Expression and Properties of Wild-Type and Mutant Forms of the Drosophila Sex Comb on Midleg (SCM) Repressor Protein

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ABSTRACT

The *Sex comb on midleg* (*Scm*) gene encodes a transcriptional repressor of the *Polycomb* group (PcG). Here we show that SCM protein is nuclear and that its expression is widespread during fly development. SCM protein contains a C-terminal domain, termed the SPM domain, which mediates protein-protein interactions. The biochemical function of another domain consisting of two 100-amino-acid-long repeats, termed "mbt" repeats, is unknown. We have determined the molecular lesions of nine *Scm* mutant alleles, which identify functional requirements for specific domains. The *Scm* alleles were tested for genetic interactions with mutations in other PcG genes. Intriguingly, three hypomorphic *Scm* mutations, which map within an mbt repeat, interact with PcG mutations more strongly than do *Scm* null alleles. The strongest interactions produce partial synthetic lethality that affects doubly heterozygous females more severely than males. We show that mbt repeat alleles produce stable SCM proteins that associate with normal sites in polytene chromosomes. We also analyzed progeny from *Scm* mutant germline clones to compare the effects of an mbt repeat mutation during embryonic *vs.* pupal development. We suggest that the mbt repeat alleles produce altered SCM proteins that incorporate into and impair function of PcG protein complexes.

DEVELOPMENT along the anterior-posterior (A-P) body axis in Drosophila is controlled by the homeotic products of the Antennapedia and bithorax complexes (Lewis 1978; Kaufman *et al.* 1980). The homeotic proteins are expressed in spatially-restricted A-P domains (White and Wilcox 1985; Celniker *et al.* 1989; Karch *et al.* 1990), and their proper deployment along this axis is crucial for A-P pattern formation.

The *Polycomb* group (PcG) genes encode a set of transcriptional repressors that mediate restricted homeotic gene expression. Mutations in PcG genes cause ectopic expression of homeotic proteins in inappropriate positions along the A-P axis (Struhl and Akam 1985; McKeon and Brock 1991; Simon *et al.* 1992). At least 13 Drosophila PcG genes are required for homeotic gene repression (McKeon and Brock 1991; Simon *et al.* 1992; Soto *et al.* 1995; see Simon 1995 and Pirrotta 1997 for reviews). Once PcG proteins become engaged in homeotic gene repression, at ~4 to 5 hr of embryogenesis (Jones and Gelbart 1990; Simon *et al.* 1992; Soto *et al.* 1995), they are continuously required to maintain repression during subsequent stages of development (Duncan and Lewis 1982; Wu *et al.* 1989; Jones and Gelbart 1990). In agreement with this, most PcG proteins examined so far are expressed throughout embryonic, larval, and pupal stages (Paro and Zink 1992; Martin and Adler 1993; DeCamillis and Brock 1994; Lonie *et al.* 1994; Carrington and Jones 1996).

A likely molecular explanation for the large number of PcG components is that they work together in multiprotein complexes. Thus, loss of one PcG protein could impair the repressive function of the entire complex. Cytological evidence for PcG complexes is provided by identical distributions of the Polycomb (PC), polyhomeotic (PH), and Polycomblike (PCL) proteins at approximately 100 polytene chromosome sites (Franke *et al.* 1992; Lonie *et al.* 1994). In addition, the Posterior sex combs (PSC) and Sex comb on midleg (SCM) protein distributions overlap extensively with these sites (Rastelli *et al.* 1993; Peterson *et al.* 1997). Biochemical evidence for PcG protein associations includes coimmunoprecipitation of PC with PH (Franke *et al.* 1992) and extra sex combs (ESC) with Enhancer of zeste [E(Z); Jones *et al.* 1998] from fly embryo extracts. There is also evidence for complexes of mammalian PcG proteins. The mouse PH and PSC homologs coimmunoprecipitate with the PC homolog (Alkema *et al.* 1997) and the human PH and PSC homologs coimmunoprecipitate and cofractionate on sucrose gradients (Gunster *et al.* 1997).

Despite accumulating data on PcG complexes, the precise biochemical roles of individual PcG members are not known. Although PcG proteins localize to specific chromosomal sites, none of those yet tested exhibits sequence-specific DNA-binding activity *in vitro.* In addition, none of the PcG proteins yet sequenced contains recognizable catalytic domains. The main functional

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clues afforded by PcG protein sequences are evolutionarily conserved domains, such as the chromodomain, which mediate PcG chromosome associations or protein interactions (Messmer *et al.* 1992; Carrington and Jones 1996; Platero *et al.* 1996; Alkema *et al.* 1997).

The SCM protein plays a key role in PcG repression because embryos that lack both maternal and zygotic SCM die with severe homeotic transformations (Breen and Duncan 1986). SCM protein contains several homology domains, two of which are also present in the PH PcG protein (Bornemann *et al.* 1996). One of these shared domains is a type of zinc finger present in SCM in two copies and in PH in one copy. SCM and PH also share a C-terminal domain of 65 amino acids, termed the SPM domain, which mediates self-binding and cross-binding of these two proteins (Peterson *et al.* 1997). SCM is even more similar to another fly protein, the product of the tumor suppressor gene *lethal (3) malignant brain tumor* [*l(3)mbt*; Wismar *et al.* 1995]. The SCM and L(3)MBT proteins share zinc fingers, the SPM domain, and a third domain consisting of 100-amino-acid long repeats. These repeats, termed mbt repeats (Wismar *et al.* 1995; Bornemann *et al.* 1996), are present in two tandem copies in SCM and three copies in L(3)MBT. The biochemical role of mbt repeats is not known.

To investigate the importance of the Scm domains *in vivo*, we have characterized molecular lesions associated with *Scm* mutant alleles. This analysis identifies a subset of *Scm* mutations that maps to the first mbt repeat and that displays especially strong genetic interactions with other PcG mutations. We used polyclonal antibodies to show that SCM protein is nuclear and to determine its temporal and spatial distribution during development. We also assessed the expression and stability of mutant SCM proteins and their accumulation at specific sites on polytene chromosomes.

MATERIALS AND METHODS

Sequence determination of mutant alleles: Genomic DNA for *Scm* mutant alleles and corresponding background chromosomes was amplified by PCR and cloned as described below. The template DNA for sequencing reactions was double-stranded plasmid DNA prepared as a mixture from 8 to 10 independent PCR clones. Sequencing was performed by dideoxy chain termination with Sequenase 2.0 (United States Biochemical, Cleveland).

$Scm^{Su(z)302}$: Genomic DNA purified from homozygous mutant $Scm^{Su(z)302}$ pupae was used as template for PCR. The 5′ one-third of the *Scm* gene was amplified using the primers 5′-ACTAATTGTGCGGCTCG-3′ and 5′-GAGATTCGCACATG CCC-3′, and the product was digested with *Ngo*MI. The resulting 1.2-kb fragment was inserted into pBluescript KSII+. The 3′ two-thirds of *Scm* was amplified using primers 5′-GCTGGATGGAAGTGACT-3′ and 5′-GAATCACGAGCAGT TGG-3′, the product was digested with *Sal*I and *Nru*I, and the resulting 1.9-kb fragment was inserted into pBluescript.

Scm^{ET50}: Template DNA for PCR amplification was prepared from Scm^{ET50}/Scm^{P12} pupae. Scm^{P12} is a deletion that removes the *Scm* gene (Bornemann *et al.* 1996). The 5′ one-third of *Scm* was amplified using primers 5′-ACTAATTGTGCGGCTCG-3′ and 5′-CGCCAACCATCGAATGT-3′, the product was digested with *Apo*I and *Xho*I, and the resulting 1.2-kb fragment was inserted into pBluescript. The 3′ two-thirds of the gene was amplified using primers 5′-GCTGGATGGAAGTGACT-3′ and 5′-GGAAACGCAATTGATAC-3′, the product was digested with *Xho*I and *Hpa*I, and the resulting 1.8-kb fragment was inserted into pBluescript. The *ET50* mutant lesion was confirmed by PCR cloning and by sequencing the same region from the *st e* background chromosome (Jurgens 1985).

Scm^{D1}, Scm^{D2}, Scm^{H1}, Scm^{M56}, Scm^{M36}, $Scm^{R5\text{-}13}$, Scm^{KM23}, and Scm^{K2}: Template DNA for PCR amplification of each allele was obtained from pharate adults of genotype $Scm^{allele}/Scm^{Su(z)302}$. The transheterozygotes were identified by presence of extra sex combs and absence of the balancer marker, *Sb*. The 3′ two-thirds of the gene was amplified using primers 5′-AGTGCGA CAACGTCATC-3′ and 5′-GAATCACGAGCAGTTGG-3′, the product was digested with *Bam*HI and *Nru*I, and the resulting 2.2-kb fragment was inserted into pBluescript. Mutant lesions were confirmed by PCR cloning and sequencing the corresponding regions from the respective background or isogenic chromosomes: *Cbx Ubx* for Scm^{D1} (Breen and Duncan 1986), $E(z)^{61}$ *e* for Scm^{H1} (R. Jones, personal communication), "47.29.1" for $Scm^{R5\text{-}13}$ (M. Muller and H. Gyurkovics, personal communication), and *ri e* for Scm^{KM23} (K. Matthews, personal communication). Since Scm^{M56} and Scm^{M36} were raised on the same *kar ry* chromosome (G. Maroni, personal communication), they each provided background sequence for the other.

Generation and purification of SCM antibodies: A 1.9-kb *Sal*I-*Nru*I fragment encoding SCM amino acids 324 to 877 was isolated from the cDNA Sc9 (Bornemann *et al.* 1996) and was inserted into the vector pGEX-BgRP3i (Jones *et al.* 1998). The resulting glutathione-S-transferase (GST)-SCM fusion protein was prepared from *Escherichia coli* inclusion bodies as described in Li *et al.* (1994), purified by preparative SDS gel electrophoresis, and used as immunogen in rabbits. Crude sera that tested positive for immunogen reactivity were affinity-purified against solubilized GST-SCM protein coupled to the Actigel ALD affinity chromatography resin (Sterogene Bioseparations, Inc., Arcadia, CA). Antibodies were bound and eluted from the Actigel column according to the manufacturer's instructions.

Drosophila protein extracts and Western blots: Protein extracts were prepared by homogenizing tissues in 2× SDS sample buffer (100 mm Tris pH 6.8, 200 mm dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) plus 1 mm phenylmethylsulfonyl fluoride. Homogenates were sonicated for 30 sec and heated to 95° for 5 min. Insoluble material was pelleted by microcentrifugation, and the supernatants were recovered. Unfertilized egg extracts were prepared using 200 eggs per 50 μl of sample buffer. Embryonic extracts were prepared using a 1:2 v:v ratio of embryos to sample buffer. Extracts from pupae and adults were generated using 10 μl of sample buffer per animal. Ovaries and adult heads were dissected from 20 females and homogenized in 40 μl of sample buffer. Extracts from larvae were prepared by first freezing animals in liquid nitrogen, followed by tissue disruption with a mortar and pestle at −20° and resuspension at a 1:2 ratio of tissue volume to sample buffer (first and second instars) or in 10 μl of sample buffer per animal (third instars). We found that mortar and pestle disruption of frozen tissues provided more efficient total protein recovery from larval stages, presumably because of greater disruption of the larval cuticle barrier. Relative concentrations of extracts were gauged by Coomassie Blue staining of proteins after SDS gel electrophoresis.

Immunodetection on Western blots was with affinity-puri-

fied rabbit anti-SCM antibody (1:1000) and goat anti-rabbit-HRP secondary antibody (1:2000; Bio-Rad, Hercules, CA). Levels of tubulin, used in some experiments as a control for lane loading, were detected using mouse anti-tubulin primary antibody (1:5000; Amersham, Piscataway, NJ) and goat anti-mouse-HRP secondary antibody (1:5000; Jackson Immunoresearch Labs., Inc., West Grove, PA). Signals were developed using the ECL detection system (Amersham).

Immunostaining of embryos, larval tissues, and polytene chromosomes: Immunostaining of embryos was performed as described in Simon *et al.* (1992) using a rabbit polyclonal abdominal-A (ABDA) antibody (Karch *et al.* 1990). Whole-mount immunostaining of larval tissues was performed essentially as described in Carrington and Jones (1996) using anti-SCM antibody at a 1:35 dilution. Polytene chromosomes were immunostained with anti-SCM as described in Peterson *et al.* (1997).

Generation of *Scm* germline clones: *Scm* mutations (*M56*, *D1*, *Su(z)302*, or *M36*) were recombined onto a third chromosome bearing a centromere-linked FRT site at cytological location 82B (Xu and Rubin 1993). *FRT(82B) Scm⁻ / TM3* females were mated to *HS-FLP/Y; FRT(82B) ovo^{D1} / TM3* males (Chou *et al.* 1993). FLP recombinase was induced by heat shocking the progeny for 1.5 hr at 36° during each of 4 consecutive days starting the fourth day after egg-laying commenced. The *FRT(82B) Scm⁻ / FRT(82B) ovo^{D1}* virgin female progeny were collected. To obtain unfertilized *Scm⁻* eggs for Western analysis, these females were mock-mated to males carrying the dominant male-sterile β-tubulin mutation, *B2t^{D}* (Kemphues *et al.* 1980). To generate embryos and larvae that contain solely the *Su(z)302* form of SCM protein from both maternal and zygotic sources, *FRT(82B) Scm$^{Su(z)302}$ / FRT(82B) ovo^{D1}* females were mated to *Scm$^{Su(z)302}$ / TM6B,Tb* males. Embryos produced from this cross were stained with ABDA antibody. Salivary glands from the non-*Tb* progeny were harvested to obtain *Scm$^{Su(z)302}$* mutant polytene chromosomes.

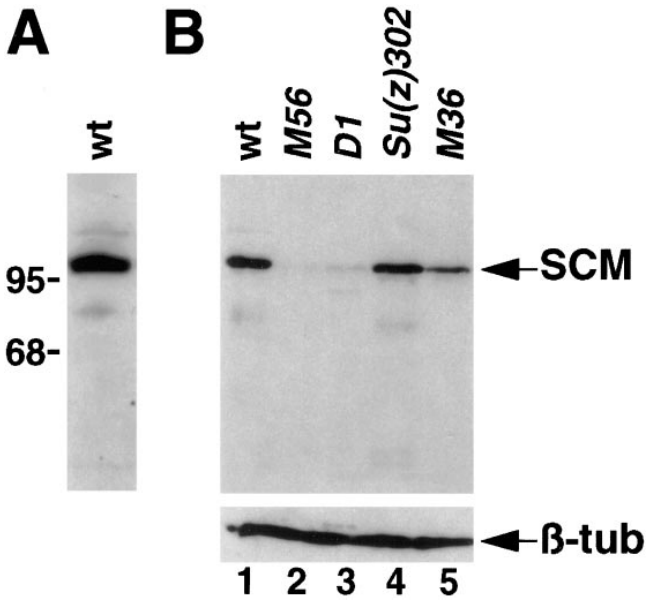

Figure 1.—Detection of SCM protein in wild-type and mutant extracts. Western blots are shown using affinity-purified polyclonal anti-SCM antibodies. (A) Extract from wild-type (*Dfw^{67c2}*) fly embryos. Numbers indicate positions of molecular weight markers. (B) Extracts from unfertilized eggs. Lane 1 contains extract from wild-type (*Dfw^{67c2}*) eggs and lanes 2 through 5 contain extracts from eggs derived from germline clones homozygous for the indicated *Scm* mutations. The faint species in lane 3 likely represent altered SCM proteins produced from the *D1* allele, which is a C-terminal frameshift (see Figure 5). (Bottom) β-Tubulin detection on the same blot as a control for lane loading.

RESULTS

Generation and specificity of SCM antibody: Rabbit polyclonal antibodies were generated against a GST-SCM fusion protein that contains SCM amino acids 324 to 877 (see materials and methods). The affinity-purified SCM antibodies detect a single major band on Western blots of wild-type fly embryonic extracts (Figure 1A). This reacting species migrates at approximately 100 kD, which is close to the 94-kD predicted size for SCM protein (Bornemann *et al.* 1996).

We wished to determine if this reacting species corresponds to SCM protein by analyzing extracts from *Scm* mutant embryos. However, embryos that are zygotically mutant for *Scm* likely contain wild-type SCM protein derived from maternal expression (Breen and Duncan 1986). Therefore, we used the dominant female sterile technique coupled with the FLP recombination system (Golic and Lindquist 1989; Chou *et al.* 1993) to generate females with germlines containing *Scm* mutant clones. Unfertilized eggs collected from these germline mosaic females should contain solely mutant maternal *Scm* product.

Animals of genotype *HS-FLP/+; FRT(82B) Scm⁻ / FRT(82B) ovo^{D1}* were heat shocked to induce recombination during larval stages (see materials and methods). To assess production of germline clones, *Scm^{D1} / TM3* fathers were mated to mothers containing putative *Scm^{D1} / Scm^{D1}* clones, and cuticles from the *Scm^{D1} / Scm^{D1}* progeny embryos were examined. We observed embryos with all segments transformed towards eighth abdominal (not shown), which appeared identical to *Scm* mutant embryos lacking both maternal and zygotic product generated by pole cell transplantation (Breen and Duncan 1986). Similar homeotic phenotypes were seen in hemizygous *Scm^{M56}* embryos produced from *Scm^{M56}* germline clone females. The severity of these phenotypes, which requires loss of maternal *Scm* function (Breen and Duncan 1986), confirms that these FRT recombinant chromosomes produce *Scm* germline clones.

Females containing *Scm* germline clones were mock-mated to males containing a dominant male sterile β-tubulin mutation (Kemphues *et al.* 1980), and unfertilized eggs were collected. Protein extracts were prepared from *Scm* mutant eggs and from wild-type unfertilized eggs collected in parallel. As shown in Figure 1B, lane 1, wild-type eggs contain the ~100-kD species that reacts with anti-SCM antibody. In contrast, little or none of this species is detected in extracts from mutant *Scm^{M56}* or *Scm^{D1}* unfertilized eggs (lanes 2 and 3). This result indicates that the ~100-kD species is SCM protein. Fig-

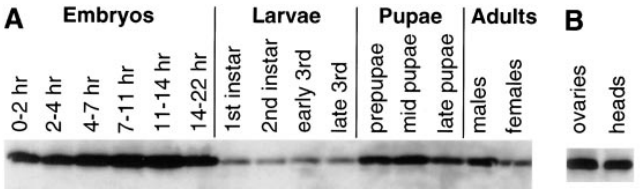

Figure 2.—Expression of SCM protein during development. (A) Detection of SCM protein by Western blot in wild-type extracts from indicated embryonic, larval, pupal, and adult stages. Approximately equal amounts of total protein were loaded per lane. (B) Detection of SCM protein in ovaries and heads dissected from females.

ure 1B, lanes 4 and 5 show that full-length SCM protein is present in $Scm^{Su(z)302}$ and Scm^{M36} unfertilized eggs. The abundance of *Su(z)302* protein appears comparable to wild type, whereas the level of *M36* protein is reduced.

Expression of SCM protein during development: Protein extracts were prepared from wild-type animals at different developmental stages, and relative levels of SCM protein were assessed on Western blots. Figure 2A shows that SCM protein is expressed throughout development, with the highest levels detected during embryonic and pupal stages and lower levels seen in the intervening larval stages. The detection of SCM protein in 0- to 2-hr embryos and in ovaries (Figure 2B), together with mRNA expression data (Bornemann *et al.* 1996), is consistent with maternal *Scm* product furnished as both protein and mRNA. The accumulation of SCM protein in adult males (Figure 2A) and in adult heads (Figure 2B) suggests that SCM might also function in terminally differentiated fly tissues. SCM protein expression in specific tissues was examined by whole-mount staining with anti-SCM antibody. Figure 3 shows that SCM protein accumulates in nuclei and that its distribution appears ubiquitous in larval tissues including the salivary gland, imaginal discs, and the central nervous system. Similar widespread nuclear accumulation has been described for several other PcG proteins (Martin and Adler 1993; DeCamillis and Brock 1994; Carrington and Jones 1996).

Properties of *Scm* alleles: Mutations in the *Scm* gene have been identified in several types of genetic screens, including screens for adults with extra sex combs (Jurgens 1985), for dominant enhancers of *Polycomb* (Kennison and Tamkun 1988), for dominant enhancers of *Contrabithorax* (Breen and Duncan 1986), for dominant suppressors of $zeste^{1}$ (Wu *et al.* 1989), and for lethal noncomplementation of deficiencies that remove *Scm* (K. Matthews, personal communication; G. Maroni, personal communication). The 11 *Scm* alleles analyzed here, and their sources, are listed in Table 1. We have previously shown that Scm^{XF24} is a deletion that removes a C-terminal portion of the *Scm* coding region (Bornemann *et al.* 1996). Each of the other 10 *Scm* alleles fails to complement the recessive lethality of *XF24*.

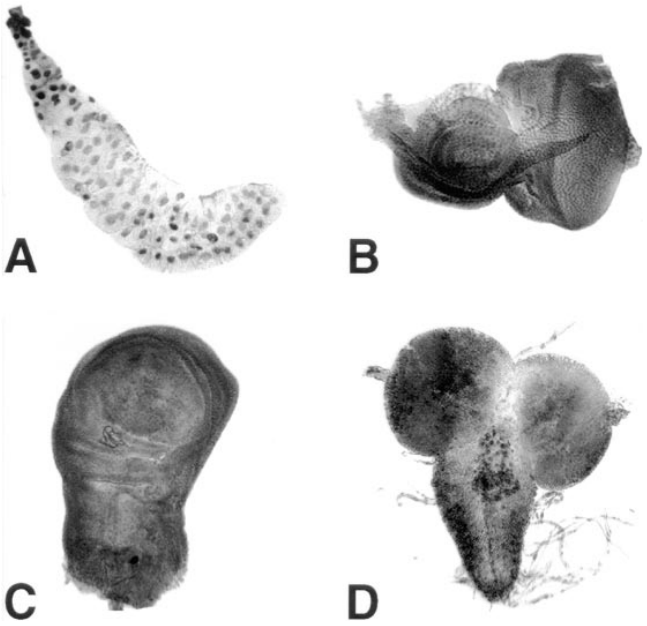

Figure 3.—Accumulation of SCM protein in nuclei of larval tissues. Wild-type whole-mount tissues immunostained with anti-SCM antibodies are shown. (A) Salivary gland. (B) Eye-imaginal disc. (C) Wing disc. (D) Larval central nervous system.

To assess the relative severities of the *Scm* alleles, homozygous mutant embryos were stained with antibodies against the ABDA homeotic protein. In cases where the homozygous mutants showed grossly abnormal morphology, presumably due to other mutations on the third chromosome, the mutant alleles were instead analyzed as hemizygotes in combination with *Df(3R)GB104* (Lindsley and Zimm 1992). In wild-type embryos, ABDA is restricted to a posterior domain encompassing parasegments 7 to 13 (Karch *et al.* 1990; Figure 4A). We found that the *Scm* alleles fall into two broad classes of severity. The first class is exemplified by Scm^{M56} homozygotes (Figure 4B), which show abundant ABDA misexpression anterior to PS7, primarily in the central nervous system. Similar misexpression patterns are seen in Scm^{D1} and Scm^{XF24} homozygotes (Simon *et al.* 1992; Bornemann *et al.* 1996), which are null or nearly null alleles according to phenotypic criteria (Breen and Duncan 1986). Based upon these results, and lethal phases during late embryonic or first larval stages (Jurgens 1985; Breen and Duncan 1986), we classify five *Scm* alleles as null or nearly null (Table 1). The second class of *Scm* alleles produced little or no ABDA misexpression, including four alleles with patterns indistinguishable from wild type. An example of slight ABDA misexpression is provided by Scm^{KM23} hemizygotes, which misexpress ABDA in ~30 to 60 cells anterior to PS7 (Figure 4C). Based upon little or no ABDA misexpression, and survival of hemi- or homozygotes to mid-larval or pupal stages, we classify six *Scm* alleles as hypomorphic (Table 1).

TABLE 1

Properties and interactions of *Scm* alleles

Scm allele	Mutagen	Reference[a]	Type of allele	Lethal phase[b]	Ectopic ABDA in embryos[c]	Effect of molecular lesion	*zeste*[1] interaction[d]	Synthetic lethal with	
								Pc^{3}[e]	Sce^{D1}[e]
XF24	X ray	1, 2	Null	E, L1	Abundant	Δ C-terminal 49 aa	Moderate	No	No
D1	X ray	3	Null	E, L1	Abundant	Franeshift	Moderate	No	Partial
D2	X ray	3	Null	E, L1	Abundant	ND	Moderate	No	No
H1	EMS	4	Null	E, L1	Abundant	W249stop	Moderate	No	No
M56	EMS	5	Null	E, L1	Abundant	Franeshift	Moderate	No	No
Su(z)302	EMS	6	Hypomorph	P	None	D215N	Strong	Partial	Partial
ET50	EMS	1	Hypomorph	P	Very slight	G275E	Moderate	Partial	Partial
R5-13	X ray	7	Hypomorph	P	None	ΔL270-P273	Moderate	Partial	Partial
KM23	EMS	8	Hypomorph	L2	Slight	V227E	Moderate	No	No
K2	EMS	9	Hypomorph	ND	None	C425Y	Moderate	No	No
M36	EMS	5	Hypomroph	P	None	C511Y	Moderate	No	No

ND, not determined.

[a] References: (1) Jurgens 1985; (2) Bornemann *et al.* 1996; (3) Breen and Duncan 1986; (4) R. Jones, personal communication; (5) G. Maroni, personal communication; (6) Wu *et al.* 1989; (7) M. Muller and H. Gyurkovics, personal communication; (8) K. Matthews, personal communication; (9) J. Kennison, personal communication.

[b] Lethal phase was determined in homozygotes or, in the cases of *ET50* and *KM23*, as hemizygotes. E, embryonic; L1, first larval instar; L2, second larval instar; P, pupal stage.

[c] The ABDA expression pattern was determined in homozygotes for *M56* and *M36*, in hemizygotes for *D2* and *KM23*, and in both homozygotes and hemizygotes for *Su(z)302*, *ET50*, *R5-13*, and *K2*. ABDA expression in *XF24*, *D1*, and *H1* homozygotes has been described (Simon *et al.* 1992; Bornemann *et al.* 1996).

[d] Strong, eye color is dark red in z^{1} w^{is}; *Scm*/+ males; moderate, eye color is orange-red in these males.

[e] No, transheterozygous progeny survive at normal frequencies from crosses of Scm^{x}/*balancer* males to Pc^{3}/*balancer* or Sce^{D1}/*balancer* females. Partial, transheterozygotes from these crosses are present but at frequencies reduced by 40–80% (see Figure 6).

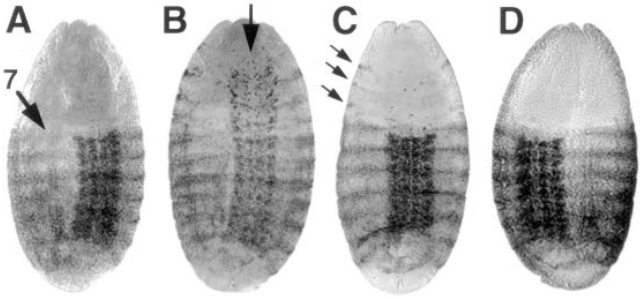

Figure 4.—Expression of ABDA protein in embryos containing null or hypomorphic *Scm* mutations. Embryos immunostained with ABDA antibody (Karch *et al.* 1990) are shown. (A) Wild-type; arrow indicates parasegment 7. (B) Scm^{M56} homozygote derived from heterozygous parents; arrow indicates misexpression in the central nervous system. (C) Scm^{KM23}/*DfGB104* hemizygote derived from heterozygous parents; arrows indicate misexpression in the lateral hypodermis. (D) $Scm^{Su(z)302}$ homozygote derived from $Scm^{Su(z)302}$ germline clone mother.

Sequence analysis of *Scm* alleles: Genomic DNA was isolated from homozygous $Scm^{Su(z)302}$ pupae, and the mutant DNA was PCR-amplified, cloned and sequenced (see materials and methods). We found that the entire *Scm* open reading frame is wild type in DNA sequence except for a single G to A transition that replaces amino acid D215 with N. This missense mutation maps within the first of the two mbt repeats (Figure 5, B and C). The *Su(z)302* mutation also fortuitously removes a *Bam*HI site that is unique within the *Scm* gene (Figure 5A). Southern blot analysis independently verified the loss of this *Bam*HI site in *Su(z)302* mutant DNA (data not shown). The loss of this site in *Su(z)302* DNA was used to design a strategy for PCR cloning and sequencing the remaining *Scm* alleles, including the embryonic lethal alleles. We found that $Scm^{x}/Scm^{Su(z)302}$ animals, where Scm^{x} represents any lethal allele, survive to pharate adulthood with phenotypes similar to *Su(z)302* homozygotes. Thus, PCR clones for most of the remaining *Scm* mutations were obtained by isolating genomic DNA from $Scm^{x}/Scm^{Su(z)302}$ pupae followed by shotgun cloning of *Bam*HI-digested PCR products (see materials and methods).

The locations of 10 *Scm* mutant lesions are shown in Figure 5B. There is good correspondence between the strengths of the alleles and their predicted effects upon SCM protein. Each of the molecular lesions for the four null alleles sequenced (Table 1) causes deletion of a substantial portion of SCM protein. Scm^{H1} is a nonsense mutation located at position 249 (Figure 5B) and thus could produce, at best, a severely truncated protein that lacks several homology domains. Similarly, Scm^{M56} produces a frameshift that deletes the C-terminal half of SCM protein. These two mutations are the most likely protein nulls among the *Scm* alleles. Indeed, Western blot analysis fails to detect SCM protein in the Scm^{M56} mutant (Figure 1B).

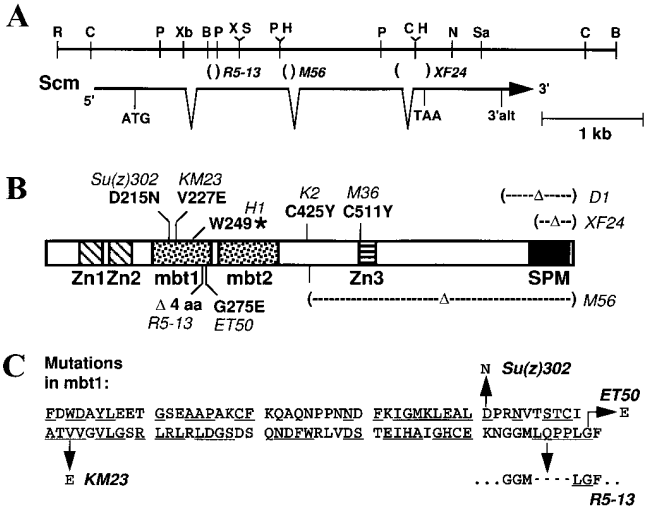

Figure 5.—Molecular lesions of *Scm* alleles. (A) A restriction map of the genomic region encompassing the *Scm* transcription unit is shown. Deletions in the *M56* and *XF24* alleles are indicated below the DNA map. A transcript map is shown with the positions of start and stop codons, an alternative 3′ polyadenylation site (Bornemann *et al.* 1996), and introns indicated. The intron sizes are 58, 65, and 61 bp, respectively. Restriction sites: R, *Eco*RI; C, *Cla*I; P, *Pst*I; Xb, *Xba*I; B, *Bam*HI; X, *Xho*I; S, *Sal*I; H, *Hin*dIII; N, *Nru*I; Sa, *Sac*I. (B) A domain map of SCM protein is shown with the SPM domain, two mbt repeats, and three potential zinc fingers indicated. Alterations in SCM protein caused by ten mutant alleles are shown. The allele names are in italic type, and the resulting molecular changes to the protein are indicated in bold type here and are listed in Table 1. The asterisk indicates replacement with a stop codon. *M56* is an 11-bp deletion that causes a frameshift and deletes the C-terminal half of SCM. *D1* is a frameshift that removes the SPM domain. (C) The amino acid sequence of the first mbt repeat and the effects of four *Scm* mutant alleles are shown. Underlined residues are conserved positions in an alignment of mbt repeats from SCM and L(3)MBT (Bornemann *et al.* 1996).

The six hypomorphic alleles are mutations that should produce altered forms of essentially full-length SCM protein (Figure 5B and Table 1); five are missense mutations, and the sixth, $Scm^{R5\text{-}13}$, is an in-frame 12-bp deletion that removes four amino acids. Among the hypomorphic alleles, four are clustered in the first mbt repeat (Figure 5, B and C). Another hypomorphic allele, Scm^{M36}, produces a cysteine to tyrosine change within a region that might form a zinc-binding domain (Zn3; Bornemann *et al.* 1996). In agreement with their identification as missense mutations, Scm^{M36} and $Scm^{Su(z)302}$ produce full-length versions of SCM protein (Figure 1B).

Three *Scm* alleles affecting the mbt repeat domain show stronger PcG genetic interactions than null *Scm* alleles: Animals doubly mutant for two different PcG genes often show phenotypes more extreme than either single mutant alone (Jurgens 1985; Adler *et al.* 1989; Cheng *et al.* 1994). It has been suggested that this phenotypic enhancement results from PcG protein complexes that are more severely impaired by simultaneous

reduction or alteration of multiple components. Having defined the molecular lesions and relative strengths of many *Scm* mutations (Figure 5 and Table 1), we wished to test the *Scm* alleles for interactions with other PcG mutations. In particular, we wished to compare genetic interactions exhibited by mutations affecting different parts of SCM protein.

We began by generating animals doubly heterozygous for each of the *Scm* alleles in Table 1 and for a lethal allele of *Polycomb*, Pc^3. We found that transheterozygous Pc^3/*Scm* adults display more severe homeotic phenotypes than Pc^3/+ adults, and that this enhancement is seen with all *Scm* alleles tested. These phenotypes include transformations of wing to haltere, antenna to leg, second and third leg to first leg, and fourth abdominal segment to fifth. However, three *Scm* alleles, *Su(z)302*, *R5-13*, and *ET50*, produced much stronger interactions with Pc^3 than did other *Scm* alleles. These three were the only *Scm* alleles to cause partial lethality in combination with Pc^3. As shown in Figure 6A, the transheterozygous progeny classes for *Su(z)302*, *R5-13*, and *ET50* are reduced to about one-third that expected for full viability. In contrast, the *Scm* null alleles *H1* and *M56* are fully viable with Pc^3. The surviving *Su(z)302*, *R5-13*, and *ET50* transheterozygous progeny also exhibited more severe homeotic phenotypes than did other Pc^3/*Scm* combinations. We observed a marked male sex bias among these survivors (Figure 6A). In the most severe case, only about 5% of the surviving Pc^3/$Scm^{Su(z)302}$ progeny were female. Similarly, partial lethality and a male sex bias were seen with the reciprocal crosses consisting of *Su(z)302*, *R5-13*, or *ET50* females mated to Pc^3 males (data not shown). These interactions likely result from the *Scm* lesions rather than mutations at other loci because these three *Scm* alleles produce similar phenotypic effects and were isolated independently on different genetic backgrounds (Jurgens 1985; Wu *et al.* 1989; M. Muller and H. Gyurkovics, personal communication). Each of the three alleles maps to the first mbt repeat in SCM protein (Figure 5, B and C). Thus, *Su(z)302*, *R5-13*, and *ET50* are hypomorphic mutations based upon their behavior as homozygotes (Table 1), yet they interact with Pc^3 more strongly than do null *Scm* alleles (Figure 6A).

We wondered if the strong *Su(z)302*, *R5-13*, and *ET50* interactions reflect a general property of these mbt repeat alleles. In particular, since Pc^3 is an antimorphic allele (Duncan and Lewis 1982), we were concerned that it might show unusual interaction patterns with *Scm* alleles. To address this, we examined genetic interactions of *Scm* alleles with mutations in other PcG genes and with other mutant alleles of the *Pc* gene.

Figure 6B shows data for interaction of several *Scm* alleles with Sce^{D1}, an allele of the *Sex comb extra* (*Sce*) gene (Breen and Duncan 1986). This is the single existing allele of the uncloned *Sce* gene; it is homozygous lethal and its precise nature has not been characterized.

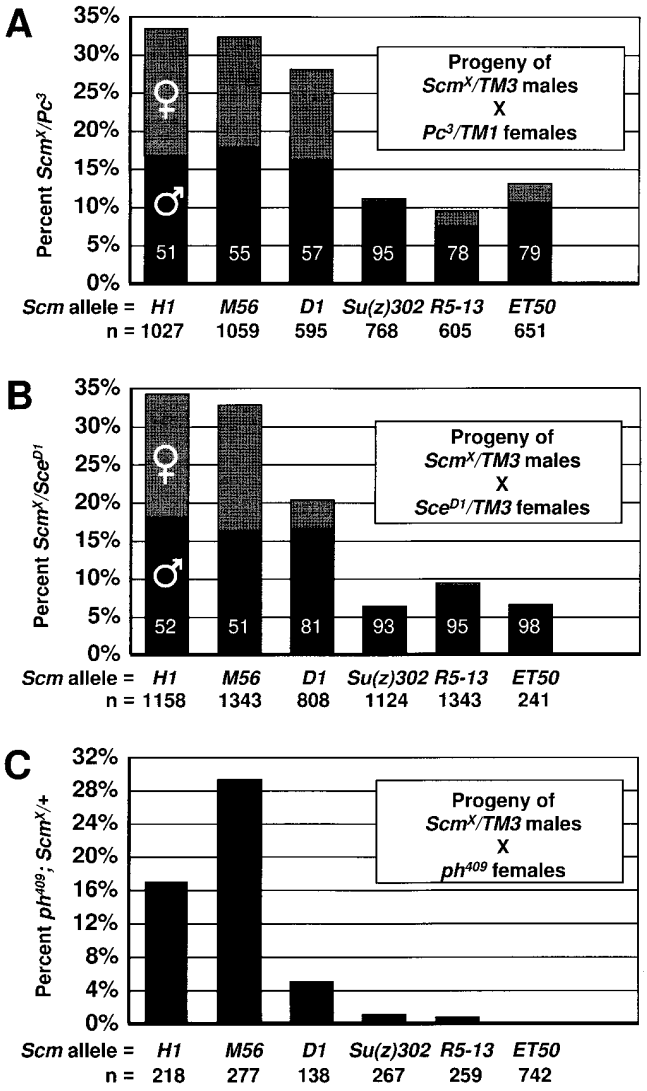

Figure 6.—Survival of adults heterozygous for *Scm* mutations and for mutations in other PcG genes. Bar graphs depict the percentage of total progeny from the indicated crosses that are doubly mutant for an *Scm* allele and another PcG allele. (A) Survival of *Scm*/Pc^3 adults. (B) Survival of *Scm*/Sce^{D1} adults. (C) Survival of ph^{409}; *Scm*/+ adults. Full viability of the double mutant progeny class corresponds to frequencies of 33% for the crosses in A and B and 50% for the cross in C. The *Scm* alleles used and the numbers of total progeny scored are indicated below each graph. Scm^x represents any *Scm* allele used. In A and B, the proportions of surviving double mutant progeny that are male or female are represented by the black or grey portions, respectively, of the bars. Numbers within these bars indicate the percentage of surviving progeny that are male. Due to *X* linkage of *ph*, only data for the male progeny are depicted in C.

As with Pc^3, there was partial lethality and male sex bias among Sce^{D1}/*Scm* transheterozygotes when the *Scm* mbt repeat alleles were used. In the most extreme example, only 1 out of 60 surviving Sce^{D1}/Scm^{ET50} adults was female. Once again, transheterozygotes with the *Scm* null alleles *H1* and *M56* were fully viable and produced progeny in the expected sex ratios (Figure 6B).

The ph^{409} mutation disrupts one of the two tandem copies of the *polyhomeotic* gene located on the *X* chromosome (Dura *et al.* 1987). Although ph^{409} is a hypomorphic, homozygous viable allele, Cheng *et al.* (1994) have shown that it is lethal or near-lethal in combination with other PcG mutations, including alleles of *Scm.* To assess *ph* interaction, we crossed homozygous ph^{409} females to males bearing *Scm* mutations and scored viability among the double mutant male progeny. We found that all *Scm* alleles (Table 1) cause partial lethality among ph^{409}*; Scm* / + males. However, *Su(z)302*, *R5-13*, and *ET50* showed much higher lethality in this combination than did *H1* and *M56* (Figure 6C).

Finally, we tested a subset of the *Scm* alleles for interaction with additional *Pc* alleles, Pc^2 and Pc^{XT109}. Pc^2 is a frameshift near the C terminus that produces detectable PC protein of about the normal size (Franke *et al.* 1995). Pc^{XT109} is associated with a 2-kb deletion, and PC protein is not detected in Pc^{XT109} mutant embryos (Franke *et al.* 1995). We found that interactions with Pc^2 were similar to interactions with Pc^3; the three mbt repeat alleles produced partial lethality in combination with Pc^2, whereas Scm^{H1}/Pc^2 and Scm^{M56}/Pc^2 animals were fully viable (data not shown). The *Scm* mutations, as a group, showed less severe enhancement with Pc^{XT109} than with Pc^2 or Pc^3. However, the same trend was observed; combinations with the three mbt repeat alleles produced more severe wing-to-haltere and extra sex comb transformations compared to the *H1* and *M56* null alleles. Thus, in tests for genetic enhancement employing different PcG genes and different *Pc* alleles, we found that three mbt repeat alleles of *Scm* consistently produced the strongest interactions.

Interactions of *Scm* alleles with *zeste*: Mutations in a subset of PcG genes, including *Scm*, have been shown to modify eye color in flies bearing the $zeste^1$ (z^1) mutation (Wu *et al.* 1989; Jones and Gelbart 1990). Indeed, these workers isolated the $Scm^{Su(z)302}$ allele as a dominant suppressor of z^1. The Scm^{XF24} null allele and a deficiency for the *Scm* locus are also dominant suppressors of z^1 eye color (Wu *et al.* 1989; Bornemann *et al.* 1996), although their effects are weaker than seen with *Su(z)302.* To further this analysis, we tested each of the additional *Scm* alleles (Table 1) in combination with the $z^1\ w^{is}$ tester chromosome (Wu *et al.* 1989). We found that, except for *Su(z)302*, heterozygosity for each *Scm* allele converts eye color in $z^1\ w^{is}$ males from light-orange to red-orange (Table 1). *Su(z)302* is unique in producing much stronger suppression, which is manifested by dark red eye color in this combination (Wu *et al.* 1989). Since *Su(z)302* is a missense mutation in mbt repeat 1 (Figure 5), this difference may reflect a role for mbt repeats in molecular interactions that contribute to $zeste^1$ suppression.

mbt repeat mutants produce stable SCM protein that associates with normal chromosomal target loci: The *Su(z)302*, *R5-13*, and *ET50* molecular lesions (Figure 5) suggest that these strongly-interacting alleles encode altered SCM proteins that are essentially full-length. To test for production of these mutant proteins, extracts were prepared from *Scm* mutant pupae and Western blots with anti-SCM antibody were performed. Figure 7 shows that homozygous *Su(z)302*, homozygous *R5-13*, and hemizygous *ET50* pupae express full-length versions of SCM protein at levels similar to wild type (lanes 1, 2, 4, and 5). Since these mutant pupae are derived from heterozygous *Scm* mutant mothers, it is conceivable that the signals could reflect maternal protein that has perdured to pupal stages. However, the increase in wild-type SCM levels during development from larval to pupal stages (Figure 2), indicates that a substantial portion of pupal SCM is newly synthesized protein. In addition, reduced signal was reproducibly seen with extracts from homozygous *M36* mutant pupae (lane 3); this indicates that maternal product, if present, does not compromise detection of reduced zygotic SCM levels at this stage. Thus, the *Su(z)302*, *R5-13*, and *ET50* mutants accumulate SCM proteins at levels comparable to wild type.

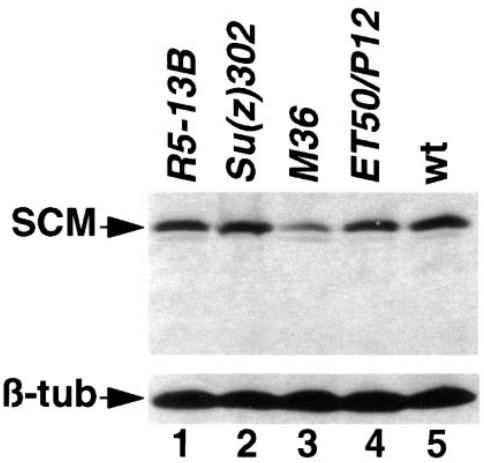

Figure 7.—Accumulation of SCM mutant proteins in pupae. (Top) Western blot using anti-SCM antibodies. Lanes contain protein extracts from $Scm^{R5\text{-}13}$ homozygous (lane 1), $Scm^{Su(z)302}$ homozygous (lane 2), Scm^{M36} homozygous (lane 3), Scm^{ET50} hemizygous (lane 4), or wild-type (lane 5) pupae. Scm^{P12} is a deletion that removes *Scm* (Bornemann *et al.* 1996). (Bottom) Immunodetection of β-tubulin on the same blot as a control for lane loading.

To assess whether these SCM mutant proteins associate with target sites *in vivo*, we used anti-SCM antibodies to stain polytene chromosomes from larvae homozygous for the *Su(z)302* or *R5-13* alleles. Figure 8A shows the wild-type distribution of SCM protein on a portion of the third chromosome that includes the bithorax complex (BX-C; arrow). Five sites of SCM protein accumulation are apparent. Figure 8, B and C show that these sites still accumulate SCM protein encoded by the *Su(z)302* and *R5-13* alleles. The number of staining sites per genome and the signal intensities were similar for these two mutants and wild type. Figure 8D shows that the

same result is obtained if both the maternal and zygotic contributions consist of *Su(z)302* mutant SCM protein. Thus, the signals are not due to perdurance of wild-type maternal SCM and must reflect the chromosome-binding properties of the mutant protein.

***Su(z)302* mutant SCM protein is sufficient for embryonic but not pupal development:** The homeotic gene misexpression and embryonic lethality seen with *Scm* null alleles (Figure 4B and Table 1) show that SCM protein is required during embryogenesis. In contrast, expression of ABDA protein in homozygous mutant $Scm^{Su(z)302}$ embryos appears normal (Table 1), and these animals survive most of pupal development to die as pharate adults with homeotic phenotypes (Wu *et al.* 1989). Thus, *Su(z)302* mutant protein appears defective in homeotic repression in pupae. Whether *Su(z)302* protein is also defective in embryonic processes is unclear because maternally provided, wild-type SCM product is present in homozygous *Su(z)302* embryos. To address this, we used the dominant female sterile technique and FLP recombination system (see materials and methods) to generate *Su(z)302* mutant embryos from *Su(z)302* germline clone mothers. As shown in Figure 4D, embryos that express solely the *Su(z)302* form of SCM protein from maternal and zygotic sources still show normal patterns of ABDA expression. Like *Su(z)302* homozygotes from *Su(z)302*/+ mothers, these animals survive to pupal stages. We conclude that *Su(z)302* mutant protein provides sufficient *Scm* function for apparently normal embryogenesis but that it is compromised in requirements for pupal development.

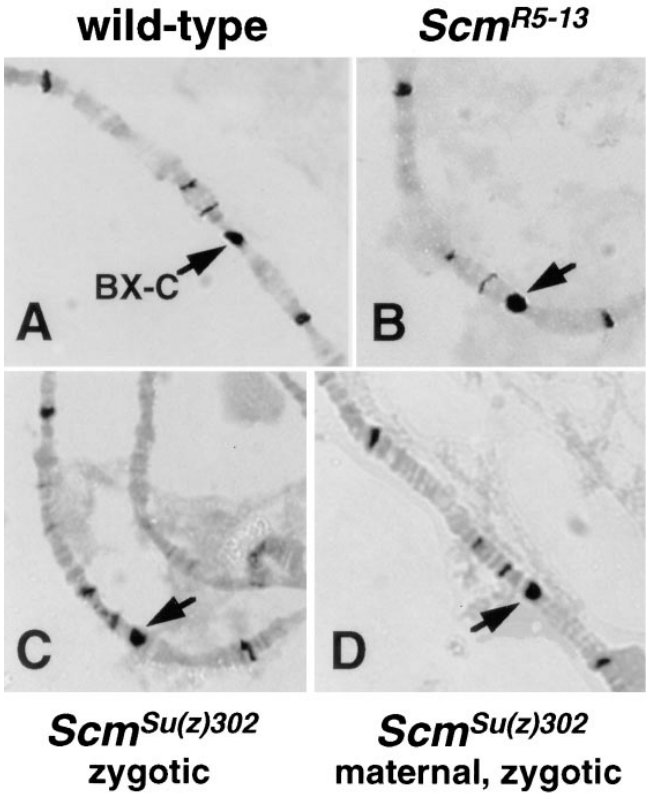

Figure 8.—Accumulation of SCM mutant proteins at sites on chromosomes. Portions of polytene chromosomes spanning the bithorax complex (BX-C, arrows) and immunostained with anti-SCM antibodies are shown. Chromosomes were obtained from (A) wild-type larvae, (B) homozygous Scm^{R5-13} larvae from heterozygous mothers, (C) homozygous $Scm^{Su(z)302}$ larvae from heterozygous mothers, or (D) homozygous $Scm^{Su(z)302}$ larvae from $Scm^{Su(z)302}$ germline clone mothers. Homozygous mutant larvae were recognized as non-*Tb* progeny of heterozygous parents containing a *Tb*-marked balancer.

DISCUSSION

Functional domains in SCM protein: The SCM protein contains multiple homology domains, including an N-terminal pair of zinc fingers, two copies of the mbt repeat, and the C-terminal SPM domain (Figure 5B; Bornemann *et al.* 1996). The SPM domain has been shown to mediate protein interaction *in vitro* between the SCM and PH proteins and also between SCM and itself (Peterson *et al.* 1997). Although it is not yet clear how SPM domain interactions contribute to PcG function *in vivo*, these properties suggest a role in PcG complex assembly or stabilization.

Little is known about the biochemical role of mbt repeats either in SCM or in the L(3)MBT fly tumor suppressor protein (Wismar *et al.* 1995). However, the fact that four *Scm* alleles cause alterations within the first mbt repeat (Figure 5B and Table 1) shows that this domain contributes to *Scm* function *in vivo*. Several of these hypomorphic alleles produce wild-type levels of SCM protein (Figure 7) that associates with normal sites in polytene chromosomes (Figure 8). This suggests that mbt repeats are not crucial for targeting SCM protein to specific loci but, rather, that they provide a biochemical activity required at the normal location in chromatin. The repeats could provide catalytic activity or a protein interaction surface needed to localize or bind another partner protein. The conservation of the two mbt repeats with 69% identity in a mouse SCM homolog (F. Randazzo, personal communication) implies that their biochemical role is key for *Scm* function. Thus, the SPM domain and the mbt repeats are two distinct functional domains in SCM protein.

Our analysis of *Scm* alleles did not identify molecular lesions that affect the N-terminal SCM zinc fingers. Their possible role in *Scm* function is unclear because they do not belong to canonical DNA-binding zinc finger classes (Bornemann *et al.* 1996) and *in vitro* assays have not detected sequence-specific DNA-binding by SCM protein (D. Bornemann and J. Simon, unpublished data). Further studies, including site-directed mutagenesis, will be needed to assess the role of the N-terminal fingers. In contrast, we did find a mutation within a third potential zinc finger of SCM. This mutation, Scm^{M36}, changes cysteine-511 to tyrosine (Figure 5B) and it appears to compromise protein stability, because *M36*

protein levels are reduced compared to wild type (Figures 1 and 7).

Genetic interactions and PcG complexes: Mutations in different PcG genes often produce phenotypic enhancement in double mutant combination (Jurgens 1985; Adler *et al.* 1989; Cheng *et al.* 1994). These genetic interactions have been interpreted in the context of PcG repressors that work together in multiprotein complexes. In this study, we compared how heterozygosity for different *Scm* mutations affects PcG function in a situation already compromised by heterozygosity for another PcG gene. Thus, the concentration or effectiveness of PcG complexes is brought near some critical threshold by the first PcG mutation, and we have assessed the impact of additional damage inflicted by changes in the SCM component.

The enhancement could result from two types of further alterations to PcG complexes. First, the *Scm* mutations could simply eliminate half of the SCM available to form complexes, resulting in haploinsufficiency for PcG complex function. Second, the mutations could produce stable, defective SCM proteins that incorporate into complexes and impair their activities. This type of dominant negative mechanism of inhibition has been described for many proteins that act in complexes (Herskowitz 1987; Khavari *et al.* 1993). Consistent with their molecular lesions (Figure 5), we found that some *Scm* mutations, such as *M56*, fail to produce detectable protein (Figure 1). Because these *Scm* null mutations do enhance mutations in other PcG genes (Figure 6), the enhancement mechanism in these cases appears to involve a haploinsufficiency effect.

However, the strongest genetic interactions were consistently seen with three *Scm* alleles that are not nulls. These alleles, *Su(z)302*, *ET50*, and *R5-13*, alter the first mbt repeat and produce stable SCM proteins. The accumulation of protein encoded by two of these alleles at normal sites in chromatin (Figure 8) strongly suggests that the altered proteins incorporate into PcG complexes. Assembly of the mutant proteins into complexes could occur through protein contacts supplied by the SPM domain (Peterson *et al.* 1997), which remains intact in the mbt repeat alleles (Figure 5). In contrast to the null alleles, we suggest that the mbt repeat mutations produce especially strong interactions by poisoning PcG complexes. A dominant negative effect is also consistent with phenotypes reported for these alleles in heterozygous single mutants; Wu *et al.* (1989) found that more than 85% of *Su(z)302*/+ or *ET50*/+ adults bear ectopic sex comb teeth as compared to only 13% of adults heterozygous for the *XF24* null allele.

The strong interactions seen in animals heterozygous for mbt repeat alleles contrast with the relatively mild loss-of-function seen in mbt repeat allele homozygotes (Figure 4 and Table 1). Most strikingly, embryos possessing solely the *Su(z)302* form of SCM protein appear to develop normally (Figure 4D), and they survive to pupal stages. This indicates that PcG complexes containing *Su(z)302* protein retain substantial function, at least in embryos. We suggest that the mbt repeat alleles encode partially functional SCM proteins that are sufficiently compromised that they exert dominant negative effects under conditions that are particularly sensitive to PcG function. We note that *Su(z)302*, *ET50*, and *R5-13* were isolated for dominant effects upon adult phenotypes that are sensitive indicators of PcG function (Jurgens 1985; Wu *et al.* 1989; M. Muller and H. Gyurkovics, personal communication). Thus, the special properties of these mbt repeat alleles probably reflect phenotypic requirements imposed by the screens employed.

Regulatory targets of SCM protein: The accumulation of PcG proteins, including SCM, at approximately 100 sites on polytene chromosomes (Franke *et al.* 1992; Rastelli *et al.* 1993; Lonie *et al.* 1994; Peterson *et al.* 1997) reveals a much larger number of PcG targets than just the two homeotic loci. Indeed, roles for PcG proteins in control of segmentation gene expression (Moazed and O'Farrell 1992; Pelegri and Lehmann 1994) and in dorsal-ventral patterning (Tiong *et al.* 1995) have been described. The presence of SCM protein in adult heads (Figure 2B) presumably also reflects a role besides control of homeotic genes.

The synthetic lethality seen with strongly interacting *Scm*-PcG double mutant combinations (Figure 6) could be explained by effects upon either homeotic or nonhomeotic target genes. We found that surviving adults from the synthetic lethal combinations also showed the most extreme homeotic transformations. However, the lethal frequencies are comparable in Scm/Pc^3 and Scm/Pc^3 $DfUbx^{109}$ animals (J. Simon, unpublished results). If lethality were due to misexpression of either *Ubx* or *abdA*, which are removed by $DfUbx^{109}$ (Karch *et al.* 1985), then the lower dosage of these products might be expected to reduce the lethal frequency. This suggests that the lethality is either associated with a homeotic gene besides *Ubx* or *abdA*, or with a nonhomeotic PcG target.

A striking outcome of the *Scm*-PcG double mutant analysis was that the frequency of synthetic lethality was much higher among female transheterozygotes than among males (Figure 6). Similarly, expressivity of antenna-to-leg and wing-to-haltere transformations among $Pc^3/+$ and $Pc^3/+$; $Pcl^1/+$ adults is greater in females than in males (Duncan 1982). We envision several possible explanations for more severe effects of PcG loss-of-function in females *vs.* males. First, the extra euchromatin present in *XX vs. XY* animals might sequester PcG proteins away from one or several of the normal euchromatic target sites. Under conditions where PcG function is already compromised, the need to assemble PcG proteins at sites on two *X* chromosomes rather than one might tip the balance below a critical threshold for function at other sites in the genome. In this titration model, sex-biased lethality could result from defects in homeotic gene control or in control of other target

loci. A second possible explanation is that PcG proteins might function directly in dosage compensation to repress global expression from the *X* chromosome in females. This seems unlikely, however, because PcG proteins including SCM are only present at about 10 sites on polytene *X* chromosomes (Franke *et al.* 1992; Peterson *et al.* 1997) and because Drosophila dosage compensation relies upon transcriptional up-regulation in males rather than down-regulation in females (Bashaw and Baker 1996 for review). A third possibility is that the sex bias reflects events at one or more specific *X*-linked loci. In this scenario, the lethality could result from misexpression of a specific *X*-linked gene that occurs when PcG repressors are compromised. In females, the extra copy of this locus might increase misexpression to levels that more frequently exceed a threshold leading to inviability. In the context of this discussion, we note that phenotypes in *Caenorhabditis elegans* mutant for PcG homologs are more severe in *XX* than in *X*O animals. Garvin *et al.* (1998) have shown that this difference depends on *X* chromosome dosage rather than on sexual phenotype. Further studies should identify which PcG target loci are responsible for differential consequences of PcG loss-of-function between the two sexes.

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