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MES-2, a maternal protein essential for viability of the germline in *Caenorhabditis elegans*, is homologous to a *Drosophila* Polycomb group protein

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SUMMARY

A unique and essential feature of germ cells is their immortality. In *Caenorhabditis elegans*, germline immortality requires the maternal contribution from four genes, *mes-2*, *mes-3*, *mes-4* and *mes-6*. We report here that *mes-2* encodes a protein similar to the *Drosophila* Polycomb group protein, Enhancer of zeste, and in the accompanying paper that *mes-6* encodes another Polycomb group protein. The Polycomb group is responsible for maintaining proper patterns of expression of the homeotic and other genes in *Drosophila*. It is thought that Polycomb group proteins form heteromeric complexes and control gene expression by altering chromatin conformation of target genes. As predicted from its similarity to a Polycomb group protein, MES-2 localizes to nuclei. MES-2 is found in germline nuclei in larval and adult worms and in all nuclei in early embryos. By the end of embryogenesis, MES-2 is detected primarily in the two primordial germ cells. The correct distribution of MES-2 requires the wild-type functions of *mes-3* and *mes-6*. We hypothesize that *mes-2* encodes a maternal regulator of gene expression in the early germline; its function is essential for normal early development and viability of germ cells.

Key words: maternal-effect sterile, Polycomb group, *Caenorhabditis elegans*

INTRODUCTION

In animals, the generation of offspring and the propagation of species rely on special cells called germ cells. Germ cells differ from somatic cells in several fundamental ways (reviewed in Marsh and Goode, 1994). (1) Gene expression appears to be controlled differently in germ cells than in somatic cells (Seydoux and Dunn, 1997; Van Doren et al., 1998). (2) Germ cells are uniquely capable of undergoing the specialized cell cycle, meiosis. (3) Germ cells are the only cells of the body that are totipotent and can generate all of the different cell types of the organism. (4) Germ cells are potentially immortal, while somatic cells can undergo only a finite number of divisions and then become senescent. Thus, there is a fundamental dichotomy between the germline and soma. In recent years, we have gained considerable knowledge of how somatic cells are specified and their development controlled. In contrast, our understanding of germline development remains quite rudimentary. In particular, the molecular mechanisms that distinguish germ cells from somatic cells and that guide development of the early germline remain mysterious. We have sought to identify early acting, germline-specific factors that participate in initiating the germline program in the nematode *Caenorhabditis elegans*.

Formation and development of the germline in *C. elegans* have been described in detail (reviewed in Schedl, 1997), providing a strong foundation for molecular genetic analysis of the essential components. During early embryogenesis, the germline is set apart from the soma via a series of four unequal divisions of the germline blastomeres (P0, P1, P2 and P3) (Fig. 1). The primordial germ cell, P4, is formed at the 16- to 24-cell stage and divides only once during embryogenesis. During larval development, the two daughters of P4 (Z2 and Z3) undergo extensive proliferation, generating ~1500 germ cells in the adult hermaphrodite and ~500 germ cells in the adult male. Germ cells enter meiosis in the L3 stage and differentiate into sperm in males and both sperm and oocytes in hermaphrodites.

To identify factors required for early germline development in *C. elegans*, we screened for maternal-effect mutations that result in sterile but otherwise healthy offspring. Exhaustive screens for such maternal-effect sterile mutations identified four genes (*mes-2*, *mes-3*, *mes-4* and *mes-6*) that produce a common mutant phenotype (Capowski et al., 1991; Paulsen et al., 1995; Garvin et al., 1998). In hermaphrodite progeny from *mes/mes* mothers, the germline undergoes only limited proliferation and then degenerates. As a result, the sterile worms contain as few as 5-10 germ nuclei and no mature gametes. Male offspring of *mes/mes* mothers display a less severe germline phenotype than hermaphrodite offspring: males generally contain hundreds of germ cells, including

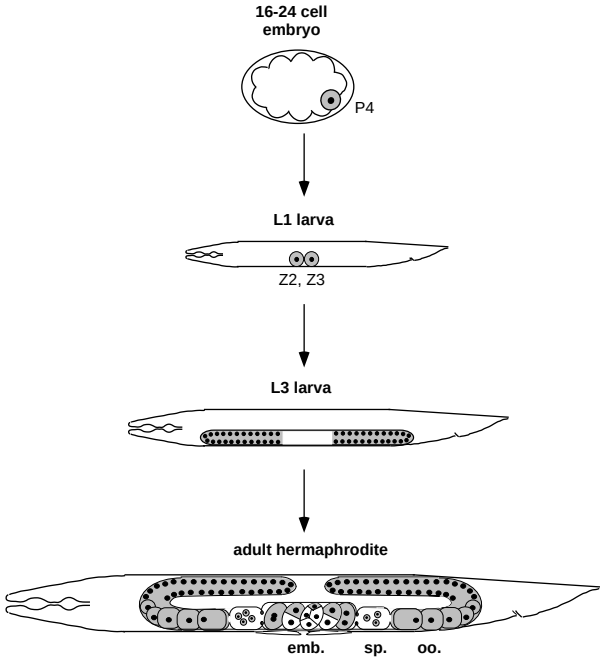

Fig. 1. Stages of germline development in *C. elegans*. The germline is shaded. The primordial germ cell, P4, is formed at the 16- to 24-cell stage of embryogenesis and divides only once before the embryo hatches into an L1 larva. Z2 and Z3 divide throughout larval development, giving rise to the ~1500 germ cells, including sperm and oocytes (labelled sp. and oo.), in an adult hermaphrodite. Embryos (emb.) are in the uterus.

mature sperm, and can be fertile. The differential response of hermaphrodite and male offspring to absence of *mes*$^{+}$ product is a result of their different X-chromosome compositions, suggesting that the *mes* genes participate in a germline process that is sensitive to chromosome dosage.

We report here that *mes-2* encodes the worm homolog of Enhancer of zeste [E(z)], which has been identified in insects, mammals and plants. *Drosophila* E(z) is a member of the Polycomb group (Pc-G) of transcriptional regulators, which maintain repression of homeotic and other developmentally regulated genes (Jones and Gelbart, 1990; Phillips and Shearn, 1990). MES-2 localizes to nuclei, specifically to germline nuclei of larvae and adults and to all nuclei in early embryos. Significantly, this staining pattern is disrupted in worms that have mutations in other *mes* genes. These results, along with recent analyses of *mes-6* and *mes-3*, suggest that survival of the germline in *C. elegans* is dependent upon a gene regulation system that shares some striking features with machinery that maintains gene-expression patterns in *Drosophila*.

MATERIALS AND METHODS

Alleles and strain maintenance

C. elegans N2 variety Bristol was the wild-type parent of all mutant strains. The following mutations, duplications, and balancers were used.

LGI: *glp-4(bn2ts), mes-3(bn21ts, bn35, bn86, bn88), dpy-5(e61), sDp2 (I,f), hDp20 (I;V,f)*

LGII: *mes-2(bn11, bn27, bn48, bn72, bn76), unc-4(e120), rol-1(e91), mnC1*

LGIII: *fem-2(b245ts)*

LGIV: *fem-3(q20ts)gf, mes-6(bn38, bn64, bn66, bn69), nT1 (IV,V), DnT1 [unc(n754)let] (IV,V)*

LGV: *mes-4(bn23, bn58, bn67, bn85, bn87), dpy-11(e224), nT1 (IV,V), DnT1 [unc(n754)let] (IV,V)*

Strains were maintained using standard techniques described by Brenner (1974).

Transformation rescue

Cosmid DNA used for transformation was prepared by a standard alkaline lysis method (Sambrook et al., 1989). Restriction fragments of cosmid DNA were isolated and purified using the Bio101 Geneclean Kit and protocol. Transgenic lines were created by germline transformation using the technique described by Mello et al. (1991). DNA to be tested was co-injected with pRF4 (100 μg/ml), a plasmid containing a dominant *rol-6* mutation as a marker, into the gonads of *mes-2(bn11)/mnC1* hermaphrodites. Heritable lines of Rol worms were assayed for rescue of the sterile phenotype in the progeny of homozygous *mes* worms. Rescue was achieved with cosmid RO6A4 (15 μg/ml) and two overlapping fragments from this cosmid [a 7 kb *Eco*RI fragment (0.6 μg/ml) and a 13 kb *Xho*I fragment (1 μg/ml)].

cDNA cloning

The 7 kb *Eco*RI and the 13 kb *Xho*I rescuing fragments were labeled with [α-^{32}P] dCTP using Boehringer Mannheim's Random Primed Labeling Kit and used as probes to screen a λZAP mixed-stage *C. elegans* cDNA library (Barstead and Waterston, 1989). Clone 15(3) hybridized to both the *Eco*RI and the *Xho*I fragments, has an insert size of 2.5 kb and also hybridizes to a 2.5 kb transcript on northern blots.

RNA-mediated interference (RNAi) analysis

Antisense (or sense) RNA to cDNA 15(3) was prepared essentially as described by Guo and Kemphues (1995), except that the MEGAscript *In Vitro* Transcription Kit by Ambion was used. The RNAs were capped with m7(5′)ppp(5′)G (Ambion). RNA (0.5 mg/ml) was microinjected into the distal gonad arm(s) of wild-type young adult hermaphrodites, and the offspring of the injected worms were examined for sterility under a dissecting microscope. The germlines of sterile worms were examined as described by Garvin et al. (1998).

Sequence analysis and 5′ end identification

cDNA 15(3) was sequenced using the protocols and reagents of the Sequitherm Long-Read Cycle Sequencing Kit-LC for LI-COR automated sequencers (Epicentre Technologies). Gels were run by the DNA Facility of the Indiana Molecular Biology Institute. Both strands of the cDNA were sequenced. The longest open reading frame obtained was identified by the DNA Strider 1.0.1 program (Marck, 1988). Sequence databases from the Genetics Computer Group (Madison, Wisconsin) package were searched using the FASTA (Pearson and Lipman, 1988), BLAST (Altschul et al., 1990) and BLITZ (Sturrock and Collins, 1993) programs. We confirmed that cDNA 15(3) contains the 5′ end of *mes-2* by doing reverse transcription PCR (RT-PCR) according to Spieth et al. (1993). Amplification of a product was successful only when using the SL1-20 primer as the upstream primer (Spieth et al., 1993). Examination of the 15(3) sequence confirmed that it contains a portion of the SL1 spliced leader. Amino acid alignments of SET domain-containing proteins were done with SeqPup program version 0.6d (D. Gilbert, personal communication, available at ftp://iubio.bio.indiana.edu/molbio/seqpup). The *mes-2* cDNA sequence has been deposited in GenBank (accession number AF011893).

Sequencing mutations

DNA template for PCR was made from ~200 sterile, homozygous

mes-2 worms. PCR primers RH4 (5′-TAGGGAAATA ACAGTTAAACA) and RH5 (5′-GGGAATGTAATCCGATGA), which had T7 and T3 extensions, respectively, on their 5′ ends, were used to amplify ~1 kb of the *mes* mutant genomic DNA (the SET domain and part of the CXC domain) in standard PCR reactions. For each allele of *mes-2*, multiple PCR reactions were pooled, purified using the QIAquick PCR Purification Kit and protocol (QIAGEN), and used as templates in standard LI-COR sequencing reactions. The cycling conditions described in LI-COR Sequencing Bulletin #14 were used.

Northern hybridization analysis

Total RNA was extracted from synchronous populations of worms according to Conrad et al. (1991). Poly(A)+ RNA was isolated using oligo(dT)-cellulose columns (Stratagene) as described by Sambrook et al. (1989). Poly(A)+ RNA (5 μg per lane) was electrophoresed on a 1.2% agarose 6% formaldehyde gel and capillary blotted to Hybond-N nylon (Sambrook et al., 1989). Blots were hybridized with *mes-2* or *rp21* probes radiolabeled with [α-^{32}P] dCTP using a Boehringer Mannheim Random Primed Labeling Kit. *rp21* (recently named *rpp-1*; Evans et al., 1997) encodes a ribosomal protein and serves as a loading control (Paulsen et al., 1995). Transcript bands were quantified using a Molecular Dynamics PhosphorImager. Results are expressed as the ratio of hybridization intensity for the *mes-2* band relative to the *rp21* band in each lane.

Antibody production and purification

Polyclonal antibodies to MES-2 were produced by injecting a MES-2-6xHis fusion protein into two rabbits. The fusion protein was created by ligating sequences that encode the amino-terminal 259 amino acids of MES-2 (which lack the SET and CXC domains) into the QIAGEN expression vector pQE-30. The fusion protein was expressed in *E. coli* and purified using a nickel column according to QIAexpressionist protocols (QIAGEN). SDS polyacrylamide gel bands of MES-2-6xHis (~30 kDa) were used for injections (Harlow and Lane, 1988). Anti-MES-2 antibodies were purified from crude antiserum using the blot affinity purification technique of Olmsted (1986). Bound antibody was eluted from the MES-2 fusion protein using 0.2 M glycine-HCl pH 2.8.

Immunostaining

Western blot analysis was done according to Towbin et al. (1979). Protein homogenates of early embryos were kindly provided by S. Kuersten. Protein was used at approximately 30 μg/lane. Primary antibody was affinity-purified rabbit anti-MES-2 diluted 1:2 or preimmune serum diluted 1:750, and secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit (Sigma) diluted 1:5000. Visualization was by chemiluminescent detection (Pierce).

To visualize the MES-2 distribution in animals, worms were cut, fixed and stained essentially as described by Strome and Wood (1983). L1, L2 and L3 larvae were not cut open but were otherwise fixed and stained as described above. Affinity-purified rabbit anti-MES-2 antibody was used undiluted as the primary antibody and was followed by affinity-purified rhodamine-conjugated goat anti-rabbit secondary (Jackson Labs) diluted 1:200. Mouse monoclonal anti-P-granule antibody, K76 (Strome and Wood, 1983), was also used in some experiments to identify the germline cells, Z2 and Z3. Diamidinophenylindole (DAPI) at 0.5 μg/ml was used to stain DNA. Samples were mounted in gelutol (Dupont) and visualized by fluorescence microscopy using a Zeiss Axioskop.

RESULTS

MES-2 is similar to *Drosophila* Enhancer of zeste, a member of the Polycomb group

To initiate cloning and molecular analysis of the *mes-2* gene, we performed transformation rescue experiments with cosmids near the *lin-7* gene (Simske et al., 1996), which maps less than 0.1 map units from *mes-2* (Capowski et al., 1991) (Fig. 2). Rescue of *mes-2* mutants was achieved with cosmid R06A4 or with the simultaneous injection of two overlapping restriction fragments from R06A4. We infer from this that the *mes-2* gene spans the region of overlap of the two fragments. These fragments, each of which hybridizes to a 2.5 kb transcript on northern blots (data not shown), were used to screen a cDNA library (Barstead and Waterston, 1989). A 2.5 kb candidate cDNA clone, which also hybridizes to a 2.5 kb transcript on northern blots, was identified in the screen.

To determine if this cDNA clone corresponds to the *mes-2* transcript, we used RNA-mediated interference with gene function (RNAi; see Rocheleau, 1997). We injected antisense RNA produced from the clone into the germlines of wild-type hermaphrodites. 50-80% of the progeny of the injected worms developed into sterile adults with severely underproliferated germlines, similar to the Mes-2 phenotype. In addition, the germ nuclei in sterile animals were enlarged and distorted, resembling the necrotic nuclei typical of *mes* mutants (Paulsen et al., 1995). Worms injected with control RNA did not show these phenotypes. As further confirmation that this cDNA clone is *mes-2*, we have identified sequence changes in the endogenous gene from worms bearing two mutant alleles (see below). RT-PCR analysis demonstrated that the *mes-2* cDNA

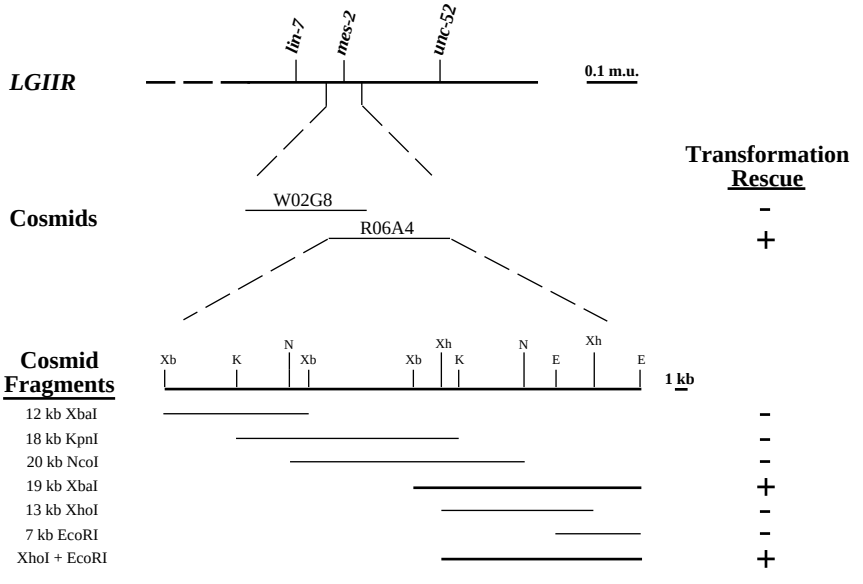

Fig. 2. Identification of the *mes-2* gene. The diagram shows the genetic and physical map in the *mes-2* region. The ability of various genomic fragments to rescue the sterile phenotype of *mes-2* mutants is shown on the right.

is full-length and that *mes-2* RNA is *trans*-spliced to SL1 (data not shown).

Sequence analysis of the *mes-2* cDNA revealed that it encodes a 773 amino acid protein with a high degree of similarity to the *Drosophila* protein, Enhancer of zeste [E(z)] (Fig. 3). Most of the similarity between MES-2 and E(z) lies in two domains found in the carboxy-terminal half of each protein (Fig. 3B). MES-2 and E(z) are 57% identical at the amino acid level in a motif of ~115 amino acids, named the SET domain for three *Drosophila* proteins: Suppressor of variegation 3-9 [Su(var)3-9] (Tschiersch et al., 1994), E(z) (Jones and Gelbart, 1993) and Trithorax (Trx) (Mazo et al., 1990) (Fig. 3C). The fact that SET domains have been found in proteins that are not otherwise similar and in organisms as diverse as plants, yeast and mammals suggests that the SET domain represents an important functional motif (Stassen et al., 1995; Hobert et al., 1996b). While the function of the SET domain is not known, all characterized SET domain proteins associate with chromatin (Tschiersch et al., 1994; Kuzin et al., 1994; Chinwalla et al., 1995; Tripoulas et al., 1996; Carrington and Jones, 1996). Currently there is no evidence that this domain binds to DNA directly; instead it has been suggested that SET-domain-containing proteins associate with their targets through protein-protein interactions (Jones and Gelbart, 1993). Consistent with the SET domain being an important functional motif, we have identified molecular lesions in highly conserved SET domain residues in two alleles of *mes-2* (see below).

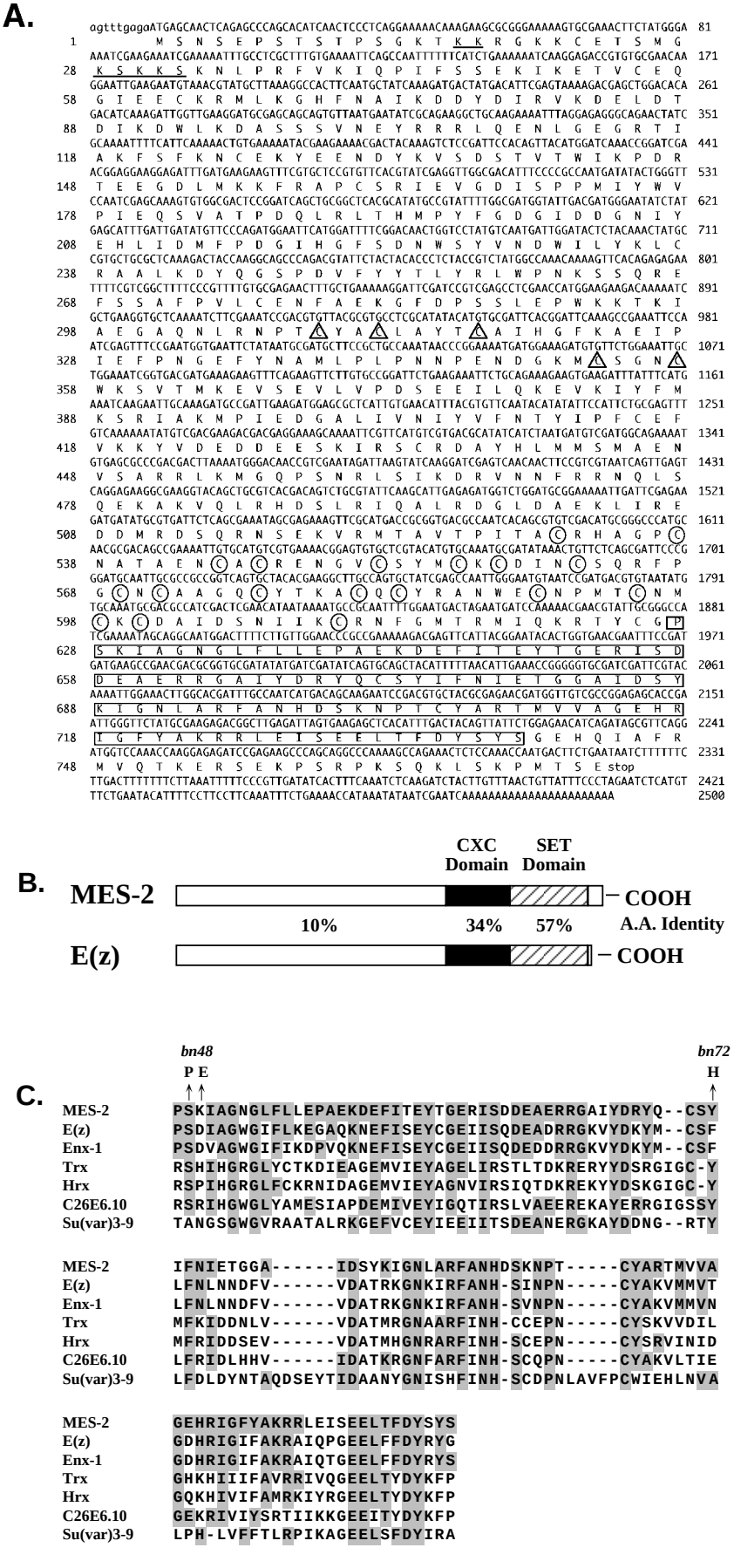

Fig. 3. MES-2 is similar to *Drosophila* E(z). (A) The *mes-2* cDNA sequence and translation of its open reading frame. The portion of the SL1 spliced leader contained in the cDNA is shown in lower case letters. A putative bipartite nuclear localization signal is underlined. Triangles indicate five cysteines that are highly conserved in E(z) homologs but not found in other SET-domain-containing proteins. The 18 cysteines of the CXC domain are circled. The 113 amino acid SET domain is boxed. (B) Schematic alignment of MES-2 and E(z) showing the relative protein lengths, domains and the percentage of amino acid identity between the two proteins. (C) Line-up of the SET domains from *C. elegans* MES-2, *Drosophila* E(z), Enx-1 [murine E(z)], *Drosophila* Trx, Hrx (human Trx), C26E6.10 (putative *C. elegans* Trx) and *Drosophila* Su(var)3-9. Shading shows amino acid identity shared with MES-2. Dashes represent gaps. Amino acid changes caused by the *mes-2* mutant alleles, *bn48* and *bn72*, are shown above the MES-2 sequence. The nucleotide changes responsible are T1882→C and A1885→G for *bn48* and T2020→C for *bn72*. *mes-2* cDNA sequence has GenBank accession number AF011893.

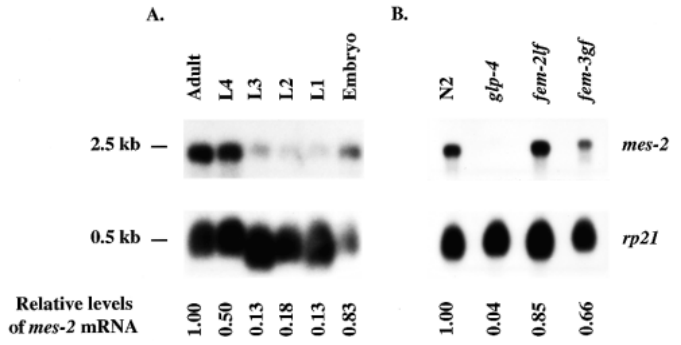

Fig. 4. Northern hybridization analysis of *mes-2*. (A) Accumulation of *mes-2* transcript throughout the life cycle. poly(A)+ RNA was isolated from wild-type hermaphrodites, which were synchronized at six different developmental stages. The *mes-2* cDNA recognizes a single 2.5 kb band in each lane. The *C. elegans* ribosomal protein gene, *rp21*, was used as a loading control. (B) Accumulation of *mes-2* transcript in the germline. This blot shows *mes-2* and *rp21* transcript levels in poly(A)+ RNA from wild-type hermaphrodites (N2), hermaphrodites with a greatly reduced germline (*glp-4*), hermaphrodites that produce only oocytes (*fem-2lf*) and hermaphrodites that produce only sperm (*fem-3gf*). The relative abundance of *mes-2* mRNA in each sample is indicated at the bottom of each lane.

MES-2 also displays 34% amino acid identity with E(z) in the CXC domain (Jones and Gelbart, 1993; Hobert et al., 1996b). This domain also has been found in E(z) homologs in plants (Goodrich et al., 1997; Grossniklaus et al., 1998) and mammals (Hobert et al., 1996a,b). The CXC domain contains 15-18 cysteines in a ~90 amino acid stretch. The spacing of these cysteine residues does not match that of any of the known finger or ring-type cysteine-rich motifs. Although the function of the CXC domain has not been determined, the fact that mutations in the CXC domain disrupt the chromosomal association and function of E(z) suggests that it is required, either directly or indirectly, for chromosome binding (Carrington and Jones, 1996). In addition to the SET and CXC domains, MES-2 residues 309 to 357 contain five highly conserved cysteines (Fig. 3A), which are found in E(z) and its mammalian and plant homologs but not in other proteins with SET domains (Carrington and Jones, 1996; Hobert et al., 1996b; Goodrich et al., 1997; Grossniklaus et al., 1998).

E(z) is a member of the Polycomb group (Pc-G) in *Drosophila*. Pc-G proteins are best known for their role in long-term maintenance of transcriptional inactivation of homeotic genes. They are thought to function cooperatively as multimeric protein complexes that associate with chromosomes at specific sites (Zink et al., 1991; DeCamillis et al., 1992; Carrington and Jones, 1996). E(z) may play a dual role in regulation of homeotic gene expression, since at certain times and in certain tissues, E(z) has been shown to participate in activating homeotic genes (LaJeunesse and Shearn, 1996). E(z) also appears to play important roles in promoting cell proliferation and in maintaining the structural integrity of chromosomes (Gatti and Baker, 1989; Phillips and Shearn, 1990; Rastelli et al., 1993).

mes-2(bn48) and *mes-2(bn72)* have mis-sense mutations in the SET domain

Sequence analysis of the 3′ end of the five mutant alleles of *mes-2* revealed that both *bn48* and *bn72* have mis-sense mutations in the SET domain (Fig. 3C). These mutations affect highly conserved SET domain residues. *bn48* results in changes in two consecutive amino acids. A serine-to-proline change at position 628 (position 2 in the SET domain) changes a residue that is conserved in 13 of 23 previously reported SET domains (Stassen et al., 1995; Hobert et al., 1996b; Tripoulas et al., 1996; Goodrich et al., 1997). Residue 629 (position 3 in the SET domain), which does not appear to be a conserved residue, is also changed from lysine to glutamic acid. *bn72* results in a tyrosine-to-histidine change at position 674 (position 48 in the SET domain). Tyrosine is found at this position in 20 of 23 reported examples. The identification of these two lesions confirms that we have cloned the *mes-2* gene and supports the idea that the SET domain is required for MES-2 function.

mes-2 transcript accumulates in the adult germline and in embryos

To gain insight into the pattern of *mes-2* gene expression, the

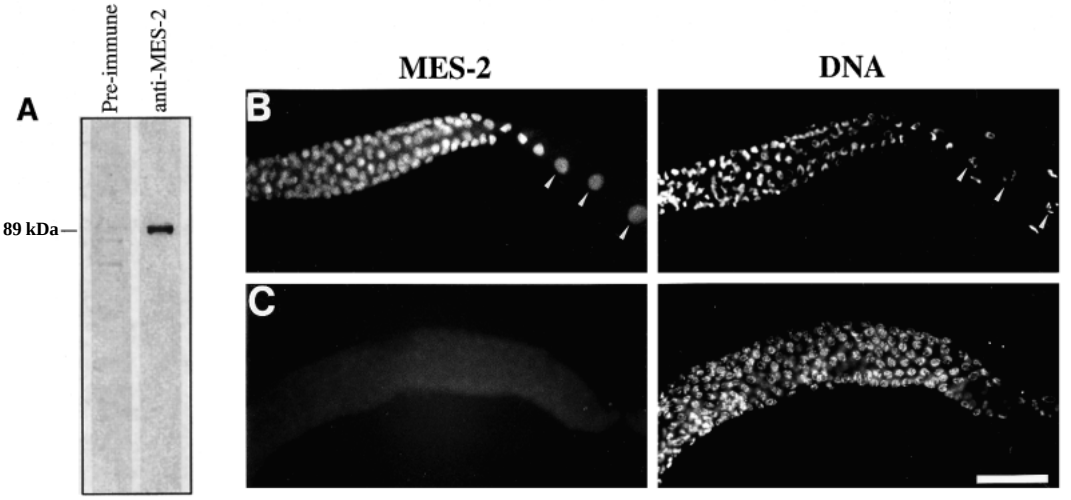

Fig. 5. MES-2 distribution in the adult germline. (A) Affinity-purified rabbit anti-MES-2 antibody and preimmune serum from the rabbit were used to stain *C. elegans* embryo homogenate on a western blot. (B,C) Extruded gonads were stained with affinity-purified anti-MES-2 antibody, and DAPI to visualize DNA. The distal region of the gonad, where germ nuclei divide mitotically and enter meiosis, is to the left, and oocytes are on the right (indicated by arrowheads in A). (B) MES-2 staining in the gonad of a wild-type adult hermaphrodite. MES-2 is localized in all germ nuclei. Sperm do not stain (not shown). (C) MES-2 staining is not detectable in the gonads of *mes-2(bn76)* adult hermaphrodites. This image is overexposed to demonstrate the lack of detectable nuclear staining. Size bar, 50 μm.

mes-2 cDNA was used to probe northern blots of mRNA from developmentally staged worms (Fig. 4A). Transcript levels are highest in adult worms and in embryos. Some accumulation also occurs in the L4 larval stage, but *mes-2* transcript is greatly reduced in earlier larval stages.

To determine if *mes-2* transcript is produced in and perhaps restricted to the maternal germline, we used the *mes-2* cDNA to probe northern blots of mRNA from hermaphrodites that have a severely reduced and undifferentiated germline [*glp-4(bn2ts)*] (Beanan and Strome, 1992), hermaphrodites that produce only oocytes [*fem-2(b245ts)*] (Kimble et al., 1984), or hermaphrodites that produce only sperm [*fem-3(q20ts)gf*] (Barton et al., 1987). *mes-2* transcript is severely reduced (4% of wild type) in worms that essentially lack a germline, but is present in worms that have a germline, regardless of the sex of the germline (66% to 85% of wild type) (Fig. 4B). This indicates that, in adult worms, *mes-2* is expressed primarily in the germline and that it is not restricted to the process of oogenesis. This is consistent with seeing transcript accumulation in the L4 larval stage, when the germline is highly proliferated and is undergoing spermatogenesis.

MES-2 is localized to all nuclei in embryos and is restricted to germ nuclei in larvae and adults

To address when and where MES-2 protein functions, we examined its subcellular localization by immunofluorescence microscopy. Affinity-purified rabbit polyclonal antibodies against the amino-terminal half of MES-2 detect a single band of the size predicted for MES-2 (89 kDa) on western blots (Fig. 5A). The specificity of the antibodies is confirmed by the fact that MES-2 staining is undetectable in worms homozygous for three of the five *mes-2* alleles, *bn11*, *bn27* and *bn76* (Fig. 5C and Table 1). Staining appears wild type in *bn48* and *bn72* homozygotes, which carry mis-sense mutations in *mes-2*, as described above. The molecular lesions associated with the three non-staining alleles have not been identified.

Immunofluorescent staining of wild-type adult hermaphrodites indicates that MES-2 is highly enriched in the germline and is located in the nuclei (Fig. 5B). All germ nuclei in the mitotic and meiotic regions are stained, with the exception of sperm (not shown). A maternal load of protein is seen in the nucleoplasm of oocytes (Fig. 5B); mature sperm lack detectable protein. Adult males also display staining in germ nuclei, which declines during spermatogenesis and is undetectable in sperm. The only adult somatic nuclei that contain detectable levels of MES-2 are those in the intestine. Staining in intestinal nuclei is extremely faint in some samples and undetectable in other samples (data not shown).

In early embryos, MES-2 is observed in the nuclei of all blastomeres (Fig. 6A-C). In blastomeres that lack an intact nuclear envelope because they are undergoing mitosis, MES-2 is predominantly cytoplasmic, but some MES-2 is clearly associated with the chromosomes (Fig. 6A). At progressively later stages of embryogenesis, the intensity of MES-2 staining in the nuclei of somatic cells decreases (Fig. 6D). Upon hatching, staining of MES-2 is significantly higher in the nuclei of the two primordial germ cells, Z2 and Z3, than in somatic nuclei (Fig. 6E). At later stages of larval development, we observe MES-2 exclusively in the germline and localized in the nuclei. Presumably, new synthesis of MES-2 accompanies proliferation of the germ nuclei.

MES-2 protein distribution is altered in other *mes* mutants

Because mutations in *mes-2*, *mes-3*, *mes-4* or *mes-6* result in nearly identical phenotypes, we have been unable to use genetic epistasis tests to establish the order in which these genes function. To perform molecular epistasis tests, we stained animals bearing several different alleles of each *mes* mutation with affinity-purified anti-MES-2 antibody. In each case, homozygous *mes*

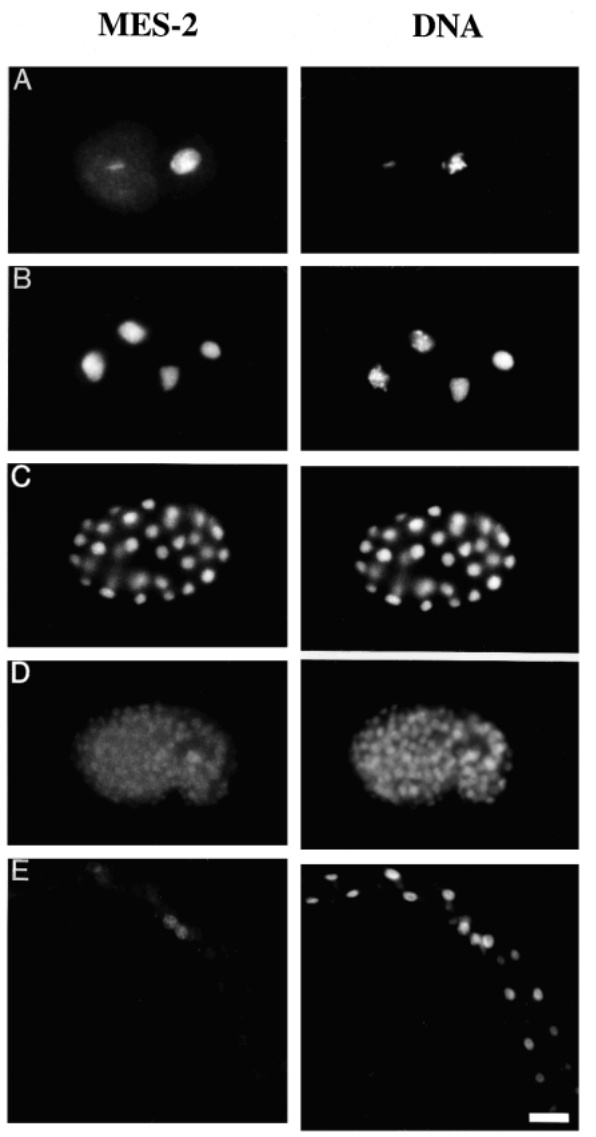

Fig. 6. MES-2 distribution in wild-type embryos and larvae. Embryos and larvae were stained with affinity-purified anti-MES-2 antibody and DAPI. For D and E, the germline cells, Z2 and Z3, were identified by co-staining with the anti-P-granule antibody, K76 (not shown). Anterior is left, ventral is down. (A) 2-cell embryo. Staining is nuclear in the P1 blastomere (right) and is both cytoplasmic and DNA-associated in the AB blastomere (left), which is in metaphase. (B) 4-cell embryo. Nuclear staining is seen in all four nuclei. (C) ~44-cell embryo. MES-2 is present in all nuclei. (D) Comma-stage embryo showing faint staining in all nuclei. The germline cells, Z2 and Z3, are more brightly stained (~1/3 from right end of embryo, near the clear zone). (E) L1 larva. Staining is detected primarily in the germline cells, Z2 and Z3 (center). Size bar, 10 μm.

Table 1. Summary of MES-2 staining in *mes* mutants

Genotype	Staining of MES-2 protein	
	Germ nuclei	Embryo nuclei
Wild-type	+	+
mes-2 (bn48)	+	+
(bn72)	+	+
(bn11)	–	–
(bn27)	–	–
(bn76)	–	–
*mes-3**	+	–
*mes-4**	+	+
*mes-6**	–	–

Staining results are summarized, with positive (+) and negative (–) results indicated for adult germline and embryonic nuclei.

*Alleles tested were as follows. *mes-3*: *bn21*, *bn35*, *bn86*, *bn88*; *mes-4*: *bn*23, *bn58*, *bn67*, *bn85*, *bn87*; *mes-6*: *bn38*, *bn64*, *bn66*, *bn69* (*bn66* stains faintly).

mutant mothers and their embryos were examined (Fig. 7, Table 1). The embryos would have developed into sterile adults.

mes-3 mutant animals (four alleles tested) display an interesting alteration in the MES-2 staining pattern: the gonads of mutant mothers display the wild-type staining pattern, but their embryos do not stain normally (Fig. 7A). MES-2 staining is undetectable until the 16- to 20-cell stage of embryogenesis, at which time weak staining appears in all nuclei of the embryo. This faint staining never reaches the intensity seen in wild-type embryos and it disappears in older embryos (>100 cells). *mes-4* mutant hermaphrodites and their embryos (five alleles tested) show the wild-type pattern of MES-2 staining (Fig. 7B). In *mes-6* mutants, MES-2 staining is undetectable at all stages of development in three of the four existing *mes-6* alleles (Fig. 7C). In the fourth *mes-6* allele, MES-2 staining is visible in the wild-type pattern but is greatly reduced in intensity compared to wild type. Thus, the wild-type distribution of MES-2 depends on *mes-3*$^+$ and *mes-6*$^+$.

DISCUSSION

mes-2 is a maternal-effect gene required for proliferation and survival of the germline in *C. elegans*. Our findings suggest that MES-2 is required for some aspect of controlling gene expression in the germline: MES-2 localizes to germ nuclei, appears to associate with chromatin and has sequence similarity to E(z), a *Drosophila* protein known to function as a transcriptional regulator. MES-2 accumulation in nuclei requires the wild-type functions of *mes-3* and *mes-6*. We hypothesize that a multimeric complex of MES proteins is required for the proper establishment of gene-expression patterns in the germline.

MES-2 is maternally required for viability of the germline

mes-2 is one of four maternal-effect genes that are required for proper development of the early germline in *C. elegans*. Previous studies showed that there is extensive germ-cell death in the progeny of *mes/mes* worms (Capowski et al., 1991; Paulsen et al., 1995). By both genetic and morphological criteria, the germline death observed in *mes* mutants is not programmed cell death; rather, it appears to be a necrotic-type death, which could be indicative of underlying physiological defects (Paulsen et al., 1995; Garvin et al., 1998). We previously tested the possibility that germ-cell death is the consequence of defects in mitosis, which could lead to aneuploidy or other chromosomal abnormalities. Our results did not support that possibility (Garvin et al., 1998). Although we have not identified the precise cause of germ-cell death, the localization of MES-2 to nuclei is consistent with death being caused by defects in regulation of gene expression.

MES-2 is similar to a E(z), a transcriptional regulator in *Drosophila*

The idea that the *mes* genes are needed for proper transcriptional regulation is supported by the finding that MES-2 is similar to E(z), a *Drosophila* protein required for the proper control of both homeotic and other developmentally regulated gene expression (Jones and Gelbart, 1990; Phillips and Shearn, 1990; Pelegri and Lehmann, 1994). Because E(z) acts as a negative transcriptional regulator of the homeotic genes of the *Antennapedia* and *Bithorax* gene complexes, it has been classified as a member of the Polycomb group. The current view is that the pattern of expression of the homeotic genes is initially established by segmentation gene products;

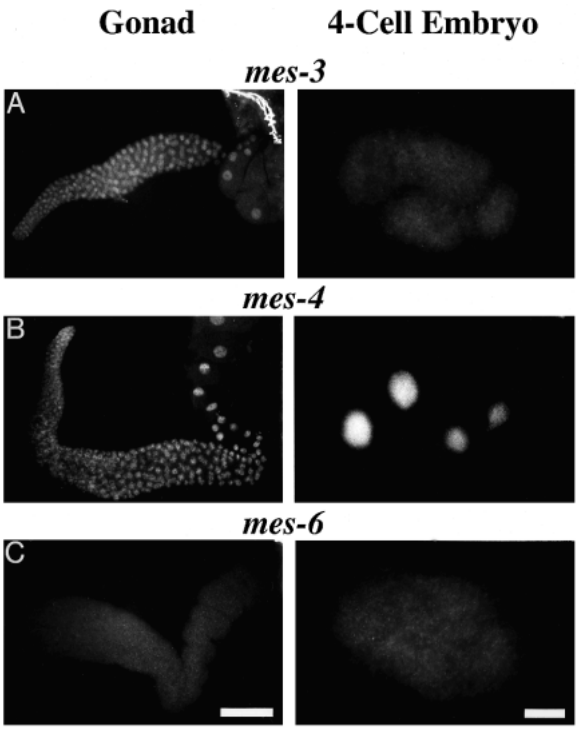

Fig. 7. Distribution of MES-2 in other *mes* mutants. Extruded gonads (left) and 4-cell embryos (right) from homozygous *mes* mothers were stained with affinity-purified anti-MES-2 and DAPI (DNA not shown). Non-staining samples were overexposed to show absence of nuclear stain. In the gonad panels, distal is to the left and oocytes are to the right. In the embryo panels, anterior is left and ventral is down. (A) *mes-3(bn86)*. MES-2 is present in germ nuclei but not detectable in embryo nuclei. Staining of the spermatheca (upper right hand corner of the gonad panel) is non-specific background staining, seen with this and other antibodies. (B) *mes-4(bn67)*. MES-2 distribution is normal. (C) *mes-6(bn38)*. MES-2 is absent from both germ nuclei and embryo nuclei. Size bars, 50 μm for gonads (left) and 10 μm for embryos (right).

maintenance of this pattern throughout development requires the gene products of the Pc-G (for maintaining gene repression) and the Trithorax group (Trx-G) (for maintaining gene activation) (reviewed in Simon, 1995; Orlando and Paro, 1995). Since Pc-G proteins have been shown to co-localize at many sites on polytene chromosomes and to immunoprecipitate as a multimeric complex (Franke et al., 1992; Rastelli et al., 1993; Martin and Adler, 1993; Lonie et al., 1994; Carrington and Jones, 1996), it is thought that Pc-G proteins act in concert to maintain genes in a repressed state. However, the mechanism by which they maintain repression is not known. Based on similarities between Pc-G repression and silencing in yeast and near heterochromatin in *Drosophila*, some current models for Pc-G action invoke modification of nucleosomes, reorganization of higher order chromatin structure and exclusion of at least some DNA-binding proteins (Simon, 1995; Orlando and Paro, 1995; McCall and Bender, 1997; Pirrotta, 1997). Alternative models are that Pc-G proteins specifically inhibit binding of transcription factors or interactions between enhancers and promoters (McCall and Bender, 1997; Pirrotta, 1997).

E(z) may serve multiple roles. It associates with distinct sites on chromosomes in a sequence-dependent manner, as do other Pc-G proteins (Carrington and Jones, 1996). In addition, a lower level of E(z) may be distributed along the lengths of the chromosomes (Carrington and Jones, 1996), perhaps to serve a role in general maintenance of chromosome structure (Gatti and Baker, 1989; Phillips and Shearn, 1990; Rastelli et al., 1993). We do not know if MES-2 associates with chromosomes in like manner, at least in part because the high level of MES-2 in the nucleoplasm impedes visualization of any specific subcellular localization. However, MES-2 does share two protein domains with E(z), suggesting that MES-2 and E(z) may use a similar targeting mechanism or have a similar function.

The SET domain is found in members of both the Pc-G [E(z)] (Jones and Gelbart, 1993) and the Trx-G (Trx) (Mazo et al., 1990; Tripoulas et al., 1996), as well as in a protein that modifies position effect variegation [Su(var)3-9] (Tschiersch et al., 1994). All three of these *Drosophila* proteins are putative modifiers of chromatin structure. The fact that MES-2 contains a SET domain may indicate that it is also a modifier of chromatin. Since mutations in the SET domain of MES-2 (*bn48* and *bn72*) do not alter the distribution of MES-2 protein, another part of the protein may be responsible for its localization. Recently, it has been proposed that chromosome localization of E(z) may be mediated by the CXC domain (Carrington and Jones, 1996).

Polycomb group regulation in *C. elegans*?

Is the function of the MES proteins in the germline to regulate gene expression by a mechanism resembling that used by the Pc-G in *Drosophila*? Several interesting parallels suggest that this may be the case. First, as demonstrated in this paper, MES-2 is similar to a Pc-G member and localizes to nuclei. Second, as demonstrated in the accompanying paper, MES-6 is similar to Extra sex combs (Esc), another member of the Pc-G, and localizes to nuclei (Korf et al., 1998). Esc is unique among the Pc-G proteins in that its function is required primarily in early embryogenesis, while other Pc-G members are needed throughout development (Struhl and Brower, 1982). It has been proposed that Esc recruits other Pc-G members into repressor complexes on DNA (Simon et al., 1995; Gutjahr et al., 1995; Jones et al., 1998). Consistent with this hypothesis, we found that wild-type *mes-6* function is required for MES-2 accumulation in nuclei. Third, MES-3 localizes to nuclei (Y. Fang and S. S., unpublished results). Although the sequence of MES-3 does not resemble that of any of the known Pc-G members, only eight of the thirteen known Pc-G genes have been sequenced (Simon, 1995; Pirrotta, 1997), and it has been estimated that considerably more Pc-G genes may exist (Jurgens, 1985). Fourth, the *mes* genes show dependence upon each other for proper localization, as do the Pc-G proteins. For example, Posterior sex combs, Suppressor 2 of zeste, Polycomb, and Polyhomeotic are not properly localized on polytene chromosomes in *E(z)* mutants (Rastelli et al., 1993; Platero et al., 1996). Similarly, correct MES-2 localization in nuclei depends on functional MES-3 and MES-6, and correct MES-6 localization in nuclei depends on functional MES-3 and MES-2 (Korf et al., 1998). This may reflect formation of a multimeric complex of MES proteins, similar to the Pc-G protein complex.

As discussed above, in *Drosophila*, the Pc-G genes regulate expression patterns of the homeotic genes in somatic tissues. The essential function of the *mes* genes that is revealed by the maternal-effect sterile phenotype is in the germline. Examination of *mes* mutants for any signs of homeotic mutant phenotypes in the soma has revealed low penetrance homeotic transformations of certain neurons and of the alae/ray boundary in males (J. Maloof and C. Kenyon, personal communication), raising the possibility that the MES proteins indeed participate in regulating expression of homeotic genes in somatic cells. Examining *Hox* gene expression patterns in *mes* mutants will clarify this issue.

Is MES protein function essential only in the germline?

The low penetrance homeotic transformations displayed by *mes* mutants (see above) suggest a non-essential role of the *mes* genes in somatic cells. An additional non-essential role in the soma is revealed by our intriguing observation that *mes* mutations feminize animals whose male sexual identity is somewhat ambiguous. Animals with an intermediate X:A ratio of 0.67 (2X/3A) normally develop as males (Madl and Herman, 1979), as do XX animals carrying mutations in the sex determination function of *sdc-3* (i.e., *sdc-3(Tra)*; DeLong et al., 1993; Klein and Meyer, 1993). However, when 2X:3A or *sdc-3(Tra)* XX animals are the offspring of *mes* mothers, they develop as hermaphrodites (Garvin et al., 1998). Mutations in the dosage compensation *dpy* genes also feminize 2X:3A and *sdc-3(Tra)* XX animals (DeLong et al., 1993). The basis for the feminization by *dpy* mutations is thought to be by disruption of the dosage compensation complex of proteins and release of SDC-2, which participates in controlling sex determination as well as dosage compensation (Davis and Meyer, 1997). Elevated levels of SDC-2 would lead to hermaphrodite development. The MES proteins are present in all nuclei during the early stages of embryogenesis when dosage compensation is established. If the MES proteins affect chromatin organization in somatic cells of early embryos, then they may affect assembly or stability of the dosage compensation complex on the X chromosomes. Even slight destabilization of the dosage compensation complex in *mes* mutants may elevate

SDC-2 levels enough to affect sex determination. Alternative models are discussed in Garvin et al. (1998).

The somatic phenotypes described above raise the issue of whether a functional MES-2,3,4,6 system is essential only in the germline, or serves a not-yet-recognized essential role in somatic cells as well. We favor the former, because maternal-effect sterility appears to be the loss-of-function, probably the null, phenotype of all four *mes* genes. (1) All mutations in the four *mes* genes cause a common phenotype, 100% maternal-effect sterility and negligible maternal-effect lethality (Capowski et al., 1991). (2) We isolated 3-4 mutant alleles of each *mes* gene in our screens of 11,219 ethyl methanesulfonate-mutagenized haploid genomes (Capowski et al., 1991). This frequency is characteristic of loss-of-function mutations in *C. elegans* (Brenner, 1974). (3) Additional mutageneses using trimethylpsoralen/UV or cesium radiation, both of which can generate deletions, yielded seven additional *mes* alleles, which display maternal-effect sterility like the EMS alleles (Paulsen et al., 1995 and our unpublished results). (4) Hemizygous *mes* mutants (i.e. *mes-3/sDf4*, *mes-4/nDf42*; *mes-6/eDf18* or *eDf19*) display the same phenotype as homozygous *mes* mutants, maternal-effect sterility and no enhancement of embryonic lethality over +/*Df* controls (Capowski et al., 1991; Korf et al., 1998; C. Garvin and S. S., unpublished). (5) In worms bearing any of three different *mes-2* mutant alleles, MES-2 protein is undetectable (this study). These observations argue that, in at least some *mes* mutant strains, MES^+ function has been eliminated, with deleterious consequences only for the germline. As a test for whether the *mes* genes act in the same process or pathway, we have analyzed double mutants, specifically *mes-2;mes-3* and *mes-4;mes-6*. These double mutants display 100% maternal-effect sterility and only background levels of lethality. Thus, the MES system appears to be devoted to an essential role only in the germline. Either somatic cells do not require MES-like functions for viability, or the essential functions served in the germline by the MES proteins are provided in the soma by different proteins.

At least some targets of the MES proteins are likely to be on the X chromosomes

If the *mes* genes function to control gene expression in the germline, what are their targets? We previously observed that male (XO) progeny of *mes* mothers contain significantly healthier germlines than their hermaphrodite (XX) siblings. By placing sex-determination mutations in combination with *mes* mutations, we demonstrated that the severity of the Mes phenotype is dependent on X-chromosome composition: the germline defects are more severe in XX than in XO mutant animals, regardless of sexual phenotype (Garvin et al., 1998). This is reminiscent of genes required for somatic dosage compensation in *C. elegans* (e.g. *dpy-26* and *dpy-27*). Mutations in these *dpy* genes cause maternal-effect death of XX animals and have no effect on XO animals (Plenefisch et al., 1989). Mutations in the *mes* genes cause maternal-effect death of the germline in XX animals and have milder effects on XO animals. Thus, by analogy to the *dpy* genes, whose function is to implement dosage compensation in somatic cells by reducing transcription of X-linked genes in XX animals (reviewed in Villeneuve and Meyer, 1990; Meyer, 1997), the *mes* genes may repress expression of X-linked genes in the germline of XX animals. In *mes* mutants, elevated transcription of X-linked genes in XX germlines may be responsible for the death of germ cells.

Despite the striking genetic similarities between the *mes* genes and the somatic dosage compensation genes, we do not think that the primary or exclusive role of the *mes* genes is dosage compensation in the germline for two reasons. First, XO animals are unaffected by mutations in most of the dosage compensation genes (Plenefisch et al., 1989), but XO progeny of *mes* mothers do display evidence of the mutant phenotype (Garvin et al., 1998). Second, dosage compensation gene products have been shown to specifically associate with the X chromosomes in XX animals (Chuang et al., 1994; Lieb et al., 1996; Davis and Meyer, 1997). We see no evidence for localization of MES-2 to the X chromosomes (R. H. and S. S., unpublished). In light of these two facts, we expect that the MES proteins serve a more general role in regulating germline gene expression and that this role is sensitive to chromosome dosage.

mes genes and control of gene expression in the germline

Our current working model is that a maternal supply of wild-type MES proteins is required for proper chromatin structure and gene expression in the nascent germline. Germline development is known to require maternal PIE-1, a transcriptional repressor that keeps somatically expressed genes turned off in the germline of early embryos (Mello et al., 1992; Seydoux et al., 1996). We have tested several somatically expressed genes for ectopic expression in the germlines of *mes* mutants and we see no evidence for ectopic expression (R. H. and S. S., unpublished). This argues that the *mes* gene products do not act in concert with or as a continuation of the PIE-1-mediated system. We hypothesize that, after PIE-mediated repression is lifted, the *mes* genes participate in establishing or maintaining germline chromatin in a conformation that ensures the proper pattern of gene expression when the germline starts its developmental program in early larvae. The chromosomes in the primordial germ cells of embryos and young larvae display a unique morphology (G. Seydoux, personal communication), which is retained in the germ cells of *mes* mutants (L. Xu and S. S., unpublished results). This suggests that the MES proteins do not operate at the level of controlling gross chromosome morphology.

Support for an involvement of the MES proteins in controlling some aspect of germline chromatin organization and gene expression comes from the findings of Kelly and Fire (1998): expression of transgenes present in many copies in extrachromosomal arrays is silenced in the germlines of wild-type worms, but desilenced in the germlines of *mes* mutants. Desilencing can also be achieved in wild-type germlines by placing the transgenes in the context of additional, complex DNA in the array. These findings suggest that the MES^+ system participates in keeping at least some genes silenced in the germline and that this is via an effect on chromatin state. We speculate that defects in the MES system result in aberrant patterns or levels of gene expression, leading to death of the germline.

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