Depletion of a Novel SET-Domain Protein Enhances the Sterility of *mes-3* and *mes-4* Mutants of *Caenorhabditis elegans*

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ABSTRACT

Four maternal-effect sterile genes, *mes-2*, *mes-3*, *mes-4*, and *mes-6*, are essential for germline development in *Caenorhabditis elegans*. Homozygous *mes* progeny from heterozygous mothers are themselves fertile but produce sterile progeny with underproliferated and degenerated germlines. All four *mes* genes encode chromatin-associated proteins, two of which resemble known regulators of gene expression. To identify additional components in the MES pathway, we used RNA-mediated interference (RNAi) to test candidate genes for enhancement of the Mes mutant phenotype. Enhancement in this assay was induction of sterility a generation earlier, in the otherwise fertile homozygous progeny of heterozygous mothers, which previous results had suggested represent a sensitized genetic background. We tested seven genes predicted to encode regulators of chromatin organization for RNAi-induced enhancement of *mes-3* sterility and identified one enhancer, called *set-2* after the SET domain encoded by the gene. Depletion of SET-2 also enhances the sterile phenotype of *mes-4* but not of *mes-2* or *mes-6*. *set-2* encodes two alternatively spliced transcripts, *set-2*$_l$ and *set-2*$_s$, both of which are enriched in the germline of adults. In the adult germline, SET-2$_L$ protein is localized in mitotic and mid-late-stage meiotic nuclei but is undetectable in early pachytene nuclei. SET-2$_L$ protein is localized in all nuclei of embryos. The localization of SET-2$_L$ does not depend on any of the four MES proteins, and none of the MES proteins depend on SET-2 for their normal localization. Our results suggest that SET-2 participates along with the MES proteins in promoting normal germline development.

GERM cells display numerous unique traits, among them the ability to undergo meiosis and the ability to generate offspring. These unique traits require unique control mechanisms. Among the specialized regulatory molecules required in the germline of *Caenorhabditis elegans* are the four maternal-effect sterile (MES) proteins, named after their maternal-effect sterile mutant phenotype (Capowski *et al.* 1991). In the progeny of homozygous *mes* mothers, the germline undergoes only limited proliferation and then degenerates, resulting in sterile but otherwise healthy adults (Paulsen *et al.* 1995; Garvin *et al.* 1998). The predicted MES protein sequences reveal that MES-2 and MES-6 are the *C. elegans* orthologs of the Drosophila Polycomb group (PcG) proteins, Enhancer of Zeste [E(Z)], and Extra Sex Combs (ESC), respectively (Holdeman *et al.* 1998; Korf *et al.* 1998). MES-3 is a novel protein with no recognizable motifs (Paulsen *et al.* 1995). MES-4 is a SET-domain protein with multiple PHD fingers (Y. Fong, L. Bender and S. Strome, unpublished results).

The similarity of MES-2 and MES-6 to PcG proteins suggests that the MES proteins are likely to function similarly to PcG proteins. PcG members associate into multiprotein complexes to repress transcription of target genes (Gould 1997; Pirrotta 1997; Preuss 1999). Their best known targets in Drosophila are homeotic genes, which function to determine the anterior-posterior body pattern (Pirrotta 1995; Simon 1995; Akam 1998). After the initial expression patterns of homeotic genes are established in the early embryo (Carroll *et al.* 1986; Celniker *et al.* 1989), maintenance of the expression patterns is controlled by the antagonistic functions of the PcG and the trxG (trithorax group). PcG proteins maintain repression of genes outside of their expression domains by promoting assembly of chromatin into a repressive state, while trxG proteins maintain active gene expression within the proper domains by remodeling chromatin into a transcriptionally active state (Owen-Hughes *et al.* 1996; Shao *et al.* 1999).

Although PcG and trxG proteins are generally thought to function antagonistically in transcriptional regulation, the distinction between them is not always clear. E(Z), for example, is a classic PcG member. Mutations in E(Z) cause anterior to posterior segment transformations that are characteristic of loss of PcG gene function (Jones and Gelbart 1990). However, E(Z) has also been implicated as a trxG protein (LaJeunesse and Shearn 1996). Animals heterozygous for both a mutation in *E(z)* and a mutation in *ash-1* (a trxG member) present homeotic transformation phenotypes similar to those displayed by *trithorax* and *ash1* double heterozygous mutants. Furthermore, in hemizygous mutants for

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amorphic mutant alleles of *E(z)*, accumulation of many homeotic gene products is lost. These results suggest that E(Z), a PcG protein, can in certain contexts display trxG characteristics and activate gene expression.

On the basis of analysis of transgene expression, the MES proteins participate in repression of gene expression in the germline of *C. elegans*, as PcG proteins do in the Drosophila soma. Transgenes present in multiple copies in extrachromosomal arrays are efficiently expressed in somatic tissues but silenced in the germline of wild-type *C. elegans* (Kelly *et al.* 1997). Such high-copy transgenic arrays are desilenced in the germlines of sterile *mes* mutants (Kelly and Fire 1998). Transgenes in arrays also can be desilenced in wild-type germlines by reducing their copy number and placing them in the context of complex DNA (Kelly *et al.* 1997). Taken together, these findings suggest that the MES proteins participate in repressing transgene expression in wild-type germlines and that this is via an effect on chromatin state.

To identify new components in the MES regulatory pathway, a powerful approach is to search for enhancers of the Mes phenotype in a sensitized genetic background. We reasoned that M+Z− *mes* mutants (M, maternal; Z, zygotic or nonmaternal) might represent such a sensitized background for this kind of search. First, M+Z− *mes* hermarphrodites (*i.e.*, *mes/mes* hermaphrodites from *mes/+* mothers) are fertile but have reduced brood sizes, indicating that their germlines are compromised (Capowski *et al.* 1991). In the case of *mes-3*, M+Z− hermaphrodites are occasionally sterile. Second, transgene desilencing occurs in the germlines of M+Z− *mes-3* and *mes-4* hermaphrodites (Kelly and Fire 1998), suggesting that the germlines of M+Z− *mes* mutants display some characteristics of the sterile M−Z− *mes* germlines. We hoped that additional mutations that enhance the Mes phenotype might induce sterility in these M+Z− *mes* worms. We used RNA-mediated interference (RNAi) to test candidate genes for enhancement of *mes-3*. We found that RNA-mediated interference with one gene, called *set-2*, caused sterility in M+Z− *mes-3* and *mes-4* mutants, but not in *mes-2* or *mes-6* mutants or in wild-type worms. *set-2* encodes two alternative transcripts, *set-2$_l$* and *set-2$_s$*, both of which are germline enriched. The larger protein product, SET-2$_L$, is distributed similarly to the MES proteins in the nuclei of germ cells and embryos.

MATERIALS AND METHODS

Alleles and strain maintenance: N2 var. Bristol was used as the wild-type *C. elegans* strain in this report. The following mutant strains were used for RNAi analyses: *mes-3(bn35) dpy-5 (e61)/mes-3(bn35)dpy-5(e61)*I/*hDp20*(I;V, f), *mes-4(bn67) dpy-11(e224)*V/*nT1 [let(m435)]*(IV,V), *mes-2(bn11) unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)]*II, and *mes-6(bn38)*IV/*DnT1[unc(n754)let]*(IV,V). Strains were maintained following standard procedures (Brenner 1974).

RNAi analyses: The cDNAs for RNAi tests were obtained from the expressed sequence tag (EST) project (Y. Kohara, National Institute of Genetics, Mishima, Japan) as λZAPII phagemid clones (yk273c7, yk11d12, yk25g10, yk40b5, yk112b11, yk52e6, yk250a4, and yk427g1). The cDNA inserts are flanked by T7 and T3 promoters in the context of linearized pBlueScript vectors. After the pBlueScript plasmids were excised from the phagemids by helper phages (Sambrook *et al.* 1989), they were transformed into *Escherichia coli* and amplified. Sense and antisense RNAs of each gene were transcribed separately *in vitro* from the cDNA by T7 or T3 polymerases, using the MEGAscript kit (Ambion, Austin, TX) followed by DNase treatment. The RNAs were heated to 85° for 5 min and cooled to room temperature to anneal.

To generate the double-stranded RNA that targets *set-2$_l$* only, an ~1-kb *set-2$_l$*-specific cDNA fragment was amplifed by RT-PCR using poly(A)$^+$ RNA prepared from wild-type worms as the template as described previously (Sambrook *et al.* 1989; Conrad *et al.* 1991). The downstream primer for reverse transcription and for PCR was 5′-AAAAGCGGCCGCTCGGTTTT TCAGCTTC-3′. The upstream primer for PCR was 5′-AAAG GATCCGCATCGGGAAGCTCTTC-3′. The PCR product was cloned into pBlueScript. The sequence of the PCR product was verified by sequencing. Double-stranded RNA from this PCR product was produced as described above.

Double-stranded RNA (~500 ng/μl) of each above cDNA was injected into heterozygous *mes* mutants that were raised at 20°. Embryos laid 12 hr and later after injection were grown to adult stage at 20° and scored for their sterility under a dissecting microscope. Worms with an empty uterus, indicating the absence of embryos, were scored as sterile.

Antibody production and immunostaining: Anti-SET-2$_L$ antibodies were generated against the N-terminal peptide of SET-2$_L$, MSTHDMNHHPPRKSHSKRDK. The peptide was synthesized and conjugated to keyhole limpet hemocyanin carrier protein by Research Genetics (Huntsville, AL). The conjugated peptide was injected into rats by Cocalico. The free peptide was crosslinked to immunopure epoxy-activated agarose (Pierce, Rockford, IL) for affinity purification of anti-SET-2$_L$ antibodies. Anti-SET-2$_L$ antibodies were eluted from the affinity column by 5 M KI and dialyzed against PBS overnight at 4°. Immunostaining was performed as previously described (Strome and Wood 1983).

Northern blot hybridization analyses: poly(A)$^+$ RNA was prepared as previously described from N2, *glp-4(bn2)*, and *fem-2 (b245ts)* mutant hermaphrodites raised at 25° (Sambrook *et al.* 1989; Conrad *et al.* 1991). It was electrophoresed on a 0.8% agarose 6% formaldehyde gel and blotted to Hybond-Nylon membrane. The blots were hybridized with a *set-2* probe, which was amplified by PCR using yk25g10 as the template and radiolabeled with [α-^{32}P]dCTP using a Boehringer Mannheim (Indianapolis) random-primed labeling kit. *rpp-1*, a ribosomal protein gene (Evans *et al.* 1997), served as a loading control. RNA bands were quantified using NIH Image software (National Institutes of Health). The relative intensity of each band was expressed as the hybridization intensity of the *set-2* band relative to the *rpp-1* band in each lane, and the ratio in the mutants was compared to that in N2, which was arbitrarily set to 1.

RT-PCR and Southern blot analyses: RT-PCR reactions were performed as previously reported (Spieth *et al.* 1993). Poly(A)$^+$ RNA prepared as described above was used as the RNA template. The downstream primers used for reverse transcription and for PCR were 5′-GAAGATGGGCTCGACAT AAG-3′ for *set-2$_l$* and 5′-CGAAGCACGTCTTCGTCGC-3′ for *set-2$_s$*. The upstream primers for PCR were SL1 (5′-GGTT TAATTACCCAAGTTTGAG-3′) and SL2 (5′-GGTTTTAACC CAGTTACTCAAG-3′). The PCR products were electropho-

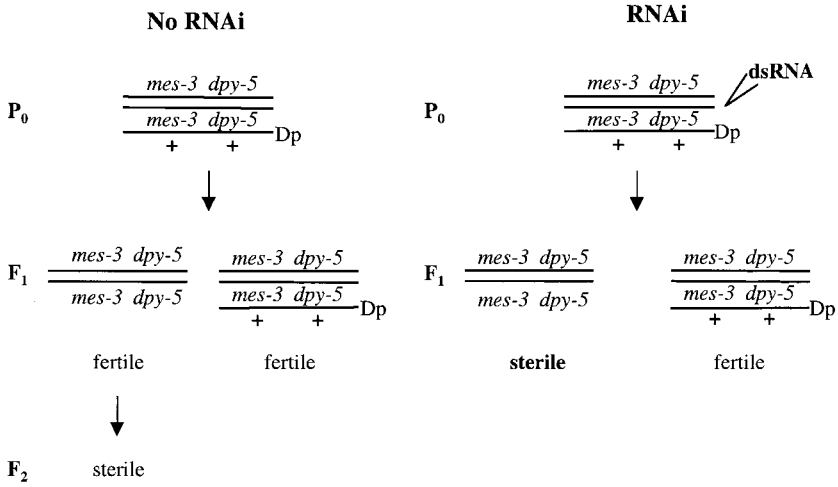

FIGURE 1.—Strategy used to search for enhancers of *mes-3*. The strain *mes-3 dpy-5/mes-3 dpy-5/hDp20* was used to search for enhancers of *mes-3*. (Left) The genotypes and phenotypes of the progeny from mothers without RNA injection. (Right) The genotypes and phenotypes of the progeny from mothers injected with double-stranded RNA made from a gene whose depletion enhances the Mes-3 phenotype. RNAi depletion of such an enhancer will cause the homozygous *mes* F_1 progeny to develop into sterile adults, while their heterozygous siblings remain fertile.

resed on a 0.8% agarose gel and probed for *set-2$_l$* or *set-2$_s$* by Southern blot, following standard procedures (SAMBROOK *et al.* 1989). The probes used were 5′-CGTACTTCACAACGT CCC-3′ to detect *set-2$_l$* and 5′-GACGACGTGGAAACTGTACG-3′ to detect *set-2$_s$*. The PCR products were cloned into pGEM-T vector and sequenced using T7 and SP6 promoter sequence as the primers.

Sequence analyses of *set-2$_l$* and *set-2$_s$* cDNAs: *set-2$_l$*-specific cDNA was amplified as three overlapping fragments by RT-PCR as described above. Fragment 1 was as described in *RT-PCR and Southern blot analyses*. For fragment 2, the downstream primer for reverse transcription and for PCR was 5′-GCT TCCCGATGCAGATTCGG-3′ and the upstream primer for PCR was 5′-CTTATGTCGAGCCCATCTTC-3′. For fragment 3, the downstream primer for reverse transcription and for PCR was 5′-AAAAGCGGCCGCTCGGTTTTTCAGCTTC-3′ and the upstream primer for PCR was 5′-AAAGGATCCGC ATCGGGAAGCTCTTC-3′.

The 231 bp of *set-2$_s$*-specific cDNA was sequenced using yk25g10 as the template and the T7 promoter sequence as the primer. yk25g10 is a cDNA clone from the EST project, which contains the *set-2$_s$*-specific sequence as well as the common region between *set-2$_l$* and *set-2$_s$*.

The common region between *set-2$_l$* and *set-2$_s$* was sequenced using yk25g10 and yk11d12 as the templates. yk11d12 is a cDNA clone from the EST project, which contains 249 bp of the *set-2$_l$*-specific region and overlaps with fragment 3 of *set-2$_l$* (see above) and the common region between *set-2$_l$* and *set-2$_s$*. Sequencing was performed using an ABI PRISM DNA sequencing kit and ABI PRISM 310 genetic analyzer (PE Applied Biosystems).

Bioinformatics: The predicted amino acid sequences of TRX, BRM, ASH-1, FSH, E(PC), and NURF p55 in Drosophila were obtained by searching GenBank (http://www3.ncbi.nlm.nih.gov/Entrez/). They were used to search for potential homologs in *C. elegans* using the Wu-BLAST program (http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml). Information on the cDNA clones available for each homolog was provided by GenBank.

The RNA recognition motif (RRM) in SET-2_L was predicted by the Pfam program (http://www.sanger.ac.uk/Software/Pfam/search.shtml). The secondary structure of this putative RRM in SET-2_L was predicted by the following programs: SSP (http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html), SSpro (http://promoter.ics.uci.edu/BRNN-PRED/), Predator (http://www.embl-heidelberg.de/cgi/predator_serv.pl), and PSIpred (http://insulin.brunel.ac.uk/psiform.html).

RESULTS

Test for enhancers of *mes-3*: To identify new components in the MES regulatory pathway, we tested candidate genes for whether depletion of gene product by RNAi enhances *mes-3* sterility. RNAi, which involves injection of gene-specific double-stranded RNA (dsRNA) into hermaphrodites, is an extremely potent and specific method of reducing gene expression and has been demonstrated to phenocopy strong or null mutations in many genes (ROCHELEAU *et al.* 1997; FIRE *et al.* 1998). Consequently, RNAi has been used extensively to examine the functions of genes with known nucleotide sequences but unknown mutant phenotypes. The parent strain we used to test for RNAi-induced enhancement of the Mes phenotype contains the *mes-3(bn35)* mutation on both chromosomes balanced by a duplication, *hDp20*, which contains a wild-type copy of *mes-3* (*i.e.*, *bn35/bn35/hDp20*; Figure 1). This strain is similar to a heterozygous *mes-3* strain and produces predominantly fertile F_1 progeny. The fertile *bn35/bn35* F_1 hermaphrodites produce all sterile F_2 progeny. We reasoned that RNAi depletion of a protein that functions with MES-3 might induce sterility in the *bn35/bn35* F_1's (Figure 1). The *bn35/+* siblings, however, might remain fertile as a result of zygotic expression of MES-3.

The genes that we selected to test for a genetic interaction with *mes-3* encode potential chromatin regulators. They included a gene encoding a protein that shares a domain with the Drosophila PcG protein Enhancer of Polycomb [E(PC)] (STANKUNAS *et al.* 1998); a gene encoding a homolog of the chromatin remodeling factor, nucleosomal remodeling factor p55 (NURF p55; MARTINEZ-BALBAS *et al.* 1998); and five *C. elegans* homologs of *trxG* genes. As described above, although PcG and trxG proteins are generally thought to function antagonistically in Drosophila, there is evidence that certain proteins belong to both groups. In particular, the PcG member, E(Z), sometimes presents trxG characteristics. Therefore, the *C. elegans* trxG homologs were consid-

TABLE 1

Tests for enhancement of *mes-3* sterility by RNAi depletion of selected genes

cDNA clones tested by RNAi	Homolog in Drosophila	Enhancement of sterility of *mes-3*[a] (%)	Enhancement of sterility of *mes-3/+*[a] (%)	Embryonic lethality[b] (%)
T12D8.1 (yk273c7)	*trx*	Yes (0–66)	No	Yes (>50)
C26E6.9a and C26E6.9b (yk11d12 and yk25g10)	*trx* SET domain	Yes (92)	Slight (11)	No
T12F5.4 (yk40b5)	*ash-1*	No	No	No
C52B9.8 (yk112b11)	*brm*	No	No	No
F57C7.1A and 1B (yk52e6)	*fsh*	No	No	No
yk250a4	*E(Pc)*	No	No	Yes (>50)
K07A1.12 (yk427g1)	*NURF p55*	Yes (83)	Yes (25)	Yes (>50)

[a] Double-stranded RNAs were injected into *mes-3(bn35) dpy-5/mes-3(bn35) dpy-5/hDp20* mothers and their *mes-3* (Dpy) progeny, and *mes-3/mes-3/hDp20* (non-Dpy) progeny were scored for sterility.
[b] Embryonic lethality was scored among the embryos of the injected mothers.

ered candidates to genetically interact with the MES proteins, two of which (MES-2 and MES-6) are PcG homologs. The *C. elegans* genome contains two genes with significant sequence similarity to *trithorax* (Stassen *et al.* 1995). One of them, T12D8.1, encodes a predicted protein that is similar to TRX throughout its length; it contains three PHD fingers and a SET domain, as does TRX. The other gene, C26E6.9, encodes two predicted proteins that have sequence similarity with TRX only in the SET domain. We also found homologs of the trxG genes *ash-1* (Tripoulas *et al.* 1996), *brm* (Tamkun *et al.* 1992), and *fsh* (Haynes *et al.* 1989).

Double-stranded RNA to each of the above genes was synthesized and injected into heterozygous *mes-3* mutants, and the F_1 progeny were scored for sterility. As summarized in Table 1, three types of RNAi effects were observed:

1. No enhancement of sterility in the *mes-3* F_1's: We did not pursue these genes further.
2. Embryonic lethality among embryos of the injected worms and also enhancement of sterility in the *mes-3* F_1's: The embryonic lethality phenotype indicated that RNAi depletion of these genes generated early somatic defects in the F_1 progeny, which led to the concern that the sterile phenotype may be a secondary consequence of somatic defects or of general sickliness. Therefore, we did not pursue these genes further.
3. Enhancement of the sterility of *mes-3* F_1's, no significant enhancement of the sterility of *mes-3/+* F_1's, and no embryonic lethality: Among the seven genes tested, one gene displayed this genetic enhancement of the *mes-3* phenotype. This gene, C26E6.9, was named *set-2* and is the focus of the remainder of this article.

***set-2* is an enhancer of *mes-3* and *mes-4*:** *set-2* was named for the SET domain in the predicted protein products. According to the GeneFinder prediction program, the locus encodes two overlapping transcripts; this was confirmed by Northern hybridization (see below). We call the longer one [open reading frame (ORF) no. C26E6.9a] *set-2*$_l$ and the shorter one (C26E6.9b) *set-2*$_s$. When both transcripts were depleted by RNAi, the sterility of F_1 *mes-3* worms was increased from 15 to 92% (Figure 2). RNAi depletion of *set-2* in wild-type worms, however, did not cause sterility or any other significant defects. The sterile germlines observed in *mes-3; set-2(RNAi)* worms were quite variable in appearance. Approximately 15% of sterile adults contained a severely underproliferated germline, resembling the phenotype of sterile *mes-3* worms from homozygous *mes-3* mothers. However, the remaining sterile worms contained fairly well-proliferated germlines and some contained gametes; none of them produced fertilized embryos. We do not know whether this variability in germline phenotype reflects variability in RNAi depletion of SET-2 or variable effects of full depletion of SET-2. This issue, as well as whether *set-2* RNAi enhancement of sterility is due to depletion of maternal or zygotic SET-2 or both, will be addressed when a null mutant of *set-2* is isolated.

To test whether *set-2*$_l$ and *set-2*$_s$ both function to enhance *mes-3*, we injected *mes-3* heterozygous worms with dsRNA that targets both isoforms or with dsRNA that targets only *set-2*$_l$. RNAi depletion of *set-2*$_l$ alone caused 53% sterility in *mes-3* F_1's, whereas RNAi depletion of both isoforms of *set-2* caused 92% sterility in *mes-3* F_1's (Figure 2). Therefore, the enhancement was more severe when both transcripts were depleted by RNAi than when only the larger one was depleted. This indicates that depletion of either *set-2*$_l$ or *set-2*$_s$ partially enhances the *mes-3* mutant phenotype.

We also tested whether RNAi depletion of *set-2* enhances the sterility caused by mutations in the other three *mes* genes. As described above, we injected *set-2* dsRNA into heterozygous *mes* mothers and analyzed

A

RNAi depletion of both *set-2$_l$* and *set-2$_s$*

Genotype of P_0 worms injected	No. of worms injected	% sterile *mes* worms in the F_1 generation	% sterile *mes*/+ worms in the F_1 generation
mes-3/+	23	92 (350/381)	11 (66/595)
mes-4/+	13	95 (92/97)	0 (1/381)
mes-2/+	9	1 (2/143)	0 (0/300*)
mes-6/+	8	0 (0/67)	0 (0/207)

Note: >10 wild-type (N2) worms were injected, and no sterile F_1s were observed.
*Estimated number.

RNAi depletion of *set-2$_l$* only

Genotype of P_0 worms injected	No. of worms injected	% sterile *mes* worms in the F_1 generation	% sterile *mes*/+ worms in the F_1 generation
mes-3/+	36	53 (560/1057)	7 (76/1141)
mes-4/+	28	53 (90/171)	0 (1/215)
mes-2/+	9	0 (2/337)	0 (0/872)
mes-6/+	6	8 (2/24)	0 (0/74)

Note: 11 wild-type (N2) worms were injected, and no sterile F_1s were observed.

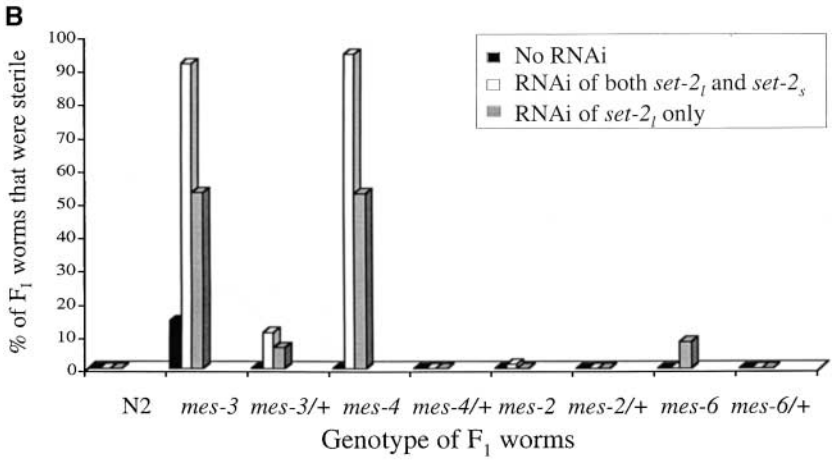

FIGURE 2.—RNAi analysis of *set-2*. Double-stranded RNA was made from a region specific to *set-2$_l$* or common to *set-2$_s$* and *set-2$_l$* and was injected into N2, *mes-2*/+, *mes-3*/+, *mes-4*/+, or *mes-6*/+ young adult hermaphrodites. The percentage of sterile F_1 progeny was calculated for the various genotypes (A) and graphed (B).

their *mes* and *mes*/+ F_1 progeny. RNAi depletion of *set-2* enhanced the sterility of *mes-4* F_1's to a similar degree as seen in *mes-3* but did not enhance the sterility of *mes-2* or *mes-6* F_1's (Figure 2).

***set-2$_l$* is *trans*-spliced to SL1:** Northern hybridization analysis demonstrated that the *set-2* locus encodes two transcripts, estimated to be 6.5 and 3.4 kb (Figure 3A). According to GeneFinder, the first four exons of *set-2$_l$* encode a GTP-binding domain and the last three exons encode a SET domain. To verify this prediction, the 5′ end of *set-2$_l$* was determined by RT-PCR followed by sequence analysis (see MATERIALS AND METHODS). We found that at least some *set-2$_l$* transcripts are *trans*-spliced to SL1 but not to SL2 (Figure 4A). *set-2$_s$* is not *trans*-spliced to either SL1 or SL2. Sequencing of the *set-2$_l$* RT-PCR product showed that, different from the GeneFinder prediction, the 5′ end of *set-2$_l$* starts after the predicted GTP-binding domain-containing exon. Consistent with our determined 5′ end, we found that RNAi using RNA directed against the GTP-binding domain did not enhance *mes-3* sterility (data not shown). Consequently, we conclude that the SET-2_L protein does not include the GTP-binding domain; this domain might be encoded by the gene upstream of *set-2$_l$*. The *set-2$_l$* cDNA was subsequently sequenced (see MATERIALS AND METHODS), and its gene structure is shown in Figure 4B. Since *set-2$_s$* appears not to be *trans*-spliced to either SL1 or SL2 (data not shown), we did not verify its 5′ end. However, our sequencing of a cDNA clone of *set-2$_s$* (yk25g10) suggests that it encodes the full-length SET-2_S protein; it includes a region of predicted 5′ UTR, the entire ORF predicted by GeneFinder, and a 3′ UTR.

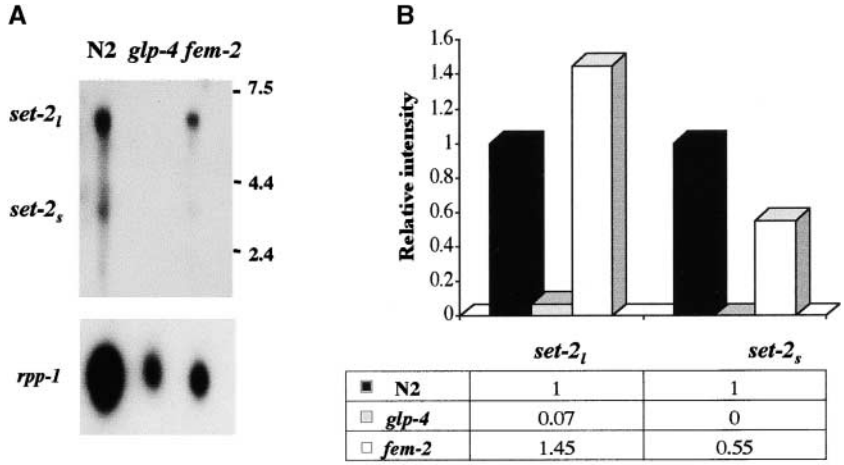

FIGURE 3.—Northern blot hybridization analysis of *set-2*. (A) poly(A)$^+$ RNA was prepared from N2, *glp-4(bn2ts)*, and *fem-2(b245ts)* adult hermaphrodites grown at 25° and probed with a region common to *set-2*$_s$ and *set-2*$_l$ on Northern blots. After growth at 25°, *glp-4* adults have a severely underproliferated germline, and *fem-2* adults produce oocytes but no sperm. The probe recognizes two bands estimated to be 6.5 and 3.4 kb. The predicted sizes of *set-2*$_l$ and *set-2*$_s$ mRNAs without poly(A) tails are ~5.2 and ~2.8 kb, respectively. The same blot was probed with the *C. elegans* ribosomal protein gene, *rpp-1*, as a loading control. The numbers on the right of the blot are in kilobases. (B) The levels of *set-2*$_l$ and *set-2*$_s$ in each strain were normalized to the loading control and are shown in the histogram with the level in N2 arbitrarily set to 1.

Protein motifs in SET-2: The predicted 1507-amino-acid SET-2$_L$ and 739-amino-acid SET-2$_s$ proteins both contain a C-terminal SET domain that displays 58% amino acid identity with the SET domain in TRX (Figure 5). The SET domain was first identified as a shared motif among Suppressor of variegation 3-9 [SU(VAR) 3-9] (TSCHIERSCH *et al.* 1994), E(Z) (JONES and GELBART 1993), and TRX (STASSEN *et al.* 1995) in Drosophila. All SET domain-containing proteins that have been characterized are associated with chromatin (KUZIN *et al.* 1994; TSCHIERSCH *et al.* 1994; CHINWALLA *et al.* 1995; CARRINGTON and JONES 1996; TRIPOULAS *et al.* 1996), suggesting that SET domain proteins are generally involved in chromatin regulation. However, the SET domains in different proteins appear to function through different mechanisms. For example, the SET domains in vertebrate homologs of SU(VAR)3-9 have histone H3 methyltransferase (HMTase) activity, but the SET

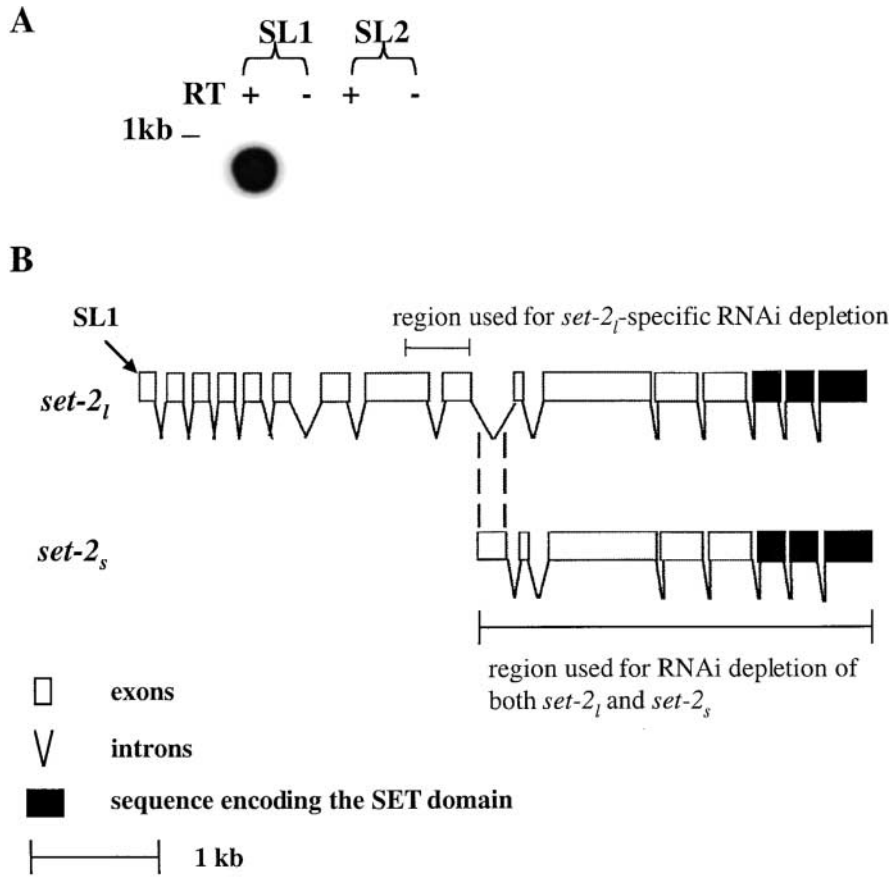

FIGURE 4.—The 5′ end of *set-2*$_l$ and the gene structures of the *set-2* isoforms. (A) RT-PCR products of *set-2*$_b$ generated using SL1 or SL2 as the upstream primer, were probed with a *set-2*$_l$-specific probe. The results show that *set-2*$_l$ is *trans*-spliced to SL1 and not to SL2. (B) Gene structures of the two predicted isoforms of *set-2*, *set-2*$_l$ and *set-2*$_s$. *set-2*$_s$ starts within intron 9 of *set-2*$_l$ and shares the 3′ ~3 kb with *set-2*$_l$. The regions used for RNAi analysis are indicated.

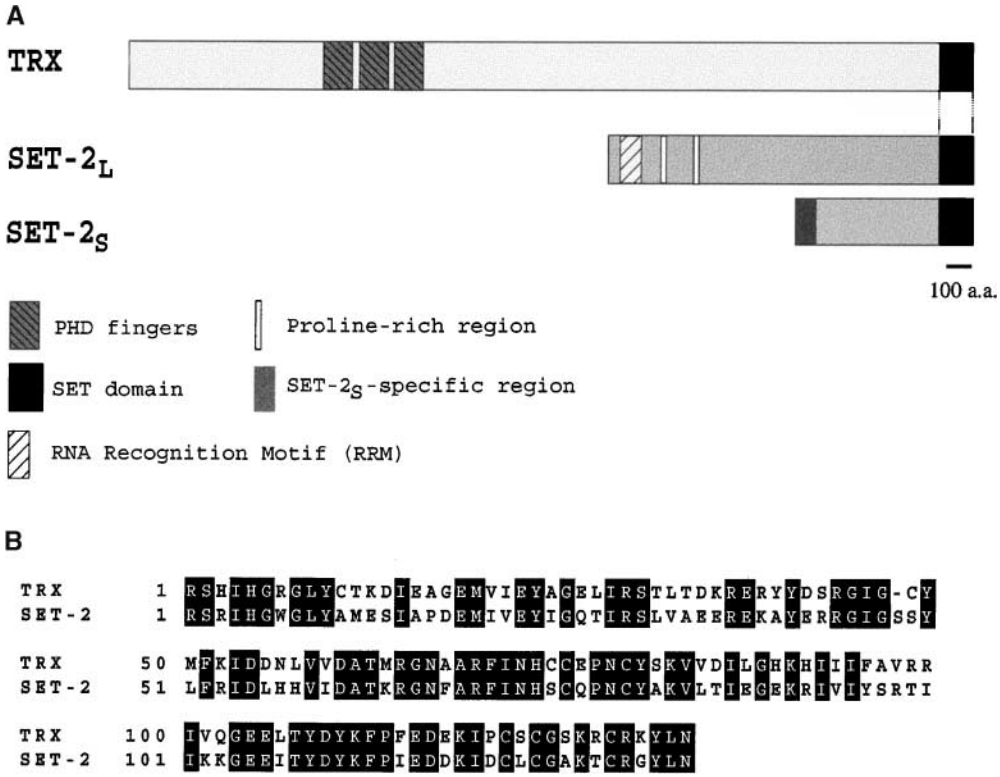

FIGURE 5.—Protein motifs in SET-2. (A) Schematic alignment of SET-2 isoforms and TRX, showing the relative protein lengths and motifs. (B) Alignment of the SET domains in TRX and SET-2. Identical residues are in black boxes. The alignment was done using NCSA BIOLOGY WORKBENCH software (http://workbench.sdsc.edu).

domains in TRX and E(Z) do not display HMTase activity (REA *et al.* 2000).

SET-2_L also contains two consecutive proline-rich regions (Figure 5A). Since proline residues enhance the flexibility of protein structures, they often appear in proteins that are involved in dynamic processes. Many proline-rich proteins function in signal transduction pathways, and the proline-rich regions in them are important for protein-protein interactions (KAY *et al.* 2000).

SET-2_L has a putative RRM on the basis of analysis by the Pfam program (see MATERIALS AND METHODS). RRMs have been found in a variety of RNA-binding proteins (SWANSON *et al.* 1987; BANDZIULIS *et al.* 1989; KENAN *et al.* 1991). The primary sequences of RRMs are not well conserved. However, their structures typically contain four β sheets and two α helices, which form two consecutive βαβ sandwiches (BURD and DREYFUSS 1994). The second βαβ sandwich, especially the last β sheet, is less well conserved. Several structure prediction programs (see MATERIALS AND METHODS) predicted that the putative RRM of SET-2_L contains the first βαβ sandwich and the βα portion of the second βαβ sandwich. Another β sheet was predicted to be adjacent to the C terminus of the predicted RRM domain. This may serve as the last β sheet to form the second βαβ sandwich and a functional RRM in SET-2_L.

***set-2* mRNAs are germline enriched:** To gain insight into the pattern of *set-2* expression, a *set-2* cDNA fragment common to both isoforms was used to probe Northern blots of poly(A)$^+$ RNA prepared from wild-type, *glp-4(bn2ts)*, and *fem-2(b245ts)* adult hermaphrodites (Figure 3). *glp-4* adult hermaphrodites, which have a severely underproliferated germline (BEANAN and STROME 1992), contain <10% of the level of *set-2$_l$* transcripts present in wild-type adult hermaphrodites. *set-2$_l$* is present at a higher level in *fem-2* hermaphrodites (~145% of wild type), which produce only oocytes (KIMBLE *et al.* 1984). However, the level of *set-2$_s$* is lower in *fem-2* hermaphrodites than in wild type. These results suggest that *set-2* mRNA is expressed predominantly in the germline. Consistent with this, *in situ* hybridization analysis showed germline-enriched accumulation of *set-2* transcript in larvae and adults (Y. KOHARA, personal communication).

SET-2_L protein is localized in the nuclei of embryos and germ cells: To determine the distribution of SET-2_L protein, antibodies were raised against the N-terminal peptide of SET-2_L. The distribution of SET-2_L in wild-type worms and embryos was determined by immunostaining. SET-2_L protein is localized in the nuclei of all cells in embryos at all stages of embryogenesis (Figure 6). In L1 larvae, SET-2_L remains visible in the nuclei of most cell types but is most prominent in Z2 and Z3, the primordial germ cells. In adults, SET-2_L staining is nuclear and is strongest in the germline (Figure 7), although it is also detectable in other cell types, such as intestinal, pharyngeal, and neuronal cells (data not shown). Interestingly, the level of SET-2_L varies in different portions of the gonad. It is present in the distal mitotic cells, diminishes to very low levels in the transition zone and early pachytene regions, and increases

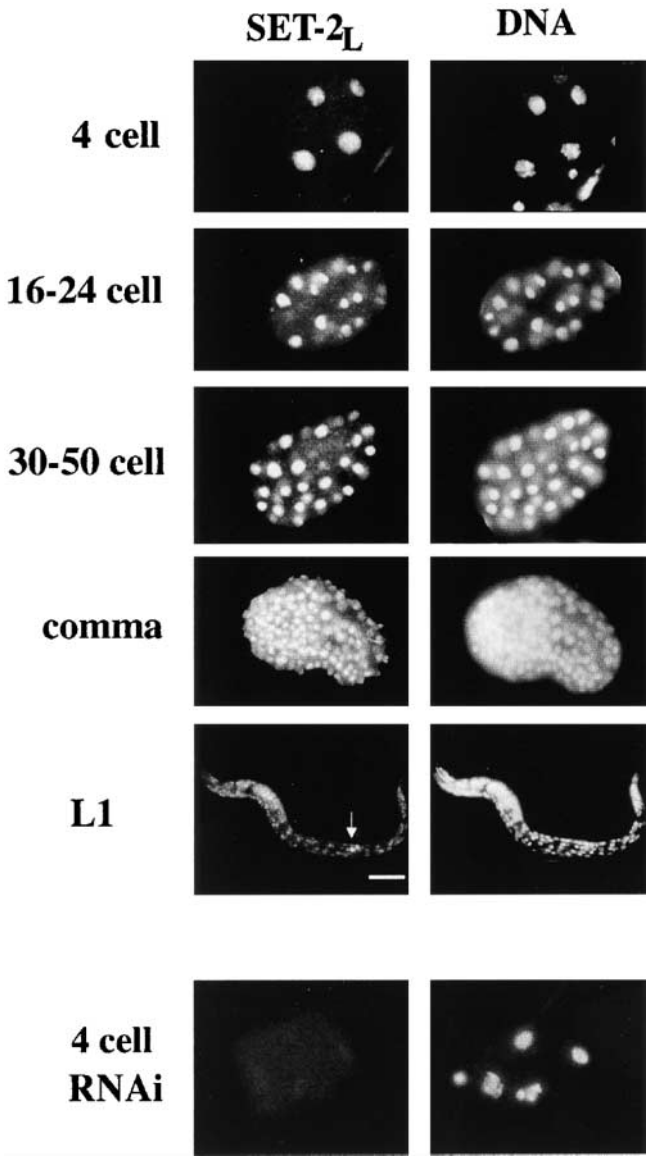

FIGURE 6.—Immunolocalization of SET-2_L in embryos and L1 larvae. Wild-type worms were stained with affinity-purified anti-SET-2_L antibody and 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA. During all stages of embryogenesis, SET-2_L is localized in the nuclei of all cells. In L1 larvae, it appears to be present at higher levels in the primordial germ cells, Z2 and Z3 (arrow). (Bottom) SET-2_L staining is not detectable in *set-2$_I$(RNAi)* embryos. Bar, 10 μm.

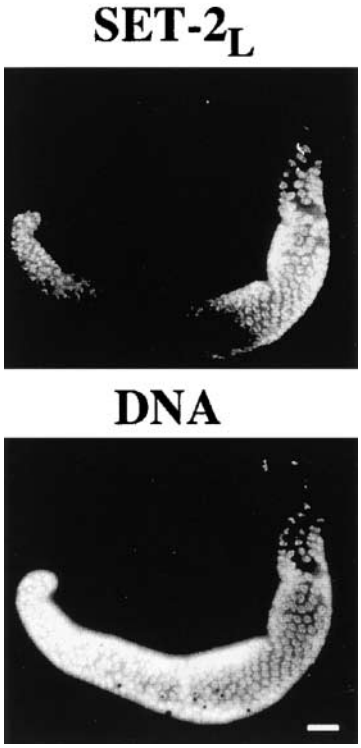

FIGURE 7.—Immunolocalization of SET-2_L in the germline. Extruded gonads of wild-type worms were stained with affinity-purified anti-SET-2_L antibody and DAPI to visualize DNA. The distal region of the gonad, where germ nuclei divide mitotically and enter meiosis, is to the left, and oocytes are in the upper right. SET-2_L is visible in the distal mitotic region of the gonad, undetectable in early pachytene nuclei, and present at high levels in mid- and late-pachytene nuclei. Its level decreases again in oocytes. Bar, 20 μm.

dramatically in mid-pachytene nuclei. In oocytes, the level of SET-2_L appears relatively low. The specificity of the antibodies was demonstrated by the observation that SET-2_L immunostaining is reduced to below detection in *set-2(RNAi)* embryos and worms (Figure 6).

SET-2_L and the MES proteins do not depend upon each other for correct nuclear localization: One hypothesis to explain *set-2* RNAi enhancement of the sterility of *mes-3* and *mes-4* is that depletion of SET-2 causes defects in the accumulation or distribution of MES-3 and/or MES-4. To test this possibility, we investigated whether the localization of MES proteins is altered in the absence of SET-2. Wild-type worms injected with *set-2$_I$*-specific dsRNA were stained with anti-MES-2, anti-MES-3, anti-MES-4, and anti-MES-6. The staining patterns of all four MES proteins resembled the staining patterns in uninjected worms (data not shown). Thus, the enhancement of sterility in *mes-3* and *mes-4* mutants by RNAi depletion of SET-2_L does not result from significant destabilization or mislocalization of MES proteins.

Conversely, to test whether the localization of SET-2 depends on any of the MES proteins, we compared the staining pattern of SET-2_L in *mes-2*, *mes-3*, *mes-4*, and *mes-6* worms and embryos with the pattern in wild type. We found that SET-2_L staining in embryos and germlines is not altered in any of the *mes* mutants (data not shown).

DISCUSSION

Screen for enhancers in a sensitized genetic background: Screening for enhancers or suppressors of mutant phenotypes in a sensitized genetic background has proven to be a powerful approach to identify new com-

ponents in a regulatory pathway. For example, Karim *et al.* (1996) took this approach to identify downstream genes of Ras1 signal transduction in Drosophila, using eye-specific expression of a mutant form of Ras1 to render eye development particularly sensitive to reduction in levels of other pathway components (Karim *et al.* 1996). As reported here, by searching for inducers of *mes-3* sterility in the M+Z− generation, which we consider to be a sensitized genetic background, we identified SET-2 as an additional participant in MES regulation of germline development. Furthermore, using RNAi as the method of gene depletion greatly accelerated the testing process. Our search for enhancers could be expanded to many more *C. elegans* genes, either by undertaking genetic screens for enhancers of *mes* sterility or by testing many genes by RNAi. The RNAi approach could be extended to the whole-genome level by systematically feeding *mes/+* worms bacteria that express dsRNA directed against nearly every *C. elegans* gene (Fraser *et al.* 2000) and screening for sterility among the *mes* progeny.

SET-2 may function redundantly with zygotic MES-3 and MES-4: MES proteins have been considered as strict maternal-effect factors. However, transgenic studies have demonstrated that zygotically synthesized MES-3 and MES-4 participate in transgene repression in the *C. elegans* germline (Kelly and Fire 1998). Furthermore, although M+Z− *mes* worms are fertile, they have reduced brood sizes (Capowski *et al.* 1991); in the case of *mes-3*, ~15% of M+Z− worms are sterile. Our findings in this article strengthen the view that zygotic MES-3 and MES-4 play important roles in the germline. RNAi depletion of *set-2* causes the majority of *mes-3* or *mes-4* homozygous progeny from heterozygous mothers to be sterile, whereas their heterozygous siblings remain fertile. Thus, depletion of SET-2 leads to sterility only when MES-3 or MES-4 is not zygotically synthesized.

One explanation for the sterility observed in *set-2 (RNAi); mes-3* and *set-2(RNAi); mes-4* hermaphrodites is that SET-2 functions redundantly with zygotic MES-3 and MES-4. When zygotic MES-3 and MES-4 are present, SET-2 is not necessary for development of a fertile germline. Conversely, when SET-2 is present, zygotic MES-3 and MES-4 are not necessary for fertility. When a combination of SET-2 and either MES-3 or MES-4 is absent, then sterility ensues. As described in the accompanying article (Xu *et al.* 2001b), analysis of the temperature-sensitive period for *mes-3* suggests that MES-3 function is not required after embryogenesis; the presence of functional MES-3 in the maternal germline and during embryogenesis is sufficient to ensure that those embryos develop into fertile adults. One scenario is that maternally supplied MES-3 induces a special state of chromatin in the germline blastomeres, which can be propagated during larval development in the absence of MES-3. The results in this article suggest that SET-2 may be involved in this epigenetic propagation. In the absence of SET-2, MES-3 may be able to participate in propagation of the germline state. In the absence of both SET-2 and MES-3, chromatin defects in the germline lead to sterility. Thus far, we have no evidence that SET-2 interacts physically with MES-3. The distributions of MES-3 and SET-2_L do not depend upon one another, and the estimated size of the MES-3/MES-2/MES-6 complex in embryo extracts (~255 kD; Xu *et al.* 2001a) predicts that the complex does not contain an additional large protein, such as SET-2_S or SET-2_L.

It is interesting that SET-2 depletion enhances the sterility of both *mes-3* and *mes-4* but not of *mes-2* or *mes-6*. Because the mutant phenotypes of the four *mes* genes are very similar in most respects (Capowski *et al.* 1991; Paulsen *et al.* 1995; Garvin *et al.* 1998) and because MES-3, MES-2, and MES-6 are assembled into and probably function in a complex in embryos, we expected that an enhancer of *mes-3* would also enhance *mes-2* and *mes-6*. This is not observed for SET-2. Instead, enhancement of sterility by SET-2 depletion appears to correlate with zygotic effects on transgene expression in the germline: *mes-3* and *mes-4* M+Z− worms show transgene desilencing (Kelly and Fire 1998) and, when depleted for *set-2*, are sterile, whereas *mes-2* and *mes-6* M+Z− worms rarely show transgene desilencing (Kelly and Fire 1998) and, when depleted for *set-2*, are not sterile. These results may be explained by different degrees of perdurance of maternally supplied *mes* gene product. Persistence of *mes-2* and *mes-6* maternal products in *mes-2* and *mes-6* M+Z− worms may enable their germlines to develop relatively normally and maintain transgene silencing, whereas more rapid disappearance of *mes-3* and *mes-4* maternal product in *mes-3* and *mes-4* M+Z− worms may compromise germline function, release transgenes from silencing, and render germline development more sensitive to depletion of other required factors, such as SET-2.

The two isoforms of SET-2, SET-2_L and SET-2_S, appear to function redundantly in enhancing the *mes-3* and *mes-4* mutant phentoype, since depletion of both SET-2_L and SET-2_S by RNAi created a more severe enhancement effect than did depletion of SET-2_L alone. Both SET-2 isoforms contain a SET domain, but only SET-2_L contains a putative RRM, which might be involved in RNA processing of target genes. It would be interesting to identify the downstream targets of SET-2_L and SET-2_S, because they may be downstream targets of zygotic MES-3 and MES-4 as well and may contribute to the sterility in *mes* mutants.

SET-2_L protein distribution: Immunostaining showed that SET-2_L is most prominently localized in the germline and in embryos, where its distribution closely parallels the distribution of MES-3 (see accompanying article). Interestingly, the levels of both proteins dramatically drop in the early meiotic region of the adult hermaphrodite germline. In the case of MES-3, this drop appears to depend on GLD-1 regulation [see accompanying article

(Xu *et al.* 2001b)]. The decline of SET-2 level does not appear to depend on either GLD-1 or MES-3; the SET-2 mRNAs do not contain consensus GLD-1-binding sites, and the distribution looks normal in M+Z− *mes-3* mutant germlines. We speculate that the drop in MES-3 and SET-2 levels as germ cells enter meiosis allows remodeling of chromatin and alteration of gene expression patterns for oogenesis.

SET-2_L is also present at easily detectable levels in various somatic cells in adults. The somatic localization of SET-2_L raises the possibility that SET-2 functions in a process common to the germline and soma but through different partners. In the germline, it may participate along with MES proteins in maintenance of proper gene expression patterns. In the soma, it may cooperate with other factors to ensure proper development of somatic tissues. SET-2_L contains proline-rich regions, which are thought to be involved in protein-protein interactions (Kay *et al.* 2000). It also contains an RRM, suggesting that its activity may involve binding RNA.

The SET domain in SET-2: SET-2 was named after the SET domain at the C termini of its two isoforms, SET-2_L and SET-2_S. The SET domains in two vertebrate homologs of SU(VAR)3-9 were recently shown to have histone HMTase activity (Rea *et al.* 2000). A catalytic motif in the SET domain and two cysteine-rich regions flanking the SET domain are important for this activity. The SET domain in EZH2 and HRX, the human homologs of E(Z) and TRX, lacks the C-terminal cysteine-rich region and the cysteine-rich region upstream of the SET domain, respectively. They do not display HMTase activity. Like TRX, SET-2 lacks the cysteine-rich region upstream of the SET domain. Therefore, SET-2 is unlikely to have HMTase activity. Instead, the SET domain in SET-2 might mediate protein-protein interactions, as the SET domain in TRX does (Cui *et al.* 1998; Rozenblatt-Rosen *et al.* 1998; Rozovskaia *et al.* 1999).

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Note added in proof: In a recent update to GenBank, the exons encoding a GTP-binding domain were removed from C26E6.9a, and C26E6.9c was added to the list of C26E6.9 transcripts. C26E6.9c is predicted to encode a protein with three extra amino acids compared to C26E6.9a. Consequently, SET-2_L is likely encoded by both C26E6.9a and C26E6.9c.

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