Long-Range Repression by Multiple Polycomb Group (PcG) Proteins Targeted by Fusion to a Defined DNA-Binding Domain in Drosophila

Robin R. Roseman,* Kelly Morgan,†,‡ Daniel R. Mallin,* Rachel Roberson,* Timothy J. Parnell,§ Douglas J. Bornemann,† Jeffrey A. Simon†,‡ and Pamela K. Geyer*,§

*Department of Biochemistry and §Genetics Program, The University of Iowa, Iowa City, Iowa 52242, and †Department of Genetics, Cell Biology and Development and ‡Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT

A tethering assay was developed to study the effects of Polycomb group (PcG) proteins on gene expression *in vivo*. This system employed the Su(Hw) DNA-binding domain (ZnF) to direct PcG proteins to transposons that carried the *white* and *yellow* reporter genes. These reporters constituted naive sensors of PcG effects, as *bona fide* PcG response elements (PREs) were absent from the constructs. To assess the effects of different genomic environments, reporter transposons integrated at nearly 40 chromosomal sites were analyzed. Three PcG fusion proteins, ZnF-PC, ZnF-SCM, and ZnF-ESC, were studied, since biochemical analyses place these PcG proteins in distinct complexes. Tethered ZnF-PcG proteins repressed *white* and *yellow* expression at the majority of sites tested, with each fusion protein displaying a characteristic degree of silencing. Repression by ZnF-PC was stronger than ZnF-SCM, which was stronger than ZnF-ESC, as judged by the percentage of insertion lines affected and the magnitude of the conferred repression. ZnF-PcG repression was more effective at centric and telomeric reporter insertion sites, as compared to euchromatic sites. ZnF-PcG proteins tethered as far as 3.0 kb away from the target promoter produced silencing, indicating that these effects were long range. Repression by ZnF-SCM required a protein interaction domain, the SPM domain, which suggests that this domain is not primarily used to direct SCM to chromosomal loci. This targeting system is useful for studying protein domains and mechanisms involved in PcG repression *in vivo*.

THE Drosophila Polycomb group (PcG) proteins are transcriptional repressors required for appropriate patterns of gene expression during development (for reviews see Simon 1995; Pirrotta 1997). Although most PcG proteins were identified on the basis of their roles as regulators of homeotic genes (Struhl 1981; Duncan 1982; Jürgens 1985; Breen and Duncan 1986; Dura *et al.* 1987), these proteins are likely to represent global regulators of gene expression. This supposition is based on the pleiotropic phenotypes of PcG mutants, the identification of regulatory targets besides homeotic genes, and the localization of PcG proteins to ~100 sites on polytene chromosomes (Dura *et al.* 1987; Zink and Paro 1989; Phillips and Shearn 1990; Adler *et al.* 1991; DeCamillis *et al.* 1992; Moazed and O'Farrell 1992; Rastelli *et al.* 1993; Lonie *et al.* 1994; Pelegri and Lehmann 1994; Carrington and Jones 1996).

PcG proteins form a family based upon their common role in gene expression rather than extensive shared homologies or structural motifs. Molecular characterization of 15 Drosophila PcG genes has demonstrated that none of the PcG proteins possesses a recognizable catalytic domain and only 1 protein, pleiohomeotic (PHO), contains a sequence-specific DNA-binding domain (Brown *et al.* 1998). The most common functional motifs found within PcG proteins are protein interaction domains (Messmer *et al.* 1992; Platero *et al.* 1996; Peterson *et al.* 1997; Kyba and Brock 1998b). The Polycomb protein (PC) contains a chromodomain, a motif shared with Heterochromatin Protein 1, that is required for its *in vivo* interaction with polyhomeotic (PH; Paro and Hogness 1991; Strutt and Paro 1997). The Sex comb on midleg (SCM) and PH proteins share a C-terminal domain, called the SPM domain (Bornemann *et al.* 1996), that can mediate heterotypic and homotypic interactions between SCM and PH *in vitro* (Peterson *et al.* 1997). Two other protein interaction domains associated with PcG proteins are the SET domain, found in the enhancer of zeste [E(Z)] protein (Jones and Gelbart 1993; Cui *et al.* 1998), and WD repeats, found in the Extra sex combs (ESC) protein (Sathe and Harte 1995; Simon *et al.* 1995; Jones *et al.* 1998; Tie *et al.* 1998). Although certain subtypes of SET domains have histone methyltransferase activity, the E(Z) SET domain apparently lacks this intrinsic catalytic function (Rea *et al.* 2000).

Mammalian homologues have been identified for

Corresponding author: Pamela K. Geyer, Department of Biochemistry, The University of Iowa, Iowa City, IA 52242.
E-mail: pamela-geyer@uiowa.edu

each of the cloned Drosophila PcG genes (Brunk *et al.* 1991; Nomura *et al.* 1994; van der Lugt *et al.* 1994; Akasaka *et al.* 1996; Schumacher *et al.* 1996; Alkema *et al.* 1997a; Core *et al.* 1997). Several of these PcG proteins repress Hox gene expression during mouse development, as mice carrying knockouts show homeotic transformations (Akasaka *et al.* 1996; van der Lugt *et al.* 1996; Takihara *et al.* 1997). In some cases, Drosophila and mammalian PcG proteins can function in heterologous systems (Bunker and Kingston 1994; Muller *et al.* 1995). For example, the mouse homologue M33 can partially rescue *Pc* mutations in Drosophila (Muller *et al.* 1995). These data indicate that mechanisms of PcG action have been evolutionarily conserved.

PcG proteins function together in multiprotein complexes. This conclusion was first suggested by studies showing that several PcG proteins are colocalized on polytene chromosomes (Franke *et al.* 1992; Rastelli *et al.* 1993; Lonie *et al.* 1994). Subsequent coimmunoprecipitation experiments, *in vitro* binding assays, and yeast two-hybrid analyses have defined binding interactions among particular PcG proteins (Peterson *et al.* 1997; Strutt and Paro 1997; Kyba and Brock 1998b; Jones *et al.* 1998; Tie *et al.* 1998). Recent biochemical studies have identified at least three discrete PcG complexes in nuclear extracts from fly embryos. The Polycomb repressive complex, PRC1, contains a subset of the identified PcG proteins including PC, PH, and posterior sex combs (PSC; Shao *et al.* 1999). PRC1 lacks E(Z) and ESC, two evolutionarily conserved binding partners (Denisenko *et al.* 1998; Jones *et al.* 1998; Sewalt *et al.* 1998; Tie *et al.* 1998; van Lohuizen *et al.* 1998). Instead, E(Z) and ESC are found in a distinct, smaller complex that does not contain PH or SCM (Ng *et al.* 2000). The bulk of embryonic SCM protein appears to be assembled into a third, distinct protein complex. Although SCM is present in PRC1, it represents a substoichiometric component (Shao *et al.* 1999; A. Saurin and R. Kingston, unpublished results). Instead, gel filtration chromatography detects the majority of SCM in an ~500-kD complex that is significantly smaller than PRC1 and displays different fractionation behavior (D. Mallin, J. Ng and J. Simon, unpublished results). Similarly, multiple mammalian PcG protein complexes have been identified. Interestingly, the biochemical separability of complexes containing the PC and PH homologues from those containing the ESC and E(Z) homologues has been conserved (Alkema *et al.* 1997a; Gunster *et al.* 1997; Hashimoto *et al.* 1998; Sewalt *et al.* 1998; van Lohuizen *et al.* 1998; van der Vlag and Otte 1999).

PcG proteins are targeted to genes by DNA sequences, known as PcG response elements (PREs; Simon *et al.* 1993; Chan *et al.* 1994). Dissection of individual PREs indicates that these elements are complex and contain multiple subregions with different silencing activities (Hagstrom *et al.* 1997; Tillib *et al.* 1999; Horard *et al.* 2000; Shimell *et al.* 2000). How PcG complexes associate with PREs is unknown, but one potential mechanism involves recruitment by PHO (Brown *et al.* 1998; Mihaly *et al.* 1998; Fritsch *et al.* 1999; Shimell *et al.* 2000). In addition, the GAGA DNA-binding protein has been implicated as a component of PRE function (Hagstrom *et al.* 1997; Horard *et al.* 2000). Once tethered, a PcG complex may establish silenced chromatin through direct interactions with nucleosomes. PC binds to nucleosomal core particles (Breiling *et al.* 1999) and this association may block the remodeling of nucleosomal arrays that accompanies gene activation (Shao *et al.* 1999). In addition, the human PcG protein embryonic ectoderm development (EED) interacts with a histone deacetylase, implicating direct nucleosome modification in the PcG repression mechanism (van der Vlag and Otte 1999). The resulting repressed chromatin state may restrict access of transcriptional activators or activator complexes to the DNA. However, *in vivo* tests show that if an inaccessible chromatin state is created, it does not exclude all DNA-binding proteins (McCall and Bender 1996). Further work is needed to assess how alterations to local chromatin structure might contribute to PcG repression.

Insights into the mechanistic basis of PcG repression have come from studies that target specific PcG proteins to reporter genes (Bunker and Kingston 1994; Muller 1995; van der Vlag *et al.* 2000). In these experiments, coding sequences of PcG proteins were fused to well-characterized DNA-binding domains, bypassing the need to use complex PREs to assess PcG protein function. In such simplified systems, direct binding of the Drosophila PC and PSC proteins to reporter genes caused repression of transcription, with more robust silencing observed in cases where reporter genes were integrated into the chromosome (Bunker and Kingston 1994; Muller 1995). Interestingly, only transient silencing of reporter genes lacking PREs occurred in the absence of the continuous production of the tethered PcG protein (Muller 1995). Furthermore, PcG silencing of a PRE reporter construct *in vivo* required the presence of an intact PRE throughout development (Busturia *et al.* 1997). These data suggest that both *trans*- and *cis*-acting PcG components are needed continuously to maintain gene repression for long periods of developmental time.

We used the method of protein targeting to determine whether individually tethered PcG proteins, which are components of distinct complexes, can confer similar levels of repression. For these experiments, we studied PC, SCM, and ESC, since these proteins are found in biochemically separable complexes. Repression by tethered PcG proteins was assayed using the *white* and *yellow* genes. These genes lack *bona fide* PREs and serve as naive reporters of PcG function. In total, the effects of the three PcG proteins tethered at nearly 40 chromosomal sites within the Drosophila genome were examined. This large collection of transgenic reporter lines

allowed us to evaluate the contributions of genomic location to targeted PcG repression, as well as to compare the levels of repression conferred by the different tethered proteins. We found that the ZnF-PcG proteins silenced gene expression at the majority of chromosomal insertion sites. Repression was observed even with separation distances of up to 3.0 kb between target promoters and binding sites for the tethered PcG proteins. We find that silencing depends upon a protein interaction domain in tethered SCM and upon the dosage of an endogenous, untethered PcG protein, PH, implying that the tethered proteins work by recruiting other PcG components. This system will be useful for dissection of additional PcG protein domains and mechanisms involved in PcG repression.

MATERIALS AND METHODS

Drosophila stocks: Flies were raised at 25°, 70% humidity on standard corn meal and agar medium. The mutations and chromosomes used in this study were described previously (Lindsley and Zimm 1992) and in Flybase (http://www.flybase.bio.indiana.edu/).

Construction of plasmids: A set of PcG fusion proteins capable of DNA binding were expressed from producer transgenes that contained the PcG coding sequences cloned downstream of the coding sequences for the 12-zinc-finger (ZnF) DNA-binding domain of the Suppressor of Hairy-wing [Su(Hw)] protein (Figure 1A). These producer transgenes were created in the following manner. A PCR fragment encoding amino acids 210–634 of the Su(Hw) protein was cloned into a modified Bluescript plasmid (RSR56) between sequences encoding a FLAG tag (MDYKDDDDKG) and an 800-bp fragment that contained the α-tubulin polyadenylation [poly (A)] sequences located at its 3′ end. Translation stop codons in every reading frame were located in the 5′ end of the 800-bp poly(A) fragment. The FLAG tag was preceded by a consensus Drosophila translation initiation sequence (Cavener and Ray 1991). The FLAG-ZnF-tubulin poly(A) plasmid was called RSR117. Full-length cDNAs for PC, SCM, and ESC were inserted into RSR117, downstream of, and in frame with, the Su(Hw) coding sequences. Insertion of an *Scm* cDNA fragment deleted for sequences encoding the last 80 amino acids, which includes the SPM domain (Bornemann *et al.* 1996), created the *ZnF-Scm*$^{\Delta SPM}$ fusion gene. DNA sequence analysis was conducted across the cloning junctions to verify the structure of each fusion gene. The *ZnF* and *ZnF-PcG* fusion genes were located within a *Not*I fragment that was removed and inserted into a modified version of pYC1.8, a *P*-element transformation vector that contains the *vermilion* gene as the selectable marker (Fridell and Searles 1991). The modified pYC1.8, called pYC-HS, contained a 0.4-kb fragment of the *hsp70* gene, encompassing the promoter. Each ZnF-PcG *Not*I fragment was inserted 3′ of the *hsp70* promoter, generating a producer transgene that encoded a heat-shock-inducible FLAG-tagged ZnF or ZnF-PcG fusion protein (Figure 1).

Germline transformation: Germline transformation was carried out as described previously (Rubin and Spradling 1983). Two host strains were used to establish producer lines carrying the FLAG-tagged *ZnF-PcG* transgenes. The v^{36f}; ry^{506} strain was used to establish all but the *ZnF-Scm*$^{\Delta SPM}$ transformed lines, where the host strain was y^2 v^1 f^1 ; *su(Hw)*^{v} *bx*^{34e}/*TM6, su(Hw)*^{f} *Ubx*. DNA concentrations used for germline transformation were 400 μg/ml of the *ZnF-PcG* construct and 200 μg/ml of the helper plasmid "Turbo" Δ2-3 (Mullins *et al.* 1989). Transformants were recognized by restoration of eye pigmentation. The number of insertions within each line and integrity of the transposons were determined by Southern blot analysis. Lines that carried a single transposon insertion were used in subsequent studies. The balancer stock y^2 v^1 f^1; *CyO, MKRS/T(2,3)ap*^{Xa} (*CyO* and *MKRS* are balancer chromosomes carrying the dominant *Cy* and *Sb* markers, respectively) was used to determine chromosome linkage of the insertions.

Western analysis: Protein extracts from five heat-shocked and five non-heat-shocked adults were isolated as described previously (Bornemann *et al.* 1996). Flies were heat shocked for 1 hr at 37° and allowed to recover 30–60 min before protein isolation. Immunodetection on Western blots used FLAG M5 antibody (1:280 dilution; Sigma, St. Louis) followed by goat anti-mouse antibody conjugated to horseradish peroxidase (1:2000 or 1:10,000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA). Signals were developed using the ECL detection system (Amersham, Pharmacia Biotech, Piscataway, NJ).

Repression assay: Three reporter transposons were used in these studies: *SUPor P*, *SUPor P-lt*, and *SUPor P-blk* (Figure 2; Roseman *et al.* 1993). These transposons contained two Su(Hw)-binding regions (BRs) placed in different positions relative to the *white* and *yellow* reporter genes. The *yellow* gene encodes a protein required for pigmentation of the cuticle structures (Geyer and Corces 1987), while the *white* gene encodes a transport protein required for the import of pigment precursors (Dreesen *et al.* 1988). Levels of eye pigmentation are well correlated with levels of *white* gene expression, such that red-eyed flies reflect high levels of transcription and yellow-eyed flies reflect low levels of transcription. The Su(Hw) BR is a 400-bp sequence that contains a cluster of 12 degenerate binding sites for the Su(Hw) protein (Spana *et al.* 1988). In *SUPor P-lt*, the Su(Hw) BRs are positioned 3.8 kb downstream and 0.3 kb upstream of the *white* promoter and 2.9 kb upstream of the *yellow* promoter. *SUPor P* contains Su(Hw) BRs positioned 3.8 kb downstream and 1.0 kb upstream of the *white* promoter and 2.9 kb upstream of the *yellow* promoter (Figure 2). Finally, *SUPor P-blk* contains Su(Hw) BRs positioned 3.8 kb downstream and 0.3 kb upstream of the *white* promoter and 3.0 kb downstream of the *yellow* promoter (Figure 2). The chromosomal insertion sites for these reporter transposons were determined previously (Roseman *et al.* 1993, 1995a,b).

The effects of the ZnF-PcG fusion proteins were tested in a *su(Hw)* mutant background. Reporter genes were crossed into a y^- ac^- w^{1118} ct^6 f^1; *su(Hw)*^{v} *bx*^{34e}/*TM6, su(Hw)*^{f} *Ubx* background, as described previously (Roseman *et al.* 1995a). This combination of *su(Hw)* alleles reverses the phenotypes associated with gypsy insertions and is female fertile. The *su(Hw)*^{v} allele is a partial deletion of the *su(Hw)* gene (Harrison *et al.* 1992) and the *su(Hw)*^{f} allele contains a point mutation in a ZnF that compromises DNA binding (Harrison *et al.* 1993). Producer lines were established that were y^+ w^+ ct^+ v^1 f^+; *ZnF-PcG/CyO*; *su(Hw)*^{E8}/*MKRS*. The *su(Hw)*^{E8} allele contains a point mutation in a ZnF that abolishes DNA binding (Harrison *et al.* 1993). The crossing scheme used to test the effects of tethered ZnF-PcG proteins on *yellow* and *white* gene expression is shown in Figure 3A. Developing progeny were given either no heat shock or heat pulses administered every day, beginning 4 days after egg laying (see below). The eye and cuticle phenotypes of the resulting *su(Hw)*$^-$ progeny were determined in flies that were aged for 1 day after eclosion. Phenotypes were compared between *su(Hw)*$^-$ siblings that carried (class I) or lacked (class III) the producer transgene and that were heterozygous for the reporter transposon.

Genetic analysis of the requirements for ZnF-PcG repres-

sion: The effects of mutations in *ph* on tethered ZnF-PcG repression were determined. In these experiments, we used ph^{409}, which is a hypomorphic allele that carries a deletion of the proximal *ph* repeat (Dura *et al.* 1987). For these studies, y^+ ac^+ ph^{409} w^{1118} ct^+ v^+ f^1/*Y ; su(Hw)*^{v} bx^{34e}/*TM6, su(Hw)*^{f} *Ubx* males were generated and were crossed to y^- ac^- ph^+ w^{1118} ct^6 v^+ f^1*; SUPor P; su(Hw)*^{v} bx^{34e}/*TM6, su(Hw)*^{f} *Ubx* females. The *SUPor P* line that was used for these analyses carried an insert at cytological location 22F. Female progeny heterozygous for both the ph^{409} mutation and *SUPor P* [y^+ ac^+ ph^{409} w^{1118} ct^+ v^+ f^1/y^- ac^- ph^+ w^{1118} ct^6 f^1*; SUPorP/+; su(Hw)*^{v} bx^{34e}/*TM6, su(Hw)*^{f} *Ubx*^{f}] were mated to y^+ ac^+ ph^+ w^+ ct^+ v^1 f^+/*Y; ZnF-PcG/ CyO*; *su(Hw)*^{E8}/*MKRS* producer males (Figure 3B). The four classes of resulting *su(Hw)*$^-$ male offspring were scored (Figure 3B). Two classes, identified by the absence of the *CyO* balancer, carried a producer transgene and differed depending upon whether they carried the ph^{409} (class I) or the ph^+ (class II) chromosome. These two classes represented the experimental classes. Two classes (III and IV), identified by the presence of the *CyO* balancer, lacked the ZnF-PcG producer transgenes and served as negative controls. The numbers and eye color phenotypes of flies in each class were determined. We note that the ph^{409} mutation reduced viability somewhat, as the numbers of progeny in classes I and III were reduced relative to classes II and IV.

RESULTS

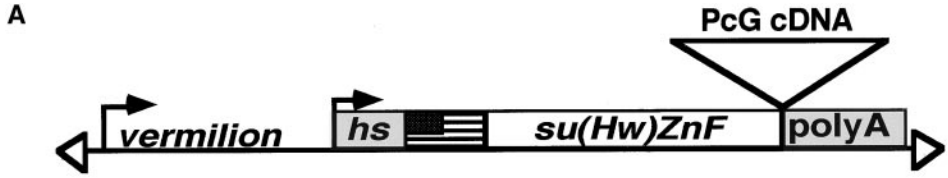
Components of the PcG DNA tethering system: The Su(Hw) zinc-finger DNA-binding domain was used to artificially target PcG complexes to reporter genes to study PcG repression. This DNA-binding domain interacts with a 400-bp cluster of 12 degenerate Su(Hw)-binding sites, known as the Su(Hw)-binding region. The Su(Hw) ZnF was chosen because its binding properties are well characterized (Spana *et al.* 1988; Spana and Corces 1990; Shen *et al.* 1994; Kim *et al.* 1996). In addition, a large number of transgenic lines that carried one of three related transposons that contained Su(Hw) BRs were available (Roseman *et al.* 1993, 1995a,b). These lines provided a useful collection of integrated reporters for assessing *in vivo* effects of targeted PcG proteins (see below).

The ZnF-PcG proteins were expressed using the *hsp70* heat-shock promoter (Figure 1A). Five producer *hsp70-ZnF* transgenes were constructed, including one that encoded only the Su(Hw) ZnF domain and four that encoded PcG fusion proteins (ZnF-PC, ZnF-SCM, ZnF-SCM$^{\Delta SPM}$, and ZnF-ESC). The fusion genes were cloned into the *vermilion* transformation vector pYC1.8 (Fridell and Searles 1991) and several independently transformed lines for each *ZnF-PcG* producer gene were obtained. Western analyses were conducted to determine whether heat-shock induction caused accumulation of appropriately sized ZnF-PcG proteins. ZnF fusion proteins were detected using the FLAG epitope tag positioned at the N terminus of each protein. We found that each producer line accumulated a ZnF-PcG protein of approximately the correct size in extracts prepared from heat-shocked flies (Figure 1B). While the ZnF-PC protein migrated more slowly than expected on the basis of molecular weight predictions, a similar retardation in mobility was observed previously for PC (Breiling *et al.* 1999). Furthermore, the amount of ZnF-PcG protein that accumulated under these conditions was similar for each producer line studied.

Three different reporter transposons, called *SUPor P-lt*, *SUPor P*, and *SUPor P-Blk*, were used to determine whether individually tethered PcG proteins conferred repression of gene expression (Figure 2). Each reporter transposon carried two Su(Hw) BRs and the *yellow* and *white* reporter genes that have been used previously in PcG repression assays (Fauvarque and Dura 1993; Chan *et al.* 1994; Kassis 1994; Gindhart and Kaufman 1995; Sigrist and Pirrotta 1997; Mallin *et al.* 1998). The three reporter transposons differed in two major ways. First, the nature of the *white* gene varied: *SUPor P-lt* carried a *mini-white* gene that lacked the eye enhancer, whereas *SUPor P* and *SUPor P-blk* contained a *mini-white* gene with the eye enhancer inserted either 300 or 700 bp upstream of the *white* promoter, respectively. This difference allowed assessment of ZnF-PcG-mediated repression of either basal or enhancer-activated *white* expression. Second, the position of the Su(Hw) BR relative to the *white* promoter differed (see materials and methods). The variable distances between the tethering sites and promoters allowed an evaluation of distance effects on ZnF-PcG-induced repression. A large collection of characterized *SUPor P*, *SUPor P-blk*, and *SUPor P-lt* lines were available for study (Roseman *et al.* 1993, 1995a,b). In this way, the influence of genomic location on PcG repression was assessed.

Effects of tethered PcG proteins on basal *white* gene expression: Effects of targeted PcG proteins were first examined using lines carrying the *SUPor P-lt* transposon. *SUPor P-lt* flies have a yellow eye color, reflecting a basal level of *white* gene expression due to absence of the eye enhancer (Roseman *et al.* 1993, 1995b). We reasoned that this low level of *white* expression should provide a sensitive system to study the effects of targeted ZnF-PcG proteins. We predicted that if tethered PcG proteins repressed *white* expression, then flies carrying the producer *ZnF-Pc*, *ZnF-Scm*, or *ZnF-Esc* transgenes should have lighter eyes than flies carrying *SUPor P-lt* alone. Furthermore, we expected that flies expressing the *ZnF* transgene should have an unchanged eye phenotype because the Su(Hw) DNA-binding domain lacks silencing activity (Harrison *et al.* 1993; Kim *et al.* 1993, 1996).

In our initial experiments, *SUPor P-lt* flies were crossed to flies containing the *hsp70-ZnF-PcG* producer transgenes and the resulting progeny were heat shocked daily, beginning in late embryogenesis. These initial studies produced two unexpected outcomes. First, no progeny were obtained from any cross that included a *ZnF-Pc* producer transgene, even though two independent *ZnF-Pc* lines were tested. These results suggest that early heat-shock induction of the ZnF-PC fusion protein is lethal. Second, the eye-color phenotype of progeny



Transgene	Protein produced	Predicted size from sequence
ZnF	Su(Hw)ZnF	51KD
ZnF-PC	Su(Hw)ZnF-Polycomb	95KD
ZnF-SCM	Su(Hw)ZnF-Sex comb on midleg	145KD
ZnF-SCM$^{\Delta SPM}$	Su(Hw)ZnF Scm C-terminal deletion	136KD
ZnF-ESC	Su(Hw)ZnF-Extra sex combs	99KD

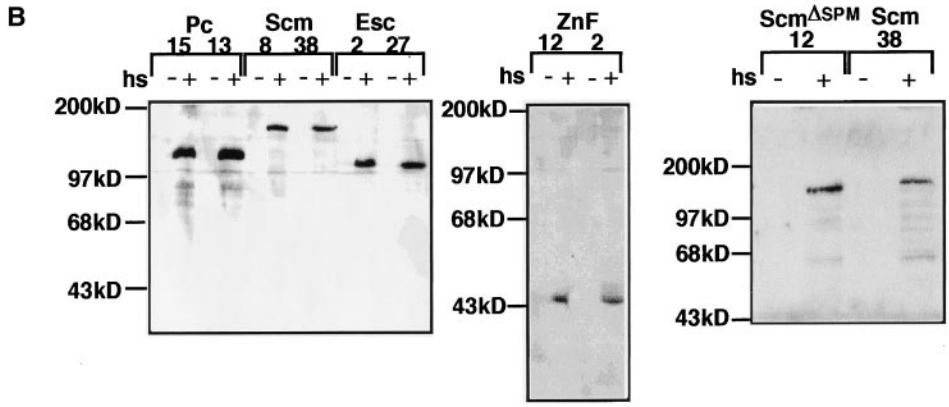


FIGURE 1.—Transgenes and ZnF-PcG fusion proteins used in PcG tethering analysis. (A) Structure of producer transgenes and predicted sizes of the proteins produced. Producer transgenes contained the *hsp70* heat-shock promoter (*hs*), a FLAG protein tag, a *su(Hw)* cDNA fragment encoding the zinc-finger-binding domain [*ZnF*], the α-tubulin polyadenylation sequence [poly(A)], and either no insertion or the insertion of PcG cDNAs (as indicated by the raised triangle). The *vermilion* gene was used as a transformation marker. Open arrowheads indicate *P*-element ends. Small arrows indicate transcription start sites. The protein sizes shown were calculated from predicted amino acid sequences. (B) Western analysis of transgenic ZnF-PcG producer lines. Adults from one or two producer lines (line number shown over lane) carrying independent insertions of the transgene were either heat shocked (hs +) or not heat shocked (hs −) and protein extracts were prepared after a 30- to 60-min recovery. After Western transfer, fusion proteins were visualized using the FLAG antibody. Protein size markers are shown on the left.

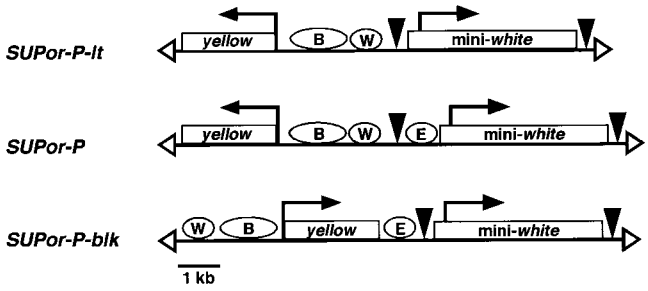
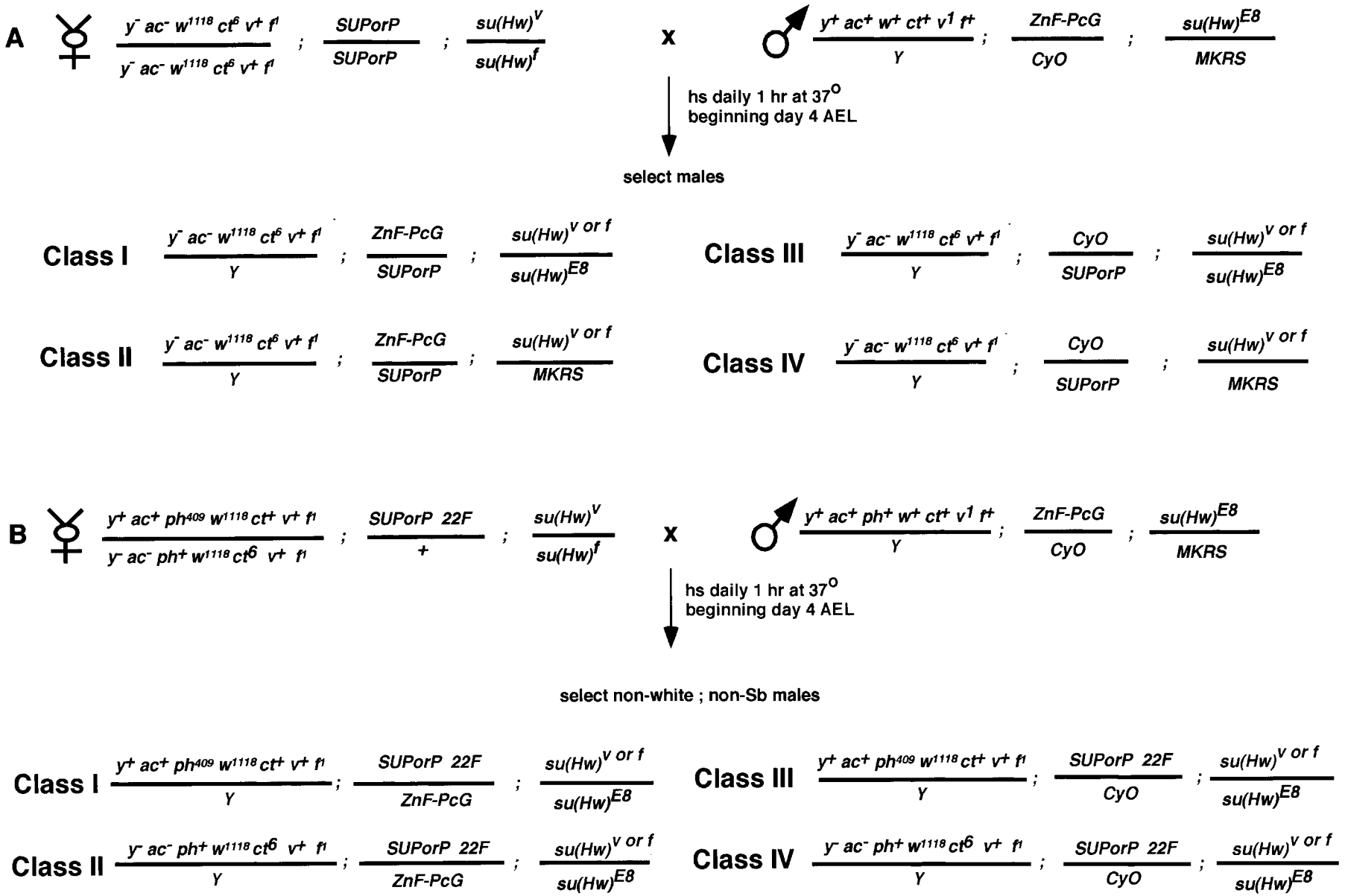


FIGURE 2.—Reporter transgenes used in ZnF-PcG tethering studies. All reporter transgenes contain the *mini-white* gene flanked by two Su(Hw)-binding regions [Su(Hw) BRs; black triangles]. *SUPor P-lt* lacked the *white* eye enhancer (oval marked E), while *SUPor P* and *SUPor P-blk* contained the eye enhancer, positioned either downstream or upstream of a second Su(Hw) BR, respectively. All three transgenes carried the intronless *yellow* gene that contained the wing (oval marked W) and body (oval marked B) enhancers. These transgenes differed in the relative orientation of the *yellow* and *white* genes. The overall structure of the transgenes is drawn to size, except for the *P*-element ends. Other symbols are as indicated in Figure 1.

expressing either ZnF-SCM or ZnF-ESC was the same as that observed in sibling *SUPor P-lt* flies that lacked the producer transgenes, indicating that under these conditions the ZnF-PcG proteins were unable to confer repression (data not shown). We reasoned that the lack of gene silencing might reflect interference by endogenous Su(Hw) protein, which could bind to the *SUPor P-lt* reporter genes in place of the ZnF-PcG proteins.

To test for interference, we used the crossing scheme shown in Figure 3A to determine whether ZnF-PcG proteins could repress *white* expression from *SUPor P-lt* in a *su(Hw)*$^{-}$ background. We found that production of the ZnF-PcG proteins repressed *white* expression in the majority of *SUPor P-lt* lines tested (Figure 4 and Table 1), whereas ZnF alone never altered *white* expression levels. These data indicate that the tethered PcG proteins, positioned 300 bp upstream of the basal *white* promoter, confer gene silencing. Furthermore, we infer that our initial failure to observe *white* repression in *su(Hw)*$^{+}$ flies was due to competition between the PcG fusion proteins and the endogenous Su(Hw) protein for *SUPor P-lt* binding, perhaps because Su(Hw) protein


A
y- ac- w1118 ct6 v+ f1 / y- ac- w1118 ct6 v+ f1 ; SUPorP / SUPorP ; su(Hw)V / su(Hw)f
x
y+ ac+ w+ ct+ v1 f+ / Y ; ZnF-PcG / CyO ; su(Hw)E8 / MKRS
hs daily 1 hr at 37°
beginning day 4 AEL
select males
Class I y- ac- w1118 ct6 v+ f1 / Y ; ZnF-PcG / SUPorP ; su(Hw)V or f / su(Hw)E8
Class II y- ac- w1118 ct6 v+ f1 / Y ; ZnF-PcG / SUPorP ; su(Hw)V or f / MKRS
Class III y- ac- w1118 ct6 v+ f1 / Y ; CyO / SUPorP ; su(Hw)V or f / su(Hw)E8
Class IV y- ac- w1118 ct6 v+ f1 / Y ; CyO / SUPorP ; su(Hw)V or f / MKRS
B
y+ ac+ ph409 w1118 ct+ v+ f1 / y- ac- ph+ w1118 ct6 v+ f1 ; SUPorP 22F / + ; su(Hw)V / su(Hw)f
x
y+ ac+ ph+ w+ ct+ v1 f+ / Y ; ZnF-PcG / CyO ; su(Hw)E8 / MKRS
hs daily 1 hr at 37°
beginning day 4 AEL
select non-white ; non-Sb males
Class I y+ ac+ ph409 w1118 ct+ v+ f1 / Y ; SUPorP 22F / ZnF-PcG ; su(Hw)V or f / su(Hw)E8
Class II y- ac- ph+ w1118 ct6 v+ f1 / Y ; SUPorP 22F / ZnF-PcG ; su(Hw)V or f / su(Hw)E8
Class III y+ ac+ ph409 w1118 ct+ v+ f1 / Y ; SUPorP 22F / CyO ; su(Hw)V or f / su(Hw)E8
Class IV y- ac- ph+ w1118 ct6 v+ f1 / Y ; SUPorP 22F / CyO ; su(Hw)V or f / su(Hw)E8

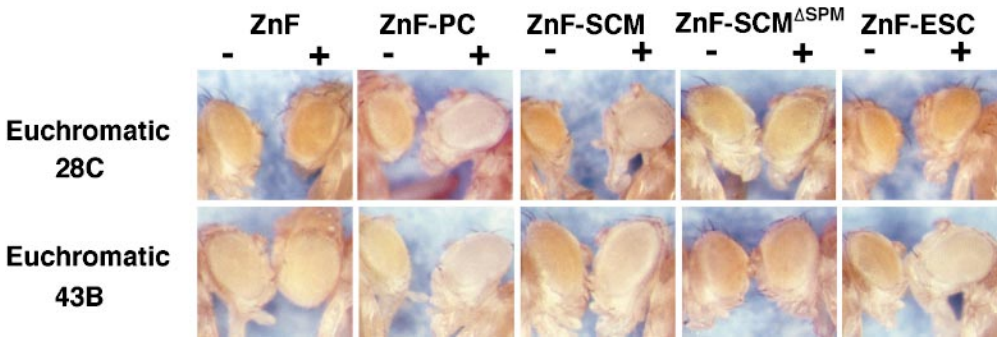


FIGURE 4.—Effects of tethered ZnF-PcG proteins on *white* expression from *SUPor P-lt* reporters. The eye phenotypes of flies carrying an insertion of the reporter transposon at two different genomic positions (28C and 43B) that contained (+) or lacked (−) the indicated ZnF-PcG protein are shown. The genomic location of the *SUPor P-lt* reporter influenced whether some (28C) or all (43B) of the ZnF-PcG proteins caused silencing.

is present at higher steady-state levels than any of the transiently supplied ZnF-PcG proteins. For this reason, all subsequent analyses were conducted in a *su(Hw)*$^-$ background.

Heat-shock induction of the *ZnF-Pc* producer was not required for *white* repression by the ZnF-PC protein. Apparently, uninduced levels of ZnF-PC expression were sufficient to mediate repression in our assay system. For this reason, as well as to minimize toxic effects of ZnF-PC overproduction, further experiments specifically employing the *ZnF-Pc* producers were performed without heat-shock induction. In contrast, repression of *white* expression by ZnF-SCM or ZnF-ESC was only observed when these fusion proteins were induced daily (data not shown), indicating that a constant presence of these ZnF-PcG proteins was required. We conclude that the ZnF-SCM and ZnF-ESC proteins decay with half-lives shorter than 24 hr and/or may be reduced in abundance during cell proliferation, necessitating a daily input of newly synthesized protein. Thus, for experiments with the *ZnF-Scm* and *ZnF-Esc* producers, we settled upon a regimen of 1-hr heat shocks administered once per day beginning on the 4th day after embryo deposition.

Although all three ZnF-PcG proteins repressed *white* expression from *SUPor P-lt*, the degrees of repression observed were not equivalent (Table 1). We found that ZnF-PC had the strongest impact on *white* expression, while ZnF-ESC had the weakest. This conclusion is based on two criteria: (1) the percentage of independent *SUPor P-lt* integration sites that were repressed by each ZnF-PcG protein and (2) the level of *white* expression associated with each tethered protein. For example, production of ZnF-PC silenced *white* expression at all seven of the *SUPorP-lt* insertion sites tested, whereas only two of the six tested *SUPorP-lt* insertions were repressed by ZnF-ESC (Table 1). Also, while ZnF-PC tethering caused complete repression of *white* expression at all *SUPor P-lt* insertion sites, *white* expression was reduced but not eliminated by targeted ZnF-SCM at some of the same sites.

Effects of tethered PcG proteins on enhancer-activated gene expression: To determine whether an enhancer would influence the repression conferred by tethered ZnF-PcG proteins, we examined the eye phenotypes of flies carrying either the *SUPor P* or *SUPor P-blk* reporters and one of the producers. The *SUPor P* and *SUPor P-blk* reporters differ in the location of the 5′ *white* eye enhancer (Figure 2). As several *SUPor P* and *SUPor P-blk* lines were available that carried insertions into centric or telomeric heterochromatin, we further addressed whether genomic regions with distinct chromatin structure influenced PcG repression.

The effects of targeted ZnF-PcG proteins at 22 independent euchromatic sites of *SUPor P* (15 sites) and *SUPor P-blk* (7 sites) were determined. In the absence of Su(Hw) protein, most flies carrying euchromatic insertions of either transposon have a red eye color, with exceptional euchromatic lines showing reduction of *white* expression due to a repressive position effect (ROSEMAN *et al.* 1995a). We found that the effects of tethered ZnF-PcG proteins on enhancer-activated *white*

FIGURE 3.—Genetic crossing schemes used in ZnF-PcG studies. (A) Crosses used to study the effect of tethered ZnF-PcG fusion proteins on *white* and *yellow* gene expression. For illustration, the crosses are shown with a *SUPor P* reporter gene located on the second chromosome. Only male progeny were examined. Class I are *su(Hw)*$^-$ males that carry both the reporter and producer transgenes, while class III are *su(Hw)*$^-$ males that carry only the reporter gene. The eye and cuticle phenotypes were compared to determine the effects of the ZnF-PcG protein. The other two classes served as negative controls. (B) Crosses used to assay the effect of a *ph* mutation on repression due to the tethered ZnF-PcG proteins. Only the four classes of *su(Hw)*$^-$ male progeny that carried the 22F *SUPor P* reporter gene are shown. These males were identified as non-white-eyed, non-Sb progeny. The eye phenotype of the class I ph^{409} males (identified by wild-type bristle pigmentation) and class II ph^+ males (identified by the lack of bristle pigmentation) were compared. Classes III and IV served as controls for the effects of the *ph* background on *white* expression in the *SUPor P* reporter.

TABLE 1

Percentage of reporter lines carrying any of three different transposons showing repression of *white* gene expression in response to ZnF-PcG fusion proteins

	ZnF	ZnF-PC	ZnF-SCM	ZnF-SCM$^{\Delta SPM}$	ZnF-ESC
SUPorP-lt					
Euchromatic	0 (0/6)	100 (6/6)	100 (5/5)	0 (0/5)	20 (1/5)
Telomeric	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
SUPorP					
Euchromatic	0 (0/14)	92 (12/13)	69 (9/13)	0 (0/8)	23 (3/13)
Euchromatic P.E.[a]	0 (0/2)	100 (1/1)	100 (2/2)	0 (0/1)	100 (2/2)
Telomeric	0 (0/6)	100 (6/6)	100 (6/6)	0 (0/3)	84 (5/6)
Centric, 4th	0 (0/4)	100 (3/3)	100 (4/4)	0 (0/1)	50 (2/4)
SUPorP-blk					
Euchromatic	0 (0/6)	100 (5/5)	100 (6/6)	0 (0/4)	50 (3/6)
Euchromatic P.E.	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)

Values in parentheses indicate the number of lines showing repression out of the total number of lines studied.

[a] P.E., position effects, transposons inserted in genomic locations that have a negative effect on gene expression.

transcription were similar to those observed for basal *white* transcription (Figure 5 and Table 1). Both the ZnF-PC and ZnF-SCM proteins showed robust repression of *SUPor P* and *SUPor P-blk white* expression at the vast majority of euchromatic sites tested, while the ZnF-ESC repression was more sensitive to the integration site of the reporter gene. ZnF alone had no effect on *white* expression from either transposon. In total, we found that *white* expression decreased at 95% (19/20) of the euchromatic sites tested for ZnF-PC, 82% (18/22) for ZnF-SCM, and 41% (9/22) for ZnF-ESC (Table 1). In general, the level of *white* expression at a given insertion site was lowest when ZnF-PC was tethered to the euchromatic *SUPor P* or *SUPor P-blk* reporter genes. While some of these sites showed a complete loss of *white* expression, the most commonly observed eye phenotype was a yellow eye color for ZnF-PC-expressing flies and an orange eye color for ZnF-SCM- and ZnF-ESC-expressing flies (Figure 5, data not shown). At insertion sites that displayed repression by both ZnF-PC and ZnF-SCM, the ZnF-PC protein reduced *white* expression more than ZnF-SCM at 61% (11/18) of the sites, showed the same degree of repression at 22% (4/18) of the sites, and showed less repression at 17% (3/18) of the sites (data not shown). In addition, silencing by ZnF-ESC at a given genomic location never exceeded that of the other ZnF-PcG proteins. These data indicate that tethered ZnF-PcG proteins can silence both enhancer-activated and basal *white* transcription. Since tethering occurred at distances of 1.0 kb upstream of the *white*

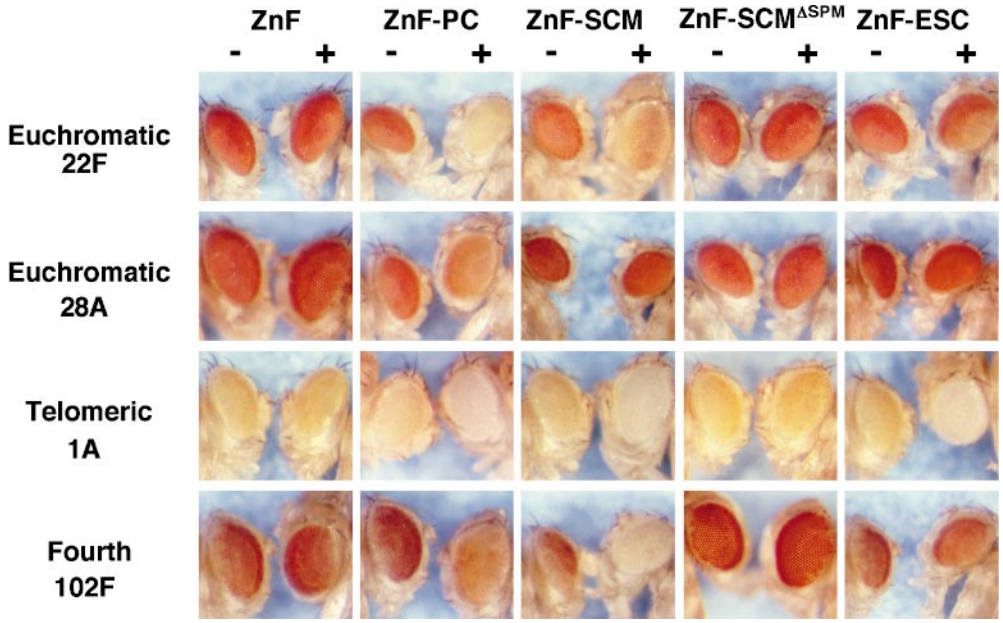


FIGURE 5.—Effects of tethered ZnF-PcG proteins on *white* expression from *SUPor P* reporters. The eye phenotypes of flies carrying an insertion of the reporter transposon at four different genomic positions (22F, 28A, 1A, and 102F) that contained (+) or lacked (−) the indicated ZnF-PcG protein are shown.

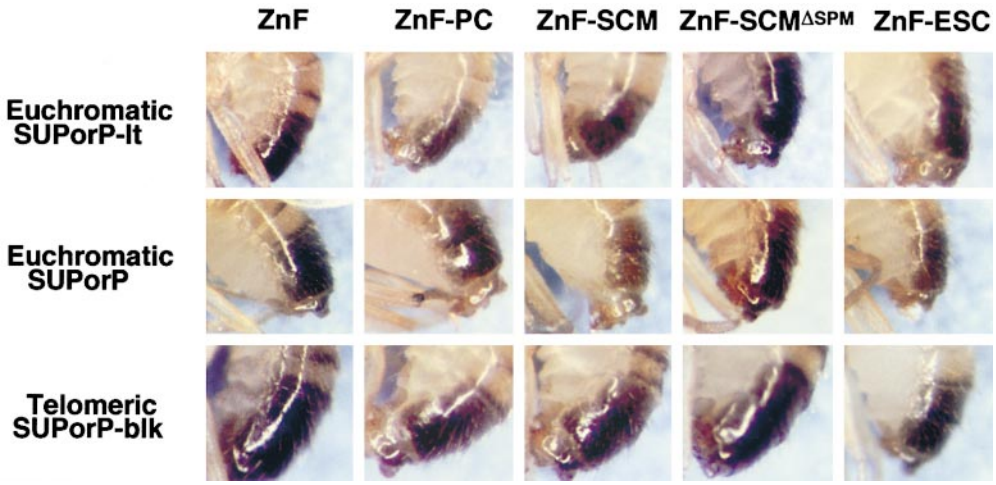


FIGURE 6.—Effects of tethered ZnF-PcG proteins on *yellow* gene expression from the *SUPor P-lt*, *SUPor P*, and *SUPor P-blk* reporters. The body phenotypes of flies carrying the indicated reporter and one of the ZnF-PcG proteins are shown.

promoter in the *SUPor P* reporter lines, our results demonstrate that ZnF-PcG silencing can be conferred over long distances.

The effects of the ZnF-PcG proteins on *white* gene expression in 10 heterochromatic *SUPor P* insertion sites were examined (Table 1). In a *su(Hw)*$^-$ background, *SUPor P* flies carrying insertions into centric or telomeric chromatin have eye phenotypes that range from a variegated red pigmentation to a light yellow color (ROSEMAN *et al.* 1995a). We found that all three ZnF-PcG proteins reduced *white* expression in lines carrying heterochromatic insertions, while ZnF alone had no effect (Figure 5). Targeting of ZnF-PcG proteins to these heterochromatic sites produced more consistent repression of gene expression than seen at euchromatic sites (Figure 5 and Table 1). This was especially notable for ZnF-ESC, where the percentage of repression of *SUPor P white* expression increased from 33% (5/15) in euchromatic insertion lines to 70% (7/10) in heterochromatic lines (Table 1). Taken together, these data indicate that tethered ZnF-ESC may not be as intrinsically strong a silencing protein as the others tested. In addition, ESC may repress more effectively at genomic sites where cooperation with local silencing proteins is possible.

Effects of tethered PcG proteins on *yellow* gene expression: To determine whether the type of promoter influenced ZnF-PcG silencing, we examined the effects of these proteins on *yellow* gene expression. The *yellow* promoter contains TATA and Initiator (Inr) regulatory sequences (GEYER and CORCES 1987; MORRIS *et al.* 1999), whereas the *white* promoter contains an Inr and downstream promoter element (DPE; OHTSUKI *et al.* 1998), but lacks a TATA sequence.

The *SUPor P-lt*, *SUPor P*, and *SUPor P-blk* transposons carry a *mini-yellow* gene that is expressed in the wing and body tissues and not in the bristles, due to lack of the intronic bristle enhancer (GEYER and CORCES 1987; MARTIN *et al.* 1989). We found that all three ZnF-PcG proteins reduced *yellow* gene expression, with the degree of repression dependent upon which tissue was examined (Figure 6 and Tables 2 and 3). Expression in the body cuticle was less sensitive to repression than in the wing cuticle. For example, among euchromatic insertion sites, ZnF-PC caused silencing at 73% (11/15) of the *SUPor P* and *SUPor P-lt* sites in the body tissue, in contrast to 93% (14/15) in the wing tissue (Tables 2 and 3). The percentage of lines of *SUPor P-blk* that showed *yellow* repression was lower relative to the other two reporters. The basis for this difference is unclear, but may reflect the smaller number of insertion sites tested. The fact that both *yellow* and *white* gene expression were repressed demonstrates that the three tethered PcG proteins can each silence promoters built from different combinations of core DNA elements.

Effects of the ZnF-PcG proteins on *yellow* expression at heterochromatic sites were similar to those observed for *white*. In general, ZnF-PcG proteins tethered at heterochromatic insertion sites more frequently repressed gene expression than when tethered to euchromatic sites (Tables 2 and 3).

Repression by tethered SCM requires the SPM interaction domain: SCM contains a highly conserved 65-amino-acid carboxyl-terminal motif, the SPM domain, that is required for SCM function (BORNEMANN *et al.* 1996; A. PETERSON and J. SIMON, unpublished results). While this domain can mediate heterotypic and homotypic interactions between the SCM and PH proteins *in vitro* (PETERSON *et al.* 1997), its role in repression *in vivo* is unclear. This domain may play a primary role in targeting SCM to the chromosome or it may be directly required for the repressive activity and/or integrity of SCM complexes. To distinguish between these possibilities, we examined whether tethered SCM lacking the SPM domain conferred silencing of the *white* and *yellow* genes. We reasoned that if this domain was solely required to target SCM to chromosomes, then a tethered ZnF-SCM protein lacking this domain should silence gene expression to a similar extent as wild-type protein because the ZnF domain would provide an alternative entry route to the chromosome.

TABLE 2

Percentage of reporter lines carrying any of three different transposons showing repression of *yellow* gene expression in the body cuticle in response to ZnF-PcG fusion proteins

	ZnF	ZnF-PC	ZnF-SCM	ZnF-SCM$^{\Delta SPM}$	ZnF-ESC
SUPor P-lt					
Euchromatic	0 (0/6)	67 (4/6)	33 (2/6)	0 (0/6)	33 (2/6)
Telomeric	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
SUPor P					
Euchromatic	0 (0/8)	75 (6/8)	38 (3/8)	0 (0/4)	13 (1/8)
Euchromatic P.E.[a]	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
Telomeric	0 (0/4)	100 (4/4)	100 (4/4)	0 (0/3)	75 (3/4)
SUPor P-blk					
Euchromatic	0 (0/5)	25 (1/4)	20 (1/5)	0 (0/4)	0 (0/5)
Euchromatic P.E.	0 (0/1)	100 (1/1)	100 (1/1)	NT[b]	100 (1/1)

Values in parentheses indicate the number of lines showing repression out of the total number of lines studied.

[a] P.E., position effects, transposons inserted in genomic locations that have negative effects on gene expression.

[b] NT, not tested.

Producer lines that expressed a truncated ZnF-SCM protein with a deletion of the last 80 amino acids, including the SPM domain, were generated (Figure 1). Western analysis demonstrated that the *hsp70-ZnF-Scm$^{\Delta SPM}$* producer lines accumulated protein with a slightly lower molecular weight than ZnF-SCM, but at a similar level (Figure 1B). The effect of ZnF-SCM$^{\Delta SPM}$ on gene expression was determined by crossing a *hsp70-ZnF-Scm$^{\Delta SPM}$* producer line to lines containing each of the three reporter transposons and examining the *white* and *yellow* phenotypes of the resulting progeny. In all cases, we found that the phenotype of the ZnF-SCM$^{\Delta SPM}$ progeny was indistinguishable from that of flies expressing ZnF alone (Figures 4–6 and Tables 1–3). These results show that the ZnF-SCM$^{\Delta SPM}$ protein does not confer repression in the tethering assay and suggest that the SPM domain is required for a gene-silencing function rather than for shepherding SCM to target loci. In addition, the requirement for the small SPM domain verifies that the tethering system recapitulates normal mechanisms of PcG repression.

Effects of alterations in PH dosage upon ZnF-PcG repression: One of the best-characterized molecular partnerships among PcG proteins is the interaction between PC and PH. These two PcG proteins show completely coincident localization patterns on chromosomes (Franke *et al.* 1992), they interact directly *in vitro* (Kyba and Brock 1998b), and they copurify from embryo extracts in the PRC1 complex (Shao *et al.* 1999). The mammalian PC and PH homologues are also physi-

TABLE 3

Percentage of reporter lines carrying any of three different transposons showing repression of *yellow* gene expression in the wing cuticle in response to ZnF-PcG fusion proteins

	ZnF	ZnF-PC	ZnF-SCM	ZnF-SCM$^{\Delta SPM}$	ZnF-ESC
SUPor P-lt					
Euchromatic	0 (0/6)	83 (5/6)	83 (5/6)	0 (0/6)	67 (4/6)
Telomeric	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
SUPor P					
Euchromatic	0 (0/8)	100 (8/8)	88 (7/8)	0 (0/4)	50 (4/8)
Euchromatic P.E.[a]	0 (0/1)	100 (1/1)	100 (1/1)	0 (0/1)	100 (1/1)
Telomeric	0 (0/4)	100 (4/4)	100 (4/4)	0 (0/3)	75 (3/4)
SUPor P-blk					
Euchromatic	0 (0/5)	75 (3/4)	60 (3/5)	0 (0/4)	80 (4/5)
Euchromatic P.E.	0 (0/1)	100 (1/1)	100 (1/1)	NT[b]	100 (1/1)

Values in parentheses indicate the number of lines showing repression out of the total number of lines studied.

[a] P.E., position effects, transposons inserted in genomic locations that have negative effects on gene expression.

[b] NT, not tested.

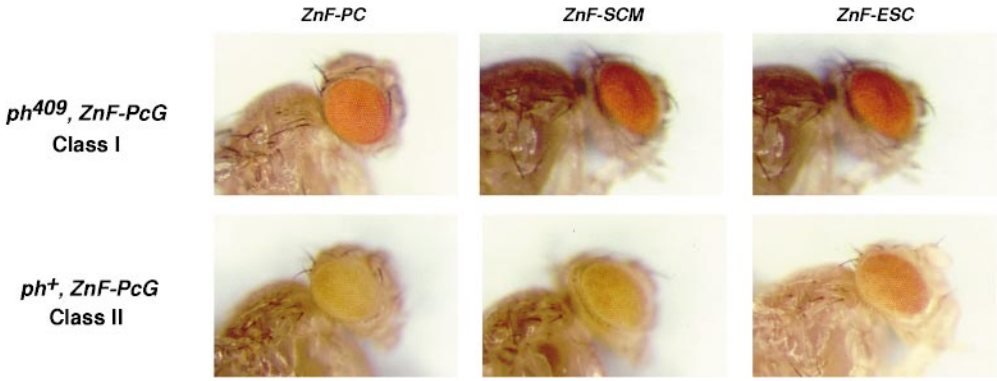


FIGURE 7.—Effects of the ph^{409} mutation on repression conferred by tethered ZnF-PcG proteins. The top row shows the eye phenotypes of class I flies carrying the ph^{409} mutation and one of the ZnF-PcG proteins. The bottom row shows the eye phenotypes of class II flies carrying the ph^{+} allele and one of the three ZnF-PcG proteins.

cally associated in multimeric complexes (ALKEMA *et al.* 1997b; GUNSTER *et al.* 1997; HASHIMOTO *et al.* 1998). This partnership prompted us to examine whether endogenous PH might be required for repression by tethered PC in our system.

To explore this possibility, we determined whether repression by ZnF-PC was compromised when levels of endogenous PH were reduced. We could not examine the effects of a complete loss of PH on ZnF-PC repression since our reporter genes were assayed in adults and *ph* null mutations are embryonic lethal. As a result, we used the ph^{409} mutation (DURA *et al.* 1987), which is a hemizygous viable allele. Since ph^{409} deletes the proximal of the two *ph* genomic copies, it reduces, but does not eliminate, the *ph* gene product. To test the effects of ph^{409} on ZnF-PC-mediated repression, we selected a *SUPor P* reporter line with an insert at the genomic location 22F. This site was chosen because it is not a normal PH-binding site (DECAMILLIS *et al.* 1992; FRANKE *et al.* 1992), as we wished to avoid contributions from endogenous PH prepositioned nearby on the chromosome. It also is an example of a euchromatic site that showed silencing by all three ZnF-PcG proteins (Figure 5, top row).

Genetic crosses involved in the ph^{409} test are outlined in Figure 3B. Females were constructed that were $su(Hw)^{-}$ and simultaneously heterozygous for both the ph^{409} mutation and for the 22F *SUPor P* reporter. These females were crossed to $su(Hw)^{-}/su(Hw)^{+}$ males carrying a *ZnF-Pc* producer construct and the eye phenotypes of ph^{409}*; SUPor P/ZnF-Pc;* $su(Hw)^{-}$ male progeny (class I) were compared with the ph^{+}*; SUPor P/ZnF-Pc;* $su(Hw)^{-}$ siblings (class II) and with siblings lacking the *ZnF-Pc* producer (classes III and IV). In the control ph^{+} flies (class II), ZnF-PC targeted to the 22F *SUPor P* caused strong silencing of *white* expression, reducing pigmentation to a light orange color (Figure 7). In contrast, the majority of ZnF-PC flies in the ph^{409} mutant background had wild-type eye color (71%, 39/55), with the remainder showing dark orange and/or variegated eye colors (Figure 7 and Table 4). These results show that, at the reporter site tested, repression by ZnF-PC requires PH function.

We tested whether repression by tethered ZnF-SCM and ZnF-ESC proteins was also sensitive to PH dosage. Crosses were performed as described above for ZnF-PC (Figure 3B), except that males contained either a *ZnF-Scm* or *ZnF-Esc* producer (Table 4). We found that the eye color of nearly all class I ph^{409}*; SUPor P/ZnF-Scm;* $su(Hw)^{-}$ flies was wild type (19/20), in contrast to the class II ph^{+}*; SUPor P/ZnF-Scm;* $su(Hw)^{-}$ siblings that had reduced and variegated eye pigmentation (Figure 7 and Table 4). These data imply that PH is also needed for the ZnF-SCM-conferred silencing of *white*. Surprisingly, targeted ZnF-ESC repression was also sensitive to a reduction in PH dosage. Although *white* repression by ZnF-ESC at 22F was more subtle (Figure 5), the ph^{409} mutation reversed this silencing in the majority (15/16) of the class I progeny (Figure 7 and Table 4). Although there is evidence for a molecular partnership *in vivo* or *in vitro* between PH and both PC and SCM, molecular links between PH and ESC have not been described. Yet, at least at this single genomic site, repression by all three tethered PcG proteins tested depended upon dosage of the PRC1 component PH.

DISCUSSION

Timing of ZnF-PcG expression: A targeting assay was developed to compare and contrast the effects of PC, SCM, and ESC on gene expression *in vivo*. In this system, fusion proteins containing the amino-terminal Su(Hw) DNA-binding domain (ZnF) and a carboxyl-terminal PcG protein were expressed from the inducible *hsp70* promoter and effects of each ZnF-PcG protein on *white* and *yellow* gene expression were studied. All analyses were conducted in a $su(Hw)^{-}$ background to prevent the endogenous Su(Hw) protein from masking the effects of the ZnF-PcG proteins. We found that expression of the *white* and *yellow* genes was silenced by tethered ZnF-PcG proteins at most of the genomic sites tested.

Repression by ZnF-SCM or ZnF-ESC required a continuous supply of ZnF-PcG proteins that involved heat-shock induction once per day beginning in late larval development. A single pulse of these ZnF-PcG proteins, either during embryogenesis or during larval develop-

TABLE 4

Effect of the ph^{409} mutation on the eye-color phenotype of four classes of *su(Hw)*$^-$, *SUPor P* male progeny generated by crossing heterozygous ph^{409} females with the indicated *ZnF-PcG* producers

		Class I	Class II	Class III	Class IV
Producer	Total male progeny	ph^{409}, *ZnF-Pc*	ph^+, *ZnF-PcG*	ph^{409}, *no ZnF-PcG*	ph^+, *no ZnF-PcG*
ZnF	452	Red (37)	Red (40)	Red (6)	Red (23)
ZnF-Scm	928	Red (19) Dk. or. (1)	Yell.-or. var (65)	Red (28)	Red (58)
ZnF-Pc	1506	Red (39) Dk. or. (9) Dk. or. var. (7)	Lt. or. (99)	Red (46)	Red (77)
ZnF-Esc	577	Red (15) Dk. red (1)	Dk. or. -red (43) Dk. Red (5)	Red (24)	Red (27)

Values in parentheses indicate the number of flies scored. A wild-type level of *white* expression produces flies with red eyes. Dk., dark; or., orange; yell., yellow; lt., light; var., variegated. The level of decreasing expression corresponding to eye color is red> dk. red> dk. or.> lt. or> yell.-or.

ment, did not yield reporter repression as scored in adults. A similar restriction could not be assessed for ZnF-PC because heat shock was not needed to confer silencing. In a previous study (Muller 1995), long-term repression of some reporters was achieved by transient production of tethered PC and was hypothesized to reflect an interaction between tethered PC and PREs present on these constructs. In contrast, the present study used naïve constructs that lacked PREs. Taken together, data from both studies reinforce proposals that the PcG-silenced state is not templated once and then maintained in the local chromatin, but that it requires continuous PRE function for maintenance during development (Busturia *et al.* 1997).

In our assay system, the phenotypes that were studied reflected the state of gene activity of the *white* and *yellow* genes during pupal development. The fact that tethered PC and SCM repressed these genes is consistent with the normal requirements for these PcG proteins during this late developmental stage (Duncan 1982; Wu *et al.* 1989; Bornemann *et al.* 1998). In contrast, ESC function is critical for embryonic but not pupal viability (Struhl 1981; Struhl and Brower 1982; Simon *et al.* 1995) and ESC is much more abundant in embryos than in pupae (Frei *et al.* 1985; Sathe and Harte 1995; Ng *et al.* 2000). In light of these developmental differences, it is notable that tethered ESC conferred silencing during larval/pupal development. Our data imply that protein partners involved in ESC repression are present and functional late in development.

Repression conferred by each ZnF-PcG protein differed: We found that a major determinant of repression was the identity of the tethered ZnF-PcG protein. This conclusion is based upon two observations. First, the percentage of genomic sites that were repressed was distinct for each ZnF-PcG protein tested. For example, the percentage of euchromatic lines showing *white* repression was 96% for ZnF-PC, 85% for ZnF-SCM, and 35% for ZnF-ESC. Second, the degree of reduction in *white* expression was specific for each ZnF-PcG protein, with ZnF-PC causing the greatest reduction in pigment levels and ZnF-ESC producing the least. Thus, in our assay system, ZnF-PC behaved as a stronger general repressor than ZnF-SCM, which was stronger than ZnF-ESC. Interestingly, the strong ZnF-PC effects were observed in the absence of heat-shock induction for two independent producer lines. It is possible that these producers were activated in a stage- or tissue-specific manner not monitored by our Western analyses due to genomic position effects. However, a more likely explanation is that low levels of ZnF-PC protein produced by leaky transcription from the *hsp70* promoter are sufficient to establish silencing.

Several possibilities may account for the observed differences in repression strengths between the three ZnF-PcG proteins. First, these ZnF-PcG proteins may have different capacities to assemble into complexes with endogenous PcG proteins. This may reflect an intrinsic difference between these proteins or may result from changes in protein behavior because of the addition of the Su(Hw) domain. For example, the much lower effectiveness observed for ZnF-ESC may indicate that the Su(Hw) fusion compromised the function or folding of the ESC protein. This possibility is unlikely since the fusion occurs at the extreme N terminus of ESC, which can tolerate addition of epitope tags (Jones and Gelbart 1990; Tie *et al.* 1998) and which is outside of the region that contributes to the β-propeller folding domain (Ng *et al.* 1997). Second, the ZnF-PcG proteins may have different abilities to recruit other complexes. Third, each ZnF-PcG protein may possess a different intrinsic repression activity. We note that, among the PcG proteins, PC has the capacity to bind directly to nucleosomes *in vitro* (Breiling *et al.* 1999). Thus, this protein may produce more effective gene silencing.

Effects of enhancers and promoters on ZnF-PcG si-

lencing: The influence of enhancer action on PcG repression was assessed by comparing the enhancer-containing *SUPor P* and *SUPor P-blk* reporter lines with the enhancerless *SUPor P-lt* lines. We found that there was a slight decrease in the percentage of enhancer-activated *white* lines that were repressed by ZnF-PC and ZnF-SCM, as compared with lines carrying a basal *white* promoter, while the percentage of enhancer-activated *white* lines repressed by ZnF-ESC actually increased. Taken together, these data indicate that *white* eye enhancer had little effect on ZnF-PcG silencing.

Tethered ZnF-PcG proteins also reduced enhancer-activated transcription of the *yellow* gene. The percentage of reporter lines showing repression of *yellow* expression in the wing cuticle was higher than in the body. This difference may simply reflect the degree of reduction of *yellow* expression required to produce an observable phenotype in these tissues. Alternatively, these data may indicate that ZnF-PcG complexes assembled in the vicinity of one enhancer do not spread uniformly to silence all linked enhancers and promoters. On the basis of previous tethering experiments, it was suggested that complex control regions subject to PcG repression have promoters and enhancers that are individually equipped with PREs to mediate silencing (Muller 1995). Our data are consistent with this proposal.

Drosophila promoters generally contain three conserved elements, the TATA box located 25–30 bp upstream of the transcription start site, the Inr centered around the start site, and the DPE located around 30 bp downstream of the start site (Arkhipova 1995; Burke and Kadonaga 1997; Smale 1997). Examination of the *white* and *yellow* promoter regions indicates that these promoters fall into different classes with respect to these elements. The *white* promoter is an example of a class II promoter because it lacks a TATA box and has an Inr and DPE, while the *yellow* promoter is an example of a class I promoter because it contains a TATA box and Inr. Repression of *yellow* transcription in the wing was qualitatively as strong as that observed for *white* repression, as judged by the percentage of genomic sites that showed repression of either gene. These results imply that both classes of promoters are susceptible to PcG repression.

An increased distance between the tethered ZnF-PcG proteins and the enhancer or promoter caused a slight decrease in repression. For example, the percentage of euchromatic insertions showing *white* repression by ZnF-PC or Zn-SCM was 100% when the tethering site was 300 bp upstream of the promoter, but dropped to 92 and 69%, respectively, when the tethering site was 1.0 kb away. Even so, consistent silencing was obtained by both ZnF-PC and ZnF-SCM at distances up to 3.0 kb. These results reinforce the notion, originally suggested by the large sizes and complexities of homeotic gene regulatory regions, that PcG proteins are long-range repressors that act over distances >500 bp (Gray and Levine 1996). The analysis of individually tethered PcG proteins establishes that PC, SCM, and ESC can each mediate long-range repression.

Influence of the genomic insertion site on ZnF-PcG silencing: Reporter transposons inserted at nearly 40 independent genomic locations were studied. It is important to note that these transgenic lines represented an unbiased collection that was obtained in the absence of any PRE sequences or PcG protein association. Thus, our findings that the majority of genomic insertion sites were repressed by ZnF-PC and ZnF-SCM are notable. ZnF-PC silenced nearly 90% of all insertion lines and ZnF-SCM silenced gene expression in ~80% of all lines. Interestingly, our data suggest that silencing by tethered PC and SCM is less dependent on genomic position than has been observed previously for constructs with *bona fide* PREs (Chan *et al.* 1994). This more widespread silencing by the ZnF-PcG proteins may reflect differences between the mechanisms employed to target the ZnF-PcG and endogenous PcG proteins to DNA. For example, ZnF-PcG tethering employs a Su(Hw) BR that contains a cluster of binding sites and interactions between recruited ZnF-PcG complexes that could help stabilize chromosomal association. Furthermore, each reporter transposon carried two Su(Hw) BRs (Figure 2), providing additional opportunities for ZnF-PcG interactions.

Telomeric and centric regions of the genome appeared to cooperate with tethered ZnF-PcG proteins to confer silencing. This is best illustrated by considering silencing of *white* expression by ZnF-ESC. Only 30% (7/24) of the euchromatic lines were repressed by ZnF-ESC, as compared to 73% (8/11) of the heterochromatic insertion lines (Table 1). This effect is not due to the inherently low basal levels of *white* expression at heterochromatic locations, because a similar low level of expression levels occurs for euchromatic *SUPor P-lt* lines, yet only 20% (1/5) of these were repressed by ZnF-ESC. It may be that association of ZnF-ESC in a region already rich in silencing complexes enhances its repressive effects. Similar trends were seen for both ZnF-PC and ZnF-SCM, suggesting that cooperation with molecular components in telomeric and centric regions may be a general feature of PcG repression.

Requirement for PH at a site repressed by all three ZnF-PcG proteins: Biochemical studies indicate that PcG repression involves multiple, distinct PcG complexes (Franke *et al.* 1992; Strutt and Paro 1997; Shao *et al.* 1999; Ng *et al.* 2000). Thus, an underlying assumption of our assay system is that gene silencing by the tethered ZnF-PcG protein involves assembly with endogenous PcG proteins at the reporter site. This hypothesis leads to the prediction that repression by a tethered ZnF-PcG protein should be compromised by loss of function for an endogenous PcG partner. It was difficult to test this prediction for the comprehensive set of endogenous PcG proteins because the basic assay

system involved generating a very complex genotype (Figure 3). Nevertheless, we investigated the requirement for endogenous PH protein, which is encoded by an *X*-linked gene and for which a hemizygous viable allele is available.

We identified a reporter integration site that normally lacks PH binding, as scored on polytene chromosomes (Franke *et al.* 1992), and that was repressed by all three ZnF-PcG proteins. Genetic tests showed that reduction in PH dosage relieved tether-based repression by PC and SCM at this site (Figures 3 and 7 and Table 4). These results can be reconciled with the PC-PH and SCM-PH molecular interactions described previously (Peterson *et al.* 1997; Kyba and Brock 1998a; Shao *et al.* 1999). Surprisingly, we also found that ZnF-ESC repression was sensitive to PH dosage (Figure 7). This result was not expected since ESC-PH interactions have not been reported and there is evidence that ESC and PH are in separate complexes in embryos (Shao *et al.* 1999; Ng *et al.* 2000).

Several explanations may account for the effect of PH dosage upon ZnF-ESC repression. First, since only a single reporter site was investigated, the PH dependency at this site may not be a general property at other genomic sites. We note, however, that this reporter site was chosen for analysis because polytene chromosome immunostaining studies indicate that it is not preequipped with endogenous PH (DeCamillis *et al.* 1992). Alternatively, it is possible that the functions of biochemically separable PcG complexes are interdependent *in vivo*, at least at certain loci. This could also explain the basic observation that PC and ESC are both required for repression at homeotic loci even though they sort into distinct complexes. An excellent example of the interplay between distinct chromatin complexes at a single locus is provided by regulation of the *HO* gene in yeast. Both the SWI/SNF nucleosome remodeling complex and the SAGA histone acetyltransferase complex are required for *HO* activation *in vivo* (Cosma *et al.* 1999). These complexes cooperate in an ordered series of events, wherein SWI/SNF action is a prerequisite for SAGA activity upon *HO* chromatin. Similarly, loci that require multiple PcG complexes for transcriptional repression may use a multistep mechanism where one PcG complex alters the chromatin template to "pave the way" for binding or action of another PcG complex. Indeed, this type of interplay could explain the observation that E(Z) function is required for association of the PRC1 components PSC and PH at many chromosomal sites (Rastelli *et al.* 1993).

Tethering and analysis of PcG functional domains: The carboxyl-terminal SPM domain of SCM is highly conserved in mammalian SCM homologues (Montini *et al.* 1999; Tomotsune *et al.* 1999). Analyses of *Scm* mutant alleles that remove the SPM domain, together with site-directed mutational analysis, have shown that the SPM domain is required for SCM function *in vivo* (Bornemann *et al.* 1998; A. Peterson and J. Simon, unpublished results). Although *in vitro* studies indicate that the SPM domain is a protein interaction module (Peterson *et al.* 1997), the functional contribution of this domain to SCM repression *in vivo* is not known. One possible role for the SPM domain would be to recruit SCM to target sites in chromatin, by analogy to the role that the conserved chromodomain plays in chromatin targeting of PC (Messmer *et al.* 1992). We found that deletion of the SPM domain in ZnF-SCM$^{\Delta SPM}$ abolished silencing in the tethering system. Although ZnF-SCM$^{\Delta SPM}$ accumulated to a similar level as wild-type ZnF-SCM (Figure 1B), its repression activity was indistinguishable from ZnF alone (Figures 4–6 and Tables 1–3). These results imply that the SPM domain does not solely provide interactions that target SCM to chromosomes, since the Su(Hw)-binding domain circumvents this targeting function. Instead, we suggest that the SPM domain is more directly involved in the repression mechanism or in maintaining integrity of SCM complexes.

One important application of a tethered-based assay is the analysis of the *in vivo* roles of PcG functional domains, as illustrated by our studies on the SPM domain of SCM. In a similar manner, a tethering system demonstrated that the carboxyl-terminal "shadow" domain of PC, but not its chromodomain, was required to silence reporter genes *in vivo* (Muller 1995). This approach can be extended for *in vivo* studies of additional SCM domains, including the pair of nonclassical zinc fingers located at the amino-terminus and the highly conserved mbt repeats (Bornemann *et al.* 1996, 1998). A tethering assay is a powerful method to identify those domains most directly involved in silencing because it short-circuits mechanisms used to target PcG proteins to chromosomes. Association of histone deacetylase enzyme activity with a mammalian PcG complex *in vitro* has been reported recently (van der Vlag and Otte 1999). Application of the tethering system to examine the potential contributions of this and other chromatin-modifying activities to PcG repression will improve our understanding of the *in vivo* functions of these domains.

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