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,¹,‡ Daniel R. Mallin,* Rachel Roberson,* Timothy J. Parnell,§ Douglas J. Bornemann,† Jeffrey A. Simon¹,‡ and Pamela K. Geyer* ,§

*Department of Biochemistry and 1Genetics Program, The University of Iowa, Iowa City, Iowa 52242, and †Department of Genetics, Cell Biology and Development and 2Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455

Manuscript received December 6, 2000
Accepted for publication February 5, 2001

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 naïve 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 zest [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 (Fea et al. 2000).

Mammalian homologues have been identified for
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 homeo-
tic transformations (Akasaka et al. 1996; van der Lugt
et al. 1996; Takihara et al. 1997). In some cases, Dro-
sophila and mammalian PcG proteins can function in
heterologous systems (Bunker and Kingston 1994;
Muller et al. 1995). For example, the mouse homol-
logue M33 can partially rescue Pc mutations in Drosoph-
ila (Muller et al. 1995). These data indicate that mechan-
isms of PcG action have been evolutionarily conserved.

PcG proteins function together in multiprotein com-
plexes. This conclusion was first suggested by studies
showing that several PcG proteins are colocalized on
duplytine chromosomes (Franke et al. 1992; Rastelli
et al. 1993; Lonie et al. 1994). Subsequent coimmuno-
precipitation experiments, in vitro binding assays, and
yeast two-hybrid analyses have defined binding interac-
tions 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 com-
plexes in nuclear extracts from fly embryos. The Pol-
ycromomb repressive complex, PRC1, contains a subset of
the identified PcG proteins including PC, PH, and poste-
rior 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 chromatog-
rphy detects the majority of SCM in an ~500-kD com-
plex that is significantly smaller than PRC1 and displays
different fractionation behavior (D. Mallin, J. Ng and
J. Simon, unpublished results). Similarly, multiple mam-
alian PcG protein complexes have been identified.
Interestingly, the biochemical separability of complexes
containing the PC and PH homologues from those con-
taining the ESC and E(Z) homologues has been con-
served (Alkema et al. 1997a; Gunster et al. 1997; Hashi-
moto 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 asso-
ciate with PREs is unknown, but one potential mecha-
nism 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 (Hag-
strom et al. 1997; Horard et al. 2000). Once tethered, a
PcG complex may establish silenced chromatin through
direct interactions with nucleosomes. PC binds to nucleo-
somal core particles (Breiling et al. 1999) and this asso-
ciation 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 com-
plexes 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 Bend-
er 1996). Further work is needed to assess how alter-
ations 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 func-
tion. In such simplified systems, direct binding of the
Drosophila PC and PSC proteins to reporter genes
causese repression of transcription, with more robust
silencing observed in cases where reporter genes were
integrated into the chromosome (Bunker and King-
ston 1994; Muller 1995). Interestingly, only transient
silencing of reporter genes lacking PREs occurred in
the absence of the continuous production of the teth-
ered 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 con-
inuously 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 teth-
ered 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 chromoso-
mal sites within the Drosophila genome were exami-
ned. 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 Hairless [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 promoter and 2.9 kb downstream of the Su(Hw) gene expressed from producer transgenes 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 Nol 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 Nol 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^cy f^ strain was used to establish all but the ZnF-ZnF-Sw^TM6 transformed lines, where the host strain was v^ f^; su(Hw)^ bx^ TM6, su(Hw)^ 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” Δ23 (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^ v^ f^; CyO, MKRS/ T(2,3)apw (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 (1:250 dilution; Jackson Immunoresearch Laboratory) for1hr at 37° and allowed to recover 30–60 min before protein isolation. Immunodetection on Western blots used FLAG M5 antibody (1:250 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 pigmentation 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 lower 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^ P-lt strain; su(Hw)^ bx^ TM6, su(Hw)^ 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)^ allele is a partial deletion of the su(Hw) gene (HARRISON et al. 1992) and the su(Hw)^ allele contains a point mutation in a ZnF that compromises DNA binding (HARRISON et al. 1993). Producer lines were established that were y~ AC w^ P-lt; ZnF-PcG/CyO; su(Hw)^/MKRS. The su(Hw)^ 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 $ph^{109}$, which is a hypomorphic allele that carries a deletion of the proximal $ph$ repeat (Dura et al. 1987). For these studies, $y^{ac}\,PH\,ph^{109}\,w^{111}\,c^{+}\,v^{+}\,f^{+}\,y^{+}\,SUP\,or\,P-lt\,TM6\,SUP\,or\,P\,Ubx\,males$ were generated and were crossed to $y^{ac}\,PH\,ph^{109}\,w^{111}\,c^{+}\,v^{+}\,f^{+}\,y^{+}\,SUP\,or\,P-lt\,TM6\,SUP\,or\,P\,UbxFemales$. The SUPor Pline that was used for these analyses carried an insert at cytological location 22F. Female progeny heterozygous for both the $ph^{109}$ mutation and $SUP\,or\,P\,[y^{ac}\,PH\,ph^{109}\,w^{111}\,c^{+}\,v^{+}\,f^{+}\,y^{+}\,PH\,ph^{109}\,w^{111}\,c^{+}\,v^{+}\,f^{+}\,y^{+}\,SUP\,or\,P\,TM6\,SUP\,or\,P\,Ubx]$ were mated to $y^{ac}\,PH\,ph^{109}\,w^{111}\,c^{+}\,v^{+}\,f^{+}\,y^{+}\,ZnF-PcG\,CyO;\,su(Hw)^{Ac}\,or\,PKRS\,producer\,males$ (Figure 3B). The four classes of resulting $su(Hw)^{Ac}$ male offspring were scored (Figure 3B).

Two classes, identified by the absence 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 $ph^{109}$ 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-SCM23M, and ZnF-ESC). The fusion genes were cloned into the $vermilion$ transformation vector pYCL1.8 (Fredell 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 $SUP\,or\,P\,Lt\,SUP\,or\,P$, and $SUP\,or\,P\,Blk$, 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; Mallis et al. 1998).

The three reporter transposons differed in two major ways. First, the nature of the white gene varied: $SUP\,or\,P\,Lt$ carried a mini-white gene that lacked the eye enhancer, whereas $SUP\,or\,P$ and $SUP\,or\,P\,Blk$ 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 $SUP\,or\,P\,SUP\,or\,P\,Blk$, and $SUP\,or\,P\,Lt$ 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 $SUP\,or\,P\,Lt$ transposon. $SUP\,or\,P\,Lt$ 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 $SUP\,or\,P\,Lt$ 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, $SUP\,or\,P\,Lt$ 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
Repressive Effects of ZnF-PcG Proteins

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 polyadénylation 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 (hs2) 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.

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 enhancer, 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...
Repressive Effects of ZnF-PcG Proteins

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 SUPor P-lt insertion sites tested, whereas only two of the six tested SUPor P-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](image-url)

**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 ph100 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.

![Figure 4](image-url)

**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.
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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Euchromatic</th>
<th>Telomeric</th>
<th>Euchromatic</th>
<th>Telomeric</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUPor P</td>
<td>0 (0/6)</td>
<td>100 (6/6)</td>
<td>100 (5/5)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>SUPor P-blk</td>
<td>0 (0/6)</td>
<td>100 (5/5)</td>
<td>100 (6/6)</td>
<td>0 (0/4)</td>
</tr>
</tbody>
</table>

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.

![Figure 5](image_url) 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.
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 factor in vitro (Peterson et al. 1997), its role in repression in vivo of these proteins on yellow gene expression is unclear. This domain may play a primary role in targeting SCM to the chromosome or it may be directly required to target 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 (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

<table>
<thead>
<tr>
<th></th>
<th>ZnF</th>
<th>ZnF-PC</th>
<th>ZnF-SCM</th>
<th>ZnF-SCM&lt;sup&gt;ΔSPM&lt;/sup&gt;</th>
<th>ZnF-ESC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P-lt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/6)</td>
<td>67 (4/6)</td>
<td>33 (2/6)</td>
<td>0 (0/6)</td>
<td>33 (2/6)</td>
</tr>
<tr>
<td>Telomeric</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/8)</td>
<td>75 (6/8)</td>
<td>38 (3/8)</td>
<td>0 (0/4)</td>
<td>13 (1/8)</td>
</tr>
<tr>
<td>Euchromatic P.E.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td>Telomeric</td>
<td>0 (0/4)</td>
<td>100 (4/4)</td>
<td>100 (4/4)</td>
<td>0 (0/3)</td>
<td>75 (3/4)</td>
</tr>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P-blk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/5)</td>
<td>25 (1/4)</td>
<td>20 (1/5)</td>
<td>0 (0/4)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Euchromatic P.E.</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 (1/1)</td>
</tr>
</tbody>
</table>

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

<sup>a</sup> P.E., position effects, transposons inserted in genomic locations that have negative effects on gene expression.

<sup>b</sup> 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 hsp<sub>70</sub>-ZnF-Scm<sup>ΔSPM</sup> 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<sup>ΔSPM</sup> on gene expression was determined by crossing a hsp<sub>70</sub>-ZnF-Scm<sup>ΔSPM</sup> 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<sup>ΔSPM</sup> progeny was indistinguishable from that of flies expressing ZnF alone (Figures 4–6 and Tables 1–3). These results show that the ZnF-SCM<sup>ΔSPM</sup> 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). (Figures 4±6 and Tables 1±3). These results show that the ZnF-SCM<sup>ΔSPM</sup> protein does not confer repression in

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

<table>
<thead>
<tr>
<th></th>
<th>ZnF</th>
<th>ZnF-PC</th>
<th>ZnF-SCM</th>
<th>ZnF-SCM&lt;sup&gt;ΔSPM&lt;/sup&gt;</th>
<th>ZnF-ESC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P-lt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/6)</td>
<td>83 (5/6)</td>
<td>83 (5/6)</td>
<td>0 (0/6)</td>
<td>67 (4/6)</td>
</tr>
<tr>
<td>Telomeric</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/8)</td>
<td>100 (8/8)</td>
<td>88 (7/8)</td>
<td>0 (0/4)</td>
<td>50 (4/8)</td>
</tr>
<tr>
<td>Euchromatic P.E.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
</tr>
<tr>
<td>Telomeric</td>
<td>0 (0/4)</td>
<td>100 (4/4)</td>
<td>100 (4/4)</td>
<td>0 (0/3)</td>
<td>75 (3/4)</td>
</tr>
<tr>
<td><strong>SUP&lt;sup&gt;or&lt;/sup&gt; P-blk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euchromatic</td>
<td>0 (0/5)</td>
<td>75 (3/4)</td>
<td>60 (3/5)</td>
<td>0 (0/4)</td>
<td>80 (4/5)</td>
</tr>
<tr>
<td>Euchromatic P.E.</td>
<td>0 (0/1)</td>
<td>100 (1/1)</td>
<td>100 (1/1)</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 (1/1)</td>
</tr>
</tbody>
</table>

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

<sup>a</sup> P.E., position effects, transposons inserted in genomic locations that have negative effects on gene expression.

<sup>b</sup> NT, not tested.
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
<br>

mutant DNA-binding domain (ZnF) and a carboxyl-terminal gene product. To test the effects of the ZnF-PcG proteins, we selected a SUM or 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
<br>

mutation on repression conferred by tethered ZnF-PcG proteins. The top row shows the eye phenotypes of class I flies carrying the ph
<br>

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.

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
<br>

flies was wild type (19/20), in contrast to the class II ph; SUM or P/ ZnF-Sem; su(Hw)

flies that had reduced and variegated eye pigmentation (Figure 7 and Table 4). These data imply that PH is also needed for the ZnF-SCM- or ZnF-ESC-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
<br>

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-
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 Î²-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-**

---

**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

<table>
<thead>
<tr>
<th>Producer</th>
<th>Total male progeny</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
<th>Class IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnF</td>
<td>452</td>
<td>Red (37)</td>
<td>Red (40)</td>
<td>Red (6)</td>
<td>Red (23)</td>
</tr>
<tr>
<td>ZnF-Scm</td>
<td>928</td>
<td>Red (19)</td>
<td>Yell-or. var (65)</td>
<td>Red (28)</td>
<td>Red (58)</td>
</tr>
<tr>
<td>ZnF-Pc</td>
<td>1506</td>
<td>Dk. or. (1)</td>
<td>Lt. or. (99)</td>
<td>Red (46)</td>
<td>Red (77)</td>
</tr>
<tr>
<td>ZnF-Esc</td>
<td>577</td>
<td>Dk. or. var (7)</td>
<td>Dk. or. -red (43)</td>
<td>Red (24)</td>
<td>Red (27)</td>
</tr>
</tbody>
</table>

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.
lencing: The influence of enhancer action on PcG repression was assessed by comparing the enhancer-containing SUPor P and SUPor P-bld 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; Smal€e 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 ZnF-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 pre-equipped with endogenous PH (деCAMILLIS 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 Sec 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 chromodomains play in chromatin targeting of PC (MESSMER et al. 1992). We found that deletion of the SPM domain in ZnF-SCM accompobilized silencing in the tethering system. Although ZnF-SCM 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 mtb 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 deaceytlyase 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.

We thank Welcome Bender for his input and sharing ideas on tethering systems and Bob Kingston for sharing his unpublished results. We thank members of the Geyer and Simon laboratories for critically reading this manuscript. This work was supported by National Institutes of Health grants to J.A.S. and P.K.G.

**LITERATURE CITED**


Burke, T. W., and J. T. Kadonaga, 1997 The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by the ATP100 of Drosophila. Genes Dev. 11: 3920–3931.


Tie, F., T. Furuyama and P. J. Harte, 1998  The Drosophila Polycomb Group proteins ESC and E(Z) bind directly to each other and co-localize at multiple chromosomal sites. Development **125**: 3483–3496.


Communicating editor: J. A. Birchler