, more than previously predicted, to communicate the distinction between one X chromosome and two. This interpretation was reinforced by our reciprocal analysis of XSE mutations in XO animals. XSE mutations only partially suppressed the male lethality caused by increasing the XSE dose through X duplications, while mutations in downstream dosage compensation genes such as *sdc-2* suppressed the lethality fully (AKERIB and MEYER 1994), further indicating that the residual male lethality was likely caused by the activity of undefined XSEs.

In their study analyzing the effects of X duplications on the sex of polyploid animals, MADL and HERMAN (1979) concluded that the right side of X likely harbors sex-determining genes that participate in X:A assessment. While it remains a possibility that XSEs are encoded on that part of X and can account in part for our undefined XSE activity, the feminizing effect described by MADL and HERMAN (1979) is equally likely due to the partial disruption of dosage compensation caused by the duplications. The right-end duplications bind the DCC, which is limiting in supply, and titrate the DCC from X (CSANKOVSZKI *et al.* 2004). Those duplications, like mutations in dosage compensation genes, can suppress the extensive masculinization of *sdc-3*(Tra) XX mutants (DELONG *et al.* 1993). Furthermore, those duplications abrogate the dosage compensation of genes not covered by them (MENEELY and NORDSTROM 1988). New genetic screens without bias for X chromosome location are needed to identify additional XSEs.

Different combinations of heterozygous XSE mutations revealed that the effects on sex determination and dosage compensation were more extreme if the XSEs with reduced dose included those that controlled *xol-1* at both transcriptional and post-transcriptional levels. These experiments were carefully controlled to ensure

that only combinations of XSEs with equivalent strengths were compared. Our results confirm the view that both transcriptional and post-transcriptional mechanisms of repression are important for *xol-1* regulation, but make it clear that more of the XSEs control *xol-1* at the transcript level.

Relative strength of XSEs: The involvement in the X:A signal of more XSEs than previously projected is coupled to the current understanding that each XSE makes a smaller contribution to the signal than anticipated by mere extrapolation from the strength of the second-discovered XSE, *sex-1*. *sex-1* null mutations cause 80% of XX animals to die, but mutations in other XSEs (*ceh-39*, *fox-1*, and *sex-2*) cause insignificant XX lethality. The relative strengths of the XSEs were determined by more sensitive assays that revealed their relative contributions to be *sex-1* > *sex-2* > *fox-1* > *ceh-39* ≥ region 1 XSE. The picture has thus emerged that multiple different X signal elements, most with a modest repressive effect on *xol-1*, act cumulatively to communicate X dose using two different mechanisms of *xol-1* repression. The modest individual contribution of each XSE to the X:A signal accounts in part for the large number of XSEs (at least five) needed to regulate *xol-1* and thereby communicate X chromosome dose.

***sex-1* regulates the sex-determination pathway at multiple steps:** *sex-1* has an unusually strong effect on sex determination and dosage compensation compared to other XSEs. Our work revealed the underlying cause of this phenomenon: *sex-1* functions in two separate capacities at different steps in the pathway. *sex-1* acts upstream in the pathway as an XSE to repress *xol-1* and downstream in the pathway at the level of *sdc* genes to activate the hermaphrodite mode of sex determination and dosage compensation.

Hints of this complexity first came from the paradox that, although *sex-1* mutations cause stronger dosage compensation phenotypes than mutations in other XSEs, they do not cause substantially greater derepression of *xol-1* transcript levels. Isolation of a *sex-1* null allele helped us to demonstrate the multi-faceted roles of *sex-1*. First, the XX lethality caused by the *sex-1* null allele is only partially suppressed by a *xol-1* null mutation, showing that while *sex-1* acts as an XSE to regulate *xol-1*, it also functions in a separate capacity. Furthermore, mutations in all XSEs enhance the XX lethality caused by partial disruption of the downstream gene *sdc-2*, but *xol-1* mutations do not suppress the enhanced lethality caused by *sex-1* mutations, although they do suppress the enhanced lethality caused by *ceh-39* and *sex-2* mutations. Thus, *sex-1*, but apparently not other XSEs, acts independently of the X signal to regulate both sex determination and dosage compensation. Finally, the XSE-independent role of *sex-1* in the sex-determination pathway acts downstream of *xol-1*, as revealed by two experiments. A *sex-1* mutation can further suppress the lethality of *xol-1* XO animals whose dosage compensation machinery has already been partially disrupted. In addition, in a *xol-1* XX mutant, a *sex-1* mutation causes derepression of *her-1* transcript levels. *her-1* is a male sex determination gene that acts downstream of *xol-1* and is repressed by *sdc* genes in XX animals (Figure 6). The downstream function of *sex-1* appears to be strong, since 44% of *xol-1*(null) *sex-1*(null) XX mutants are dead.

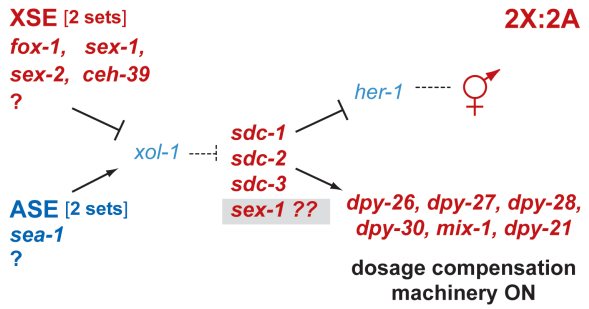

FIGURE 6.—Current model of the sex-determination and dosage compensation pathway in *C. elegans*. Regulation of the sex-determination pathway is more complex than previously thought. The XSE *sex-1* functions in two separate capacities at different steps in the pathway, accounting for its more potent effects on sex determination and dosage compensation than any other XSE. *sex-1* acts upstream in the pathway as an XSE to repress *xol-1*. It also acts downstream of *xol-1* in the pathway to activate the hermaphrodite mode of sex determination and dosage compensation. Although the nature of the XSE-independent function is unknown, it appears to act at the level of *sdc* genes. *sex-1* could, for example, control an *sdc* gene or its protein activity, or it could function with an undefined gene at the same step as the *sdc* genes. Alternatively, it could control the sex-determination and dosage compensation branches of the pathway independently.

Although the nature of the XSE-independent *sex-1* activity is unknown, it is functionally similar to the activities of *sdc* genes (Figure 6). *sex-1* might, for example, control the activity of an *sdc* gene or the stability of the SDC protein complex, or it could function with undefined genes in the sex-determination pathway at the same step as the *sdc* genes. Alternatively, *sex-1* could control the sex-determination and dosage compensation branches of the pathway independently. Regardless of the mechanism, the dual functions of *sex-1* likely account for its more potent impact on sex determination and dosage compensation than any other XSE. These functions act synergistically. The synergistic lethality caused by the loss of both the XSE function and the XSE-independent function of *sex-1* is analogous to the synergistic lethality caused by the loss of a weaker XSE (*ceh-39* or *fox-1*) and a weak dosage compensation disruption due to *sdc-2*(RNAi).

Dosage compensation and the regulation of XSEs: The use of an X-chromosome-counting mechanism to determine sex creates the potential paradox that the very genes, the XSEs, that communicate X chromosome dose, could themselves be subjected to the process that they regulate, X chromosome dosage compensation. If

XSEs become dosage compensated, the primary signal that communicates the difference in X chromosome dose between the sexes by repressing *xol-1* in XX animals would be reduced and would likely cease to exist during embryogenesis. Either the XSEs must therefore escape dosage compensation in XX embryos or the commitment to sexual fate must be firmly established by the onset of dosage compensation.

We found the XSEs to be dosage compensated as judged by two criteria: XSE expression was equivalent in XX and XO embryos that had already determined their sex, and XSE transcript levels were elevated in dosage-compensation-defective XX mutants. Thus, the process of dosage compensation influences the X signal in a temporal manner, and after the onset of dosage compensation, the XSEs cannot be used as a reference to guide sexual differentiation. Downstream genes in the sex-determination pathway that are turned on or off in response to *xol-1*'s activity state must maintain the initial choice of sexual fate.

The discovery that XSEs are dosage compensated fits well with the previous observation that, toward the end of gastrulation, synthesis of *xol-1* becomes dispensable in XO embryos and inconsequential in XX embryos (Rhind *et al.* 1995). These results suggested that an irreversible commitment to sexual fate had occurred by then and that assessment of the X:A signal was no longer necessary. Our results substantiate this view and further establish that the X:A signal cannot function after dosage compensation has been implemented, a time that precedes the end of gastrulation. The partial repression of *xol-1* expression in XX embryos by the dosage compensation process would help keep *xol-1* properly regulated until its level of activity was no longer of consequence to the embryo. Thus far, all lines of experiments have indicated that the X:A signal is assessed during a brief time window in embryogenesis and is not assessed continuously throughout the rest of development.

Finally, we found that the expression of XSEs is derepressed by more than twofold in embryos deficient in dosage compensation, implying that, in XX embryos, the XSEs are downregulated more than twofold by the DCC. The simplest interpretation of these data is that expression of XSEs differs by greater than twofold between XX and XO animals prior to dosage compensation. The probable mechanisms underlying such surprising signal amplification are not known and might range from gene-specific forms of control such as autoregulation to more general forms of regulation, perhaps related to the strategy for dosage compensation. Whatever the mechanism, such amplification would make assessment of X chromosome dose between XX and XO embryos a more robust process.

We thank M. Van Gilst for guidance with quantitative RT–PCR, T. Cline for discussions, and M. Jow and A. Severson for critical comments on the manuscript. This work was supported by National Institutes of Heath grant GM30702 to B.J.M., a National Science Foundation Predoctoral Fellowship to J.M.G., and the American Cancer Society Postdoctoral Fellowship PF-06-027-01 to B.F. B.J.M. is an investigator of the Howard Hughes Medical Institute.

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Communicating editor: D. I. Greenstein