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Functional analysis of the chromo domain of HP1

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Heterochromatin protein 1 (HP1) is a non-histone chromosomal protein in *Drosophila* with dosage-dependent effects on heterochromatin-mediated gene silencing. An evolutionarily conserved amino acid sequence in the N-terminal half of HP1 (the 'chromo domain') shares >60% sequence identity with a motif found in the Polycomb protein, a silencer of homeotic genes. We report here that point mutations in the HP1 chromo domain abolish the ability of HP1 to promote gene silencing. We show that the HP1 chromo domain, like the Polycomb chromo domain, has chromosome binding activity, but to distinct chromosomal sites. We constructed a chimeric HP1–Polycomb protein, consisting of the chromo domain of Polycomb in the context of HP1, and show that it binds to both heterochromatin and Polycomb binding sites in polytene chromosomes. In flies expressing chimeric HP1–Polycomb protein, endogenous HP1 is mislocalized to Polycomb binding sites, and endogenous Polycomb is misdirected to the heterochromatic chromocenter, suggesting that both proteins are recruited to their distinct chromosomal binding sites through protein–protein contacts. Chimeric HP1–Polycomb protein expression in transgenic flies promotes heterochromatin-mediated gene silencing, supporting the view that the chromo domain homology reflects a common mechanistic basis for homeotic and heterochromatic silencing.

Keywords: chromo domain/heterochromatin/homeotic silencing

Introduction

The chromosomes of higher eukaryotes are composed of euchromatin and heterochromatin. Heterochromatin is distinguished from euchromatin in that it remains condensed through interphase (Heitz, 1928), replicates late in the cell cycle (Holmquist, 1987), is enriched for repetitive sequences (John and Miklos, 1979), and is relatively poor in the number of genes (Pimpinelli *et al.*, 1986).

If a gene that normally resides in euchromatin is placed next to heterochromatin as a consequence of a chromosomal rearrangement, it will undergo cell-specific silencing, giving rise to a variegated phenotype. This phenomenon is known as position–effect variegation (PEV; reviewed by Spofford, 1976; Eissenberg, 1989; Henikoff, 1990; Grigliatti, 1991; Reuter and Spierer, 1992).

A number of second site modifiers alter the extent of variegated silencing. Suppressors of variegation [*Su(var)*] restore the wild-type phenotype. Enhancers of variegation increase the extent of silencing. In *Drosophila*, genetic analyses have identified more than 50 different modifiers of PEV (Sinclair *et al.*, 1983; Locke *et al.*, 1988; Wustmann *et al.*, 1989). One of these loci, *Su(var) 205*, encodes the heterochromatin-associated protein HP1 (Eissenberg *et al.*, 1990). HP1 is associated predominantly with the heterochromatic chromocenter in interphase nuclei (James and Elgin, 1986; James *et al.*, 1989) and exerts dosage-dependent effects on PEV (Eissenberg *et al.*, 1990, 1992). In HP1-like proteins from diverse species, two regions show a high degree of amino acid sequence conservation (Singh *et al.*, 1991; Clark and Elgin, 1992; Epstein *et al.*, 1992; Saunders *et al.*, 1993). The carboxy-terminal half of HP1, containing one of these regions of homology, is sufficient for nuclear localization and heterochromatin binding (Powers and Eissenberg, 1993). The other highly conserved region is found within the amino-terminal half of HP1. Paro and Hogness (1991) first noted the high degree of conservation between Polycomb (Pc) and HP1 in this region, and termed it the chromo domain, for chromosome organization modifier. In Pc, this domain is necessary and sufficient for nuclear localization and chromosome binding (Messmer *et al.*, 1992). Despite their structural relatedness, HP1 and Polycomb bind to distinct chromosomal sites (James *et al.*, 1989; Zink and Paro, 1989).

The conservation of the chromo domain between HP1 and Pc has led to the inference that the silencing associated with heterochromatic PEV employs a similar mechanism to that used to repress homeotic genes (Paro, 1990). In an effort to understand this mechanism and the role of the HP1 chromo domain in these processes, we performed a mutational analysis of the HP1 chromo domain.

Mutational analysis identified two sites in the HP1 chromo domain to be necessary for the heterochromatin-mediated silencing. The N-terminal half of HP1, which includes the chromo domain, is sufficient to direct heterochromatin binding of a β-galactosidase fusion protein *in vivo*. A substitution of the Pc chromo domain into HP1 yields a chimeric HP1–Pc protein that is competent to bind to both HP1 and Pc target sites in the genome and to promote heterochromatin-mediated position–effect silencing. These results support the view that the chromo domain homology confers properties of chromatin binding and gene-silencing activity on these distinct proteins, consistent with a common mechanistic basis for homeotic and heterochromatin gene silencing.

Binding of a HP1–Polycomb chimeric protein results in mislocalization of (i) endogenous HP1 to Pc binding

sites in euchromatin and (ii) endogenous Pc protein to heterochromatin. Thus, both of these proteins are capable of stable chromosome binding mediated by protein–protein interaction, consistent with an assembly-driven model of heterochromatin and homeotic gene silencing.

Results

Identification of the Su(var) 2-5^{02} mutation

The *Su(var) 2-5^{02}* allele was identified as an EMS-induced dominant suppressor of variegation that failed to complement the recessive lethality associated with HP1 mutations (G.Reuter, unpublished results). Northern blot analysis detected no aberrant HP1 transcripts in mRNA from heterozygous flies, and quantitation of the HP1 message in *Su(var) 2-5^{02}* heterozygotes indicated no difference from the level seen in wild-type flies (data not shown). HP1 coding sequences were amplified by the polymerase chain reaction (PCR; Saiki *et al.*, 1988), and direct sequencing of the PCR product revealed a single mutation in the open reading frame: a G→A transition in the first nucleotide of codon 26, resulting in the substitution of methionine for valine. The site of this mutation is within the chromo domain, and the corresponding position is occupied by a valine residue in HP1-like proteins from *Drosophila virilis* (Clark and Elgin, 1992), mealy bug (Epstein *et al.*, 1992), mouse (Singh *et al.*, 1991) and human (Singh *et al.*, 1991; Saunders *et al.*, 1993). This result suggests that mutation in the chromo domain disrupts HP1 function. The *Su(var) 2-5^{02}* allele is the only missense mutation in HP1 among the four Su(var) mutations affecting HP1 that have been characterized molecularly (Eissenberg *et al.*, 1990, 1992). In order to pursue further mutational analysis of the chromo domain, we employed site-directed mutagenesis to target specifically the chromo domain of HP1.

Creation of chromo domain mutations and intracellular localization of mutant protein

We selected sites for mutagenesis by identifying evolutionarily conserved residues among the different HP1 homologs, and by targeting sequences resembling motifs found in other proteins. The amino acid sequences of chromo domains from a variety of proteins and organisms are depicted in Figure 1A. The chromo domain of *Drosophila melanogaster* HP1 and the different mutational substitutions made for this study are shown in Figure 1B. The EEE→AAA and the Y→F substitutions were chosen because of the resemblance of these sequences to a protein tyrosine kinase target motif found in other proteins (Neil *et al.*, 1981; Baldwin *et al.*, 1983). The RR→QQ and the K→Q substitutions were designed to reduce the overall basic charge in this interval of the chromo domain, an evolutionarily conserved feature of this region. Amino acid substitutions were chosen based upon predicted minimal conformational interference (Chou and Fasman, 1978). The V→M substitution at codon 26 is the mutation found in the *Su(var) 2-5^{02}* allele, and was included in this study as a negative control.

Previous studies showed the HP1 chromo domain to be dispensable for nuclear localization and heterochromatin binding (Powers and Eissenberg, 1993). In contrast, point mutations in the Polycomb chromo domain abolish

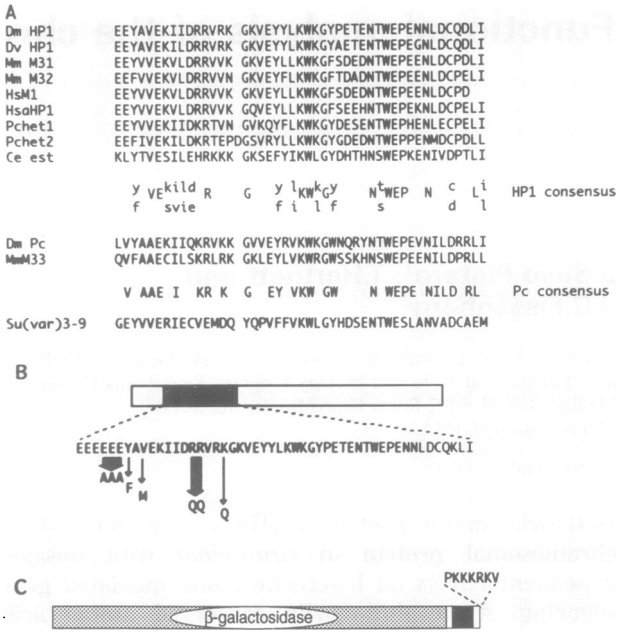

Fig. 1. (**A**) Amino acid comparisons of chromo domains. Dm, *Drosophila melanogaster* (James and Elgin, 1986); Dv, *D.virilis* (Clark and Elgin, 1992); Mm, *Mus musculus* (Singh *et al.*, 1991); HsM1 and HsaHP1, *Homo sapiens* (Singh *et al.*, 1991; Saunders *et al.*, 1993); Pchet1 and Pchet2, *Planococcus citri* (mealy bug, Epstein *et al.*, 1992); Ce est, *Caenorhabditis elegans* (nematode, Waterston *et al.*, 1992); Dm Pc, *D.melanogaster* Polycomb (Paro and Hogness, 1991); MmM33, mouse Polycomb (Pearce *et al.*, 1992); Su(var)3-9 (dominant suppressor of PEV in *D.melanogaster*; Tschiersch *et al.*, 1994). Lowercase letters in the HP1 consensus sequence indicate positions at which one of two alternative amino acids are found. (**B**) The chromo domain mutations used in this study, shown below the amino acid sequence of the wild-type chromo domain of *D.melanogaster* HP1. Horizontal bar represents HP1 protein; filled portion indicates the relative position of the HP1 chromo domain. (**C**) Diagram showing the structure of the N-terminal 95 amino acids of HP1 (open bar) containing the chromo domain (solid bar), fused to the nuclear localization signal of the SV-40 large T antigen (sequence shown above the diagram) and to β-galactosidase (shaded bar).

chromosome binding (Messmer *et al.*, 1992). Thus, it was a formal possibility that chromo domain mutations could result in a loss of heterochromatin binding and/or nuclear localization through allosteric structural changes. To confirm that the mutant proteins retained their nuclear targeting and heterochromatin binding activity, each mutant HP1 was expressed as a β-galactosidase fusion in transgenic flies and salivary glands were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Figure 2 shows that in every case, fusion proteins were predominantly nuclear and concentrated in one major site (occasionally one or two minor sites, e.g. panel C) within each nucleus, indistinguishable from that obtained with a full-length HP1 protein fused to β-galactosidase (panel A). Previous experiments have shown that this staining pattern is also characteristic of heterochromatin binding (Powers and Eissenberg, 1993). Thus, all the site-directed mutations retain the ability to localize to the nucleus and bind to heterochromatin.

The N-terminal half of HP1, containing the chromo domain, is sufficient for binding to heterochromatin

The chromo domain of Pc has nuclear targeting and chromosomal binding activities (Messmer *et al.*, 1992).

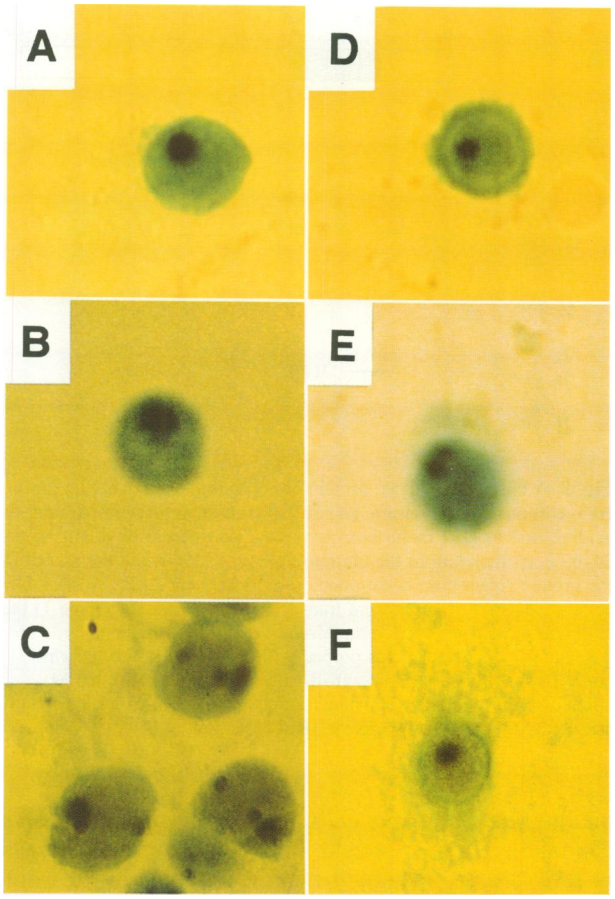

Fig. 2. Intranuclear localization of β-galactosidase fusion protein in polytene tissue. Third instar larval salivary glands of transgenic lines expressing wild-type or mutant HP1 fused to β-galactosidase, were stained with X-gal. (**A**) Wild-type HP1 fusion; (**B**) Y→F mutation fusion; (**C**) K→Q mutation fusion; (**D**) EEE→AAA mutation fusion; (**E**) V→M mutation fusion; (**F**) RR→QQ mutation fusion (see text for sites of amino acid substitutions). Additional minor spots of staining seen in (C) are an occasional feature of wild-type HP1 fusions as well (Powers and Eissenberg, 1993).

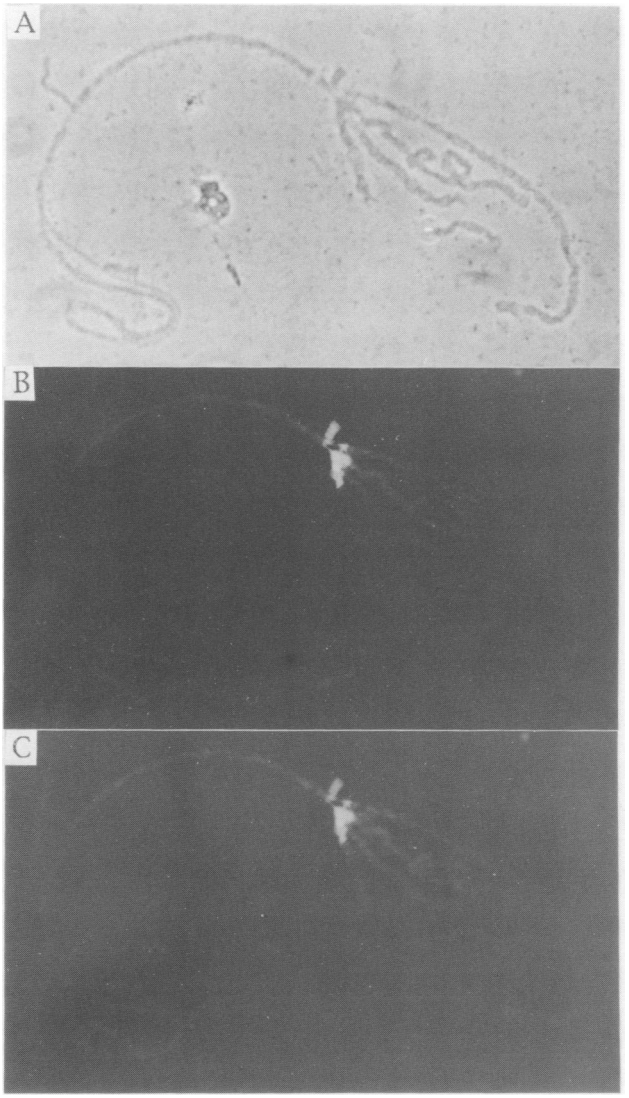

Fig. 3. The N-terminal half of HP1 encodes a heterochromatin-binding domain. (**A**) Phase-contrast micrograph of a fixed polytene chromosome squash from a transgenic line expressing the N-terminal half of HP1, fused to the SV40 T-antigen nuclear localization signal and to β-galactosidase. (**B**) Same chromosomes as in (A), stained with a rabbit antiserum specific for HP1 and an anti-rabbit FITC-coupled secondary serum. (**C**) Same chromosome as in (A), stained with a mouse anti-β-galactosidase serum and a Texas red-labeled anti-mouse secondary serum.

Powers and Eissenberg (1993) demonstrated that in HP1, these activities were associated with the C-terminal half of the HP1 protein. The latter study could not directly test whether the N-terminal half of HP1, containing the chromo domain, was capable of binding heterochromatin, because a fusion protein with the N-terminal half of HP1 failed to enter the nucleus. To test whether the N-terminal half of HP1 also has chromosome binding activity, an oligonucleotide encoding the nuclear localization signal of the SV-40 large T antigen protein (Figure 1C) was ligated downstream of a cDNA encoding the first 95 amino acids of HP1 and inserted into the pVβ/206 vector (Powers and Eissenberg, 1993). This allows the N-terminal half of HP1 fused to β-galactosidase, to be directed into the nucleus. The resulting plasmid, pvβ2NLS, was introduced into the germ line of *Drosophila* by P-element mediated transformation. Stable transgenic lines were established and upon heat-shock induction of β-galactosidase fusion protein expression, third instar larval polytene chromosomes were stained simultaneously with anti-β galactosidase antibody and anti-HP1 antibody. The staining with β-galactosidase will localize the fusion protein, while the HP1 antiserum localizes native HP1 in the same preparation. Double-label immunofluorescence analysis of a chromosome squash preparation stained with antibodies specific for β-galactosidase and HP1 is presented in Figure 3. The anti-β-galactosidase staining is concentrated in the pericentric heterochromatin (Figure 3C), and this distribution coincides with that found using HP1-specific antibody (Figure 3B). These results show that there is a redundant heterochromatin-targeting activity associated with the N-terminal half of HP1. Since the N- and C-terminal halves of HP1 have no obvious structural homology, it seems likely that the mechanism of localization for these distinct and autonomously acting domains is through separate macromolecular contacts.

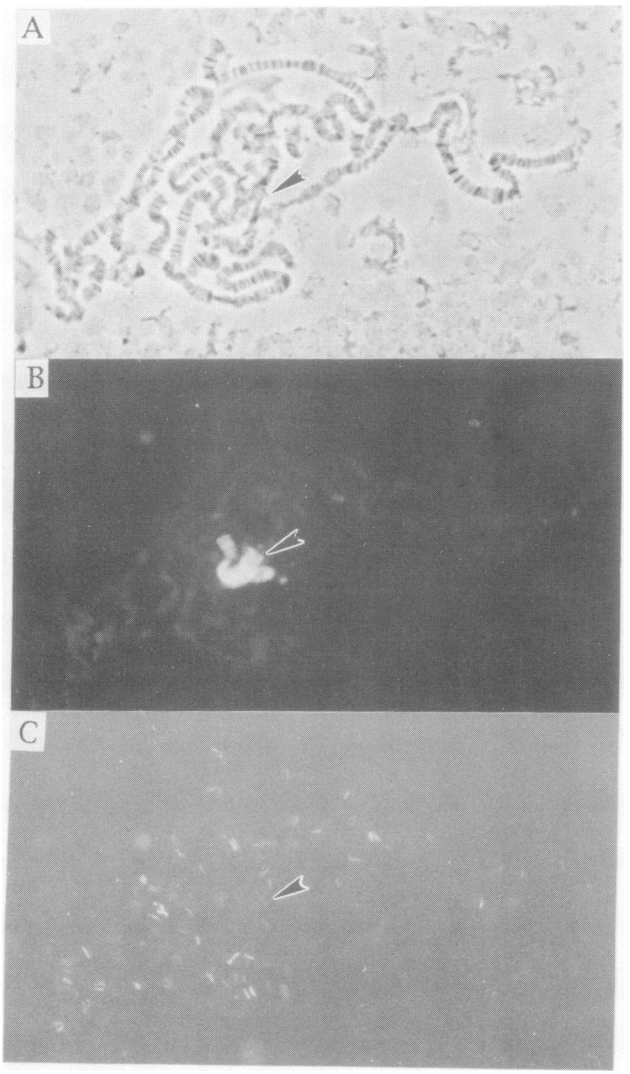

Fig. 4. HP1 and Polycomb proteins have non-overlapping distributions in wild-type polytene chromosomes. (**A**) Phase-contrast of fixed wild-type (Canton S) polytene chromosome. (**B**) Same chromosome as in (A), stained with a mouse antiserum specific for HP1 (the C1A9 monoclonal antibody; James and Elgin, 1986) and a Texas red-labeled anti-mouse secondary serum. (**C**) Same chromosome as in (A), stained with a rabbit antiserum specific for Pc followed by an anti-rabbit FITC-coupled secondary. Arrowheads point to the heterochromatic chromocenter in each panel.

A chimeric HP1–Pc protein binds heterochromatin and Pc binding sites

Although the chromo domains of both Pc and HP1 are sufficient to localize the proteins to their respective chromosomal binding sites, the distribution patterns of these proteins in polytene chromosomes are distinct. Figure 4 shows wild-type polytene chromosomes (Canton S) stained with HP1 and Pc antibodies, followed by Texas red- and fluorescein-labeled secondary antibodies, respectively. While HP1 is concentrated predominantly in the heterochromatic chromocenter (Figure 4B), Pc is associated with numerous sites throughout the euchromatic chromosome arms (Figure 4C), representing the probable targets of Pc regulation (Zink and Paro, 1989).

Previous data showing that the Pc chromo domain is sufficient to target a β-galactosidase fusion protein to Pc-binding sites (Messmer *et al.*, 1992), led us to test whether a chimeric HP1–Pc protein, in which the chromo domain of HP1 is substituted by the chromo domain of Pc, would also be able to bind to the Pc binding sites. To construct a chimeric HP1–Pc protein, we used a modified version of the 'PCR splicing by overlap extension' (PCR SOEing; Horton *et al.*, 1989). This procedure allowed us to effect a precise chromo domain swap without disrupting the rest of the HP1 protein (see Figure 5A for the final construct).

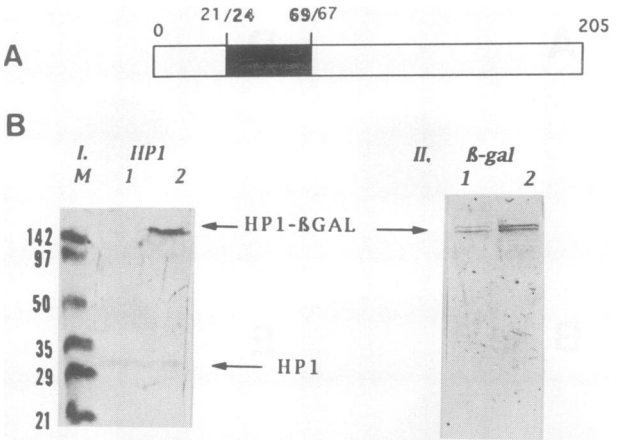

Fig. 5. A chimeric HP1–Pc protein is not immunoreactive with anti-HP1–chromo domain serum. (**A**) HP1–Pc chimeric protein. Amino acids 21–67 of HP1 (solid box) have been substituted by amino acids 24–69 corresponding to the chromo domain of Pc, while the rest of the protein (open box) corresponds to HP1 sequence. (**B**) Western blot of total adult fly proteins from transgenic lines expressing (lane 1) the chimeric protein or (lane 2) the V→M substitution, fused to β-galactosidase. Panel I was probed with an anti-HP1 serum, while panel II was probed with an anti-β-galactosidase serum. Lane M contains pre-stained size standards; molecular weight of each size standard ($\times 10^{-3}$) is shown to the left of the blot.

To test chromosome binding activity of the chimeric protein, polytene chromosomes from transgenic larvae expressing the chimeric protein fused to β-galactosidase were stained with antibodies specific for β-galactosidase followed by a Texas red-labeled secondary antibody (Figure 6B and E). The chromocenter of these flies stain in a pattern very similar to that observed with HP1. Although in these figures the staining seems to concentrate at the edges of the chromocenter, suggesting that developmentally delayed expression of HP1 results in the accumulation of additional HP1 at only a subset of heterochromatic sites, this is not a consistently observed behavior. In addition to the heterochromatin-staining characteristic of HP1, numerous sites throughout the euchromatic chromosome arms also stain. The euchromatic staining sites correspond to Pc binding sites, as demonstrated by the double labeling of the same chromosome with antibodies that recognize Pc (Figure 6C). Therefore, the chimeric protein localizes to both HP1 and Pc binding sites, indicating that the Pc chromo domain retains its chromosome binding properties in an HP1 context. Interestingly, expression of chimeric HP1–Pc also results in recruitment of endogenous Pc protein to the heterochromatic chromocenter (arrow in Figure 6C). This suggests that the Pc chromo domain is capable of directly or indirectly promoting endogenous Pc chromosome binding through protein–protein contact.

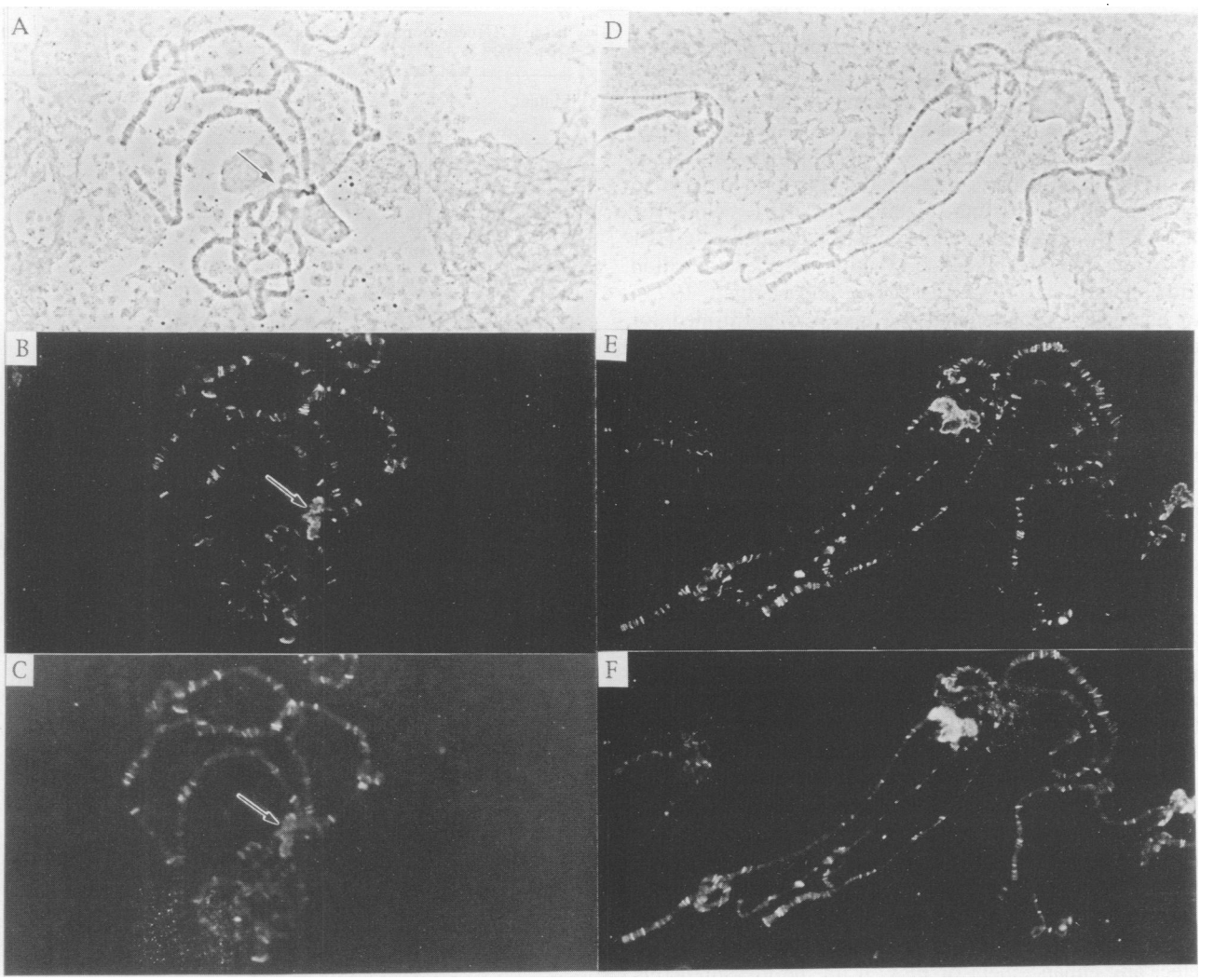

Fig. 6. Chimeric HP1–Pc protein binds both to heterochromatin and to Polycomb euchromatic sites, and recruits endogenous HP1 and Polycomb proteins to ectopic binding sites. (**A**) Phase-contrast micrograph of fixed polytene chromosomes from a transgenic line expressing a chimeric HP1–Pc β-galactosidase fusion protein. (**B**) Same chromosome as in (A), stained with a mouse anti-β-galactosidase serum followed by a Texas red-coupled anti-mouse secondary serum. (**C**) Same chromosome as in (A), stained with an anti-Pc serum, followed by a fluoresceinated secondary antiserum. (**D**) Phase-contrast micrograph of fixed polytene chromosomes from a transgenic line expressing a chimeric HP1–Pc β-galactosidase fusion protein. (**E**) Same chromosome as in (D), stained with a mouse anti-β-galactosidase serum and a Texas red-coupled anti-mouse secondary serum. (**F**) Same chromosome as in (D), stained with an HP1-specific rabbit antibody and a FITC-coupled anti-rabbit secondary serum. Arrows point to the heterochromatic chromocenter. The extensive chromosome tangling below the chromocenter in (A) and above the chromocenter in (D) is due to the presence of the multiply inverted balancer chromosome *In(2LR)CyO* in this stock.

The binding of chimeric HP1–Pc protein to Pc sites results in the recruitment of wild-type HP1 to Pc sites as well. Figure 6E and F show a polytene chromosome from a transgenic larva expressing the chimeric protein fused to β-galactosidase, stained simultaneously with anti-β-galactosidase and anti-HP1. Note that the distribution patterns of the chimeric fusion protein and wild-type HP1 are identical. We believe the anti-HP1 serum is not cross-reacting with the chimeric HP1–Pc fusion protein for three reasons: (i) the HP1 antibody used in this study is directed against a synthetic peptide based on amino acids 25–47 of HP1. These amino acids correspond to part of the chromo domain of HP1, sequences not present in the chimeric protein; (ii) this antibody does not normally stain Pc sites in polytene chromosome squash preparation (compare Figures 3C and 4C); and (iii) the HP1 antibody directed against the HP1 chromo domain does not cross-react with the chimeric protein in western immunoblot analysis (Figure 5B). The HP1 antibody recognizes the native HP1 in the chimera-expressing transgenic line (band labeled 'HP1' in Figure 5B, panel I) but fails to immunostain the chimeric protein fused to β-galactosidase (Figure 5B, panel I). The transgenic line carrying the V→M substitution, fused to β-galactosidase was used as a positive control and mobility standard, and this fusion protein is immunodetectable with the same anti-HP1 serum (Figure 5B, panel I). Both fusion proteins are immunodetectable with anti-β-galactosidase (Figure 5B, panel II).

Thus, the chimeric fusion protein can recruit HP1 to ectopic sites in the chromosomes. This ectopic association could be the result of chimeric protein-driven heterochromatin complex formation, or simply the formation of heterodimers between wild-type HP1 and the chimeric HP1–Pc fusion protein.

Genetic complementation of Su(var) 205

By cytological criteria, all of the mutant HP1 proteins localize to heterochromatin, and the chimeric HP1–Pc protein is found both in heterochromatin and at Pc binding sites. To test the functional significance of mutant protein binding to heterochromatin, we examined the ability of the different mutations and of the chimeric protein to complement an existing mutation of HP1. Wild-type HP1 cDNA under heat-shock control can support HP1 overexpression and enhance position–effect variegation (Eissenberg *et al.*, 1992; Eissenberg and Hartnett, 1993). An analogous complementation test was performed for each of the mutant HP1 proteins expressed in transgenic animals. Each of the mutant cDNAs was cloned downstream of the *Hsp 70* heat-shock promoter and inserted into the P-element transformation vector pYC1.8 (Fridell and Searles, 1991). Transgenic flies carrying each of the mutations or the chromo domain swap were mated to flies carrying the dominant suppressor of PEV *Su(var)205*. Progeny were heat-shocked for 30 min daily through eclosion, then eye pigments were extracted and quantitated spectrophotometrically (Ephrussi and Herold, 1944). The results are shown in Table I. The eye phenotypes correlated with the pigment values detected; lines in which less red pigment was measured showed a visible enhancement of variegation. In this assay, a heat-shock-driven wild-type HP1 cDNA causes a 2–5-fold enhancement of variegation (Eissenberg *et al.*, 1992). Any fold difference above 1.5 was discernible on inspection.

As anticipated, the V 26 M substitution produced an inactive protein. Thus, overexpression of a mutant protein does not result in hypomorphic activity. Interestingly, one other mutant, the Y→F mutation, also appears to produce a protein that has little or no gene silencing activity by this assay. In two out of three lines tested, we could not detect any enhancement of PEV. If all the measurements using this mutation are pooled, there is no significant enhancement compared with control siblings ($P < 0.01$; Student's *t*-test). Because of the conservative nature of this substitution and its position in a protein tyrosine kinase motif, it seems likely that the absence of activity is due to a requirement for tyrosine phosphorylation at this site.

The rest of the mutations, EEE→AAA, RR→QQ and K→Q resulted in active proteins, as demonstrated by the enhancement observed in variegation (Table I), indicating that in this assay, these mutations behave similarly to wild-type HP1.

The mutational substitutions in each case do not appear to interfere with accumulation of mutant protein, since heat-shock treatment results in the accumulation of additional HP1 protein to levels ~2–3-fold over that seen in non-transgenic controls, detectable by Western immunoblot analysis (not shown). This level of overexpression is similar to that previously reported for wild-type HP1 cDNA under Hsp 70 control (Eissenberg *et al.*, 1992; Eissenberg and Hartnett, 1994). Interestingly, in no case did mutant HP1 overexpression result in significant suppression of PEV. Therefore, there is no detectable antimorphic activity associated with any of the mutants.

A most striking result is the genetic complementation with the chimeric protein. This protein contains all of the chromo domain of Pc, yet it is able to promote heterochromatin-mediated PEV. This demonstrates that while the Pc chromo domain retains the ability to target fusion protein to Pc sites within an HP1 context, it can nevertheless substitute for the HP1 chromo domain in a functional assay for heterochromatin-mediated gene silencing.

Table I. Genetic complementation of *Su(var)205* by heat-shock-driven HP1 cDNAs

Mutant		Eye pigment value	Fold difference
HP1^{+} [a]	Line *HSHP1.85F*	13.0 ± 5.0	
	Control	34.0 ± 4.0	2.6
HP1–Pc	Line 1	15.3 ± 7.0	
	Control	32.0 ± 7.0	2.0
	Line 2	23.0 ± 9.0	
	Control	45.0 ± 4.0	2.0
	Line 3	21.0 ± 5.0	
	Control	34.0 ± 6.0	1.6
V→M	Line 1	33.0 ± 4.0	
	Control	41.0 ± 9.0	None
	Line 2	46.0 ± 7.0	
	Control	53.0 ± 4.0	None
	Line 3	35.0 ± 3.0	
	Control	37.0 ± 6.0	None
RR→QQ	Line 1	17.6 ± 3.2	
	Control	29.0 ± 3.0	1.7
	Line 2	21.0 ± 7.0	
	Control	42.0 ± 8.0	2.0
	Line 3	17.0 ± 1.0	
	Control	40.0 ± 2.6	2.3
K→Q	Line 1	22.0 ± 5.0	
	Control	43.0 ± 2.0	1.9
	Line 2	18.0 ± 2.0	
	Control	48.0 ± 3.0	2.6
Y→F	Line 1	38.0 ± 2.0	
	Control	46.0 ± 4.0	1.2
	Line 2	49.0 ± 8.0	
	Control	41.0 ± 2.0	None
	Line 3	59.0 ± 5.0	
	Control	59.0 ± 2.0	None
EEE→AAA	Line 1	12.0 ± 3.0	
	Control	32.0 ± 6.0	2.6
	Line 2	3.2 ± 0.4	
	Control	29.0 ± 5.0	9.0
	Line 3	13.0 ± 1.0	
	Control	20.0 ± 2.0	1.5

[a]Eissenberg *et al.* (1992).
'None' indicates no statistically significant difference with a 95% certainty using the Student's *t*-test.

Discussion

Significance of the HP1 chromo domain

The N-terminal half of HP1, containing the chromo domain, is able to target a β-galactosidase fusion protein to heterochromatin. Thus, HP1 has two redundant but non-homologous heterochromatin binding domains, one in the N-terminal half and one in the C-terminal half (Powers and Eissenberg, 1993). Interestingly, HP1 has no detectable DNA binding activity (Singh *et al.*, 1991), suggesting that these two domains participate in two distinct kinds of protein–protein interactions. One could hypothesize that the carboxy terminal region is involved in dimerization of HP1, while the chromo domain is

interacting with other proteins. Phosphorylation of HP1 (Eissenberg *et al.*, 1994) may act to regulate such interactions, or to promote conformational changes in HP1-dependent complexes.

Mutations in the chromo domain of HP1 produce proteins that are unable to promote heterochromatin-mediated gene silencing. Specifically, the Y 24 F and the V 26 M mutations are evidence for the importance of this domain. In addition, the valine→methionine change is associated with dominant suppression of position–effect variegation and recessive lethality. By comparing the chromo domains of other HP 1 homologs, tyrosine 24 is substituted by phenylalanine in HP1-like proteins from two species, mouse and mealy bug, but these organisms also contain an additional HP1 homolog with a tyrosine at the corresponding position, perhaps emphasizing the importance of tyrosine at that position. The inactivity of the tyrosine→phenylalanine mutation supports the idea that the tyrosine is a target for phosphorylation. HP1 is multiply phosphorylated during development (Eissenberg *et al.*, 1994) and HP1 hyperphosphorylation is correlated with heterochromatin assembly. Although most phosphorylation in HP1 occurs at serine and threonine, Tyr24 could be a target for transient phosphorylation *in vivo* that may be important for the formation of functional heterochromatin.

The other amino acid substitutions in the HP1 chromo domain resulted in proteins that were functional in our complementation assay. Functionality of the K→Q substitution is consistent with the observation that the *Su(var)3-9* protein chromo domain contains a glutamine in the homologous position as the K 36 Q substitution (Figure 1A), supporting the notion that a glutamine at that position in the chromo domain is tolerated in a protein associated with heterochromatic silencing.

Tartof and colleagues (Locke *et al.*, 1988) have proposed a mass action model of heterochromatin assembly to explain how heterochromatin-mediated silencing can be extremely sensitive to the dosage of several different genes. If proteins that form a subunit of heterochromatin associate with a stoichiometry that requires two or more molecules of a given protein per subunit, the effect of small dosage changes for that protein is greatly magnified. HP1 has been shown to have strong dosage-dependent effects on PEV (Eissenberg *et al.*, 1992). Our data showing HP1 recruited to ectopic sites of chimeric HP1–Pc protein suggests either (i) the ability of chimeric HP1–Pc to promote the assembly of HP1-containing complexes, or (ii) direct HP1 self-association.

Significance of the structural homology between HP1 and Polycomb

The ability of chimeric HP1–Pc to recruit Polycomb protein to the heterochromatic chromocenter suggests either Pc chromo domain-driven complex assembly or chromo domain-mediated self-association. The mutually complementary localization activities revealed by the chimeric protein binding underscores a further functional parallel between these otherwise unrelated chromo domain proteins: they both can promote stable and specific macromolecular complex formation through protein–protein interaction. In the case of Polycomb, the chromo domain appears to be sufficient to confer this specificity.

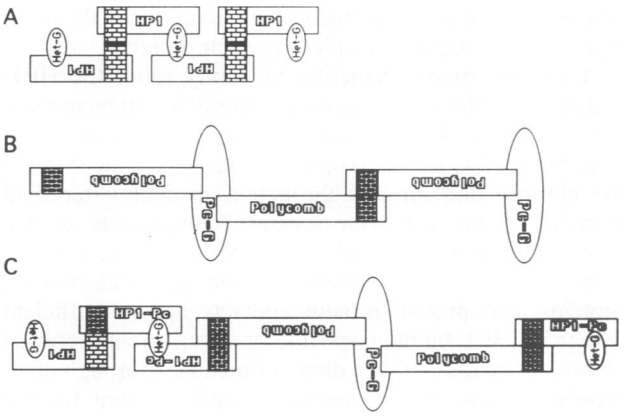

Fig. 7. Models of tandemly reiterated HP1- and Polycomb-dependent complexes in wild-type and transgenic flies. (**A**) In heterochromatin of wild-type flies, two molecules of HP1 are shown depicted as being complexed through the chromo domain (open bricks). C-terminal domains of HP1 are depicted as associating with another heterochromatin-group (Het-G) protein. (**B**) At Polycomb silencing complexes, Polycomb is depicted as self-associating directly through the chromo domain (filled bricks), while the C-terminus of Polycomb is shown associating with another member of the Polycomb-group (Pc-G) proteins. (**C**) In transgenic flies expressing the HP1–Pc chimeric protein, protein complexes in heterochromatin and at Polycomb binding sites are depicted as containing HP1, chimeric HP1–Pc and Polycomb protein, as well as potentially including other members of Het-G and Pc-G proteins. In this model, the Pc chromo domain (filled bricks) is competent to interact stably and productively with the HP1 chromo domain, when positioned in the complex through the C-terminal association with Het-G. The chimera chromo domain also remains competent to interact with its normal partner in euchromatin (shown here as the endogenous Pc chromo domain). This model accommodates the observations, reported here, that the chimeric protein is found at HP1 and Pc binding sites, can recruit HP1 to Pc sites and Pc to heterochromatin, and can participate in heterochromatin-mediated gene silencing. Of course, many other models are possible.

Consistent with a targeting role for the Polycomb chromo domain, Müller has reported that a GAL4–Pc fusion protein can establish stable and mitotically heritable silencing of a reporter gene associated with GAL4 binding sites. Furthermore, a point mutation in the chromo domain that otherwise blocks both Polycomb silencing and chromosome binding activity does not abolish silencing activity in the GAL4–Polycomb fusion protein, suggesting that the chromo domain targeting activity can be bypassed if the protein is tethered by the GAL4 DNA binding domain (Müller, 1995).

Of particular interest is the ability of the chimeric HP1–Pc protein to promote heterochromatin-mediated silencing, demonstrating that the chromo domain of Pc can substitute functionally for the chromo domain of HP1. This further supports the notion that heterochromatin-mediated silencing and homeotic gene silencing share mechanistic similarities. Conceivably, the chromo domain provides a surface needed for the maintenance of a stable complex of proteins, both in heterochromatin and in homeotic gene silencing. Insofar as the general charge and the structure of this domain is maintained, the complexes will form and will be able to act in other contexts. By this model, the structural and functional similarity between the HP1 and Pc chromo domains implies a common component, presumably one or more proteins for both heterochromatin and homeotic silencing complexes. Further molecular

genetic dissection of heterochromatin and of the Pc group genes will be required to identify such proteins.

A cartoon model depicting tandemly reiterated HP1- and Pc-dependent complexes, and possible interactions to account for the new data reported here, is shown in Figure 7. In Figure 7A, HP1 is proposed to self-associate through the chromo domain and through a distinct C-terminal domain, to interact with separate components of the heterochromatin-group (Het-G) protein family. One or more of these Het-G proteins could be DNA-binding proteins, but protein–protein contacts appear sufficient to confer HP1 binding specificity. Interactions between chromo domains could be direct or mediated through other proteins. These distinct contacts could account for the separable heterochromatin-binding activities in the C-terminal (Powers and Eissenberg, 1993) and N-terminal (this report) halves of HP1. In Figure 7B, an analogous complex is illustrated for Pc-containing complexes. The Pc chromo domain is depicted as mediating Pc self-association directly, but this could just as well occur through one or more adaptor proteins. As with HP1, the specificity of Pc chromosome binding is shown as depending primarily on protein–protein contacts. Only the N-terminal chromo domain of Pc has been shown to confer specific chromosome binding (Messmer *et al.*, 1992). Figure 7C illustrates how HP1–Pc chimeric protein in transgenic flies could result in the recruitment of endogenous HP1 and Pc to complexes in both heterochromatin and euchromatin. The compatibility between the Pc chromo domain and HP1 and/or other elements of the Het-G is implied by the ability of the chimeric protein to promote heterochromatic silencing.

While this model is certainly simplistic, it nevertheless makes certain testable predictions. The implication that other members of the Pc–G protein family would be recruited along with Pc to heterochromatin may be tested in chimera-expressing flies using antibodies to other Pc–G proteins. The suggestion that productive silencing complexes may also be formed at Pc binding sites could be tested by complementation of Pc mutation with the chimeric transgene.

Materials and methods

Identification of the mutation in Su(var) 2-5^{02}

Genomic DNA was purified from *Su(var) 2-5^{02}/InCyRoi* flies and a 1.2 kb fragment containing the HP 1 coding sequence was amplified by PCR (Saiki *et al.*, 1988). The protein coding sequences were determined for this fragment by asymmetric amplification and direct sequencing of PCR products as described (Eissenberg *et al.*, 1992)

Plasmid constructs

The following oligonucleotides were used to introduce each of the site-specific mutations into HP1 cloned in M13mp18 by the oligonucleotide-mediated method of Kunkel *et al.* (1987):

Y→F: 5′-GAGGAGGAGTTCCCCGTGGA-3′
K→Q: 5′-GGGTGCGCCAGGGAAAGGTG-3′
EEE→AAA: 5′-AGGAGGCGGCGGCGTACGCC-3′
R→Q: 5′-CATCGACCAGCAGGTGCGCA-3′

All of the different double-stranded M13mp18 containing the mutations were digested using unique *Bgl*II sites found in the cDNA of HP1 and substituted for the corresponding wild-type *Bgl*II fragment in a P-element based construct (pYC1.8, Fridell and Searles, 1991) containing HP1 cDNA under the *Hsp 70* heat-shock promoter. DNA sequencing confirmed the introduction of the mutations.

To generate vectors containing the mutations fused to β-galactosidase, an HP1 cDNA under the *Hsp 70* heat-shock promoter was introduced into a pUC 19 vector in which the *Sca*I site had been previously removed. We named this vector SP1. Each of the double-stranded M13mp18 DNAs, containing the mutations, was digested with *Sca*I and *Bgl*II, and DNA fragments containing the mutations were substituted into the SP1 vector previously digested with the same enzymes. Digestion of these new vectors with *Kpn*I produced restriction fragments containing the mutations, which were then introduced into the pvβ206 vector (Powers and Eissenberg, 1993).

For the V26M mutation, a cDNA was amplified by RT-PCR from total RNA of *Su(var)2-5^{02}/b lt rl* flies. Sequencing of such clones revealed a G→A transition in the first nucleotide of codon 26 in a subset of clones, the same mutation detected by direct sequencing of genomic PCR products. An entire cDNA carrying this mutation was introduced into SP1. Digestion with *Kpn*I produced a DNA fragment containing the mutation which was inserted into pvβ206. Also, as previously described, the mutation was introduced into the pYC1.8 vector containing the cDNA of HP1. Sequencing verified the introduction of the mutation.

To construct the pvβ2NLS vector, the following oligonucleotides were synthesized: 5′-CGCCAAGGCCCCTAAGAAGAAGAGGAAGGTCG-AGGACCCGTAACTTAAGCA-3′ and 5′-TGCTTAAGTTACGGGTCC-TCGACCTTCCTCTTCTTCTTAGGGGCCTTGGCG-3′.

The oligonucleotides were allowed to anneal, and double-stranded DNA was cut with the restriction enzymes *Sty*I and *Afl*II. The SP1 vector was cut with *Sty*I and *Afl*II and ligated to the oligonucleotides. A *Kpn*I fragment of this construct, containing the first 437 base pairs of HP1 and the oligonucleotides, was introduced into the *Kpn*I site of pvβ206. We called this final plasmid pvβ2NLS. Sequencing confirmed the addition of only the desired sequences.

Construction of the chimeric protein

For PCR amplification and PCR SOEing, we used the primers:

#1′: 5′-GCCGAAGAGGAGGAGCTAGTGTACGCGGCT-3′
#1 : 5′-CTCCTCCTCTTCGGC-3′
#2′ : 5′-CTTGCGGCTCGCCTCGTACTGCTGGATGAGGCGGCGATC-3′
#2 : 5′-GAGGCGAGCCGCAAGGA-3′
#6 : 5′-ACCAATTTAGCTGCGTGCAT-3′
#7 : 5′-GCCACTGAGGAGGGCACCAT-3′

Three independent PCRs were carried out. The first reaction was with primers #6 and #1 using an HP1 cDNA as a template. The second reaction was with primers #2 and #7 with the same template. The third reaction was with primers #1′ and #2′ with a *Pc* cDNA as a template. Primers #1, #1′ and #2, #2′ are complimentary at their 5′ ends. As a consequence, the product of the first PCR is complementary in its 3′ end to the 5′ end of the product of the third reaction. Also, the 5′ end of the product of the second PCR, is complementary to the 3′ end of the third reaction. All the products, after agarose gel isolation, were pooled for a final PCR with primers #6 and #7. The final product was cut with *Bgl*II and introduced into the pYC1.8 vector containing the heat-shock-driven HP1 cDNA. DNA sequencing determined that no additional mutations were introduced during the different PCR. This restriction fragment was also cloned into a pUC19 vector containing the cDNA of HP1 and subsequently introduced into pvβ206 as described previously.

Germ line transformation

v^{36F}; ry^{506} embryos were injected with each of the constructs, together with the helper plasmid pπ25.7wc (Karess and Rubin, 1984), as described by Spradling (1986). G_0 survivors were mated to *v^{36F}; ry^{506}* flies. F_1 adults were screened based on the rescue of the *vermilion* eye color (Fridell and Searles, 1991). Upon the identification of the chromosome in which the transgene had inserted, stable transformed lines were established by crossing the transformed lines to either second (*v^{36F};CyO/Sco;ry^{506}*) or third chromosome (*v^{36F};Sb/TM2,ry^{506}*) balancer stocks.

β-galactosidase activity staining

Third instar larvae were collected from each of the transformed lines carrying the pvβ206 constructs. Heat-shock treatment for 30 min was followed by a 1 h recovery period. Salivary glands were dissected and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the assay buffer described by Simon *et al.* (1985). Stained tissue was placed on a microscope slide and mounted in 95% glycerol, 5% PBS.

Immunostaining of polytene chromosomes

Third instar larvae were heat-shocked for 30 min and allowed to recover at room temperature for 1 h. Salivary glands were dissected in Cohen

and Gotchel (1971) gland medium, then incubated for 1 min in 2% formaldehyde solution (2% formaldehyde, 2% Triton X-100, 10 mM sodium phosphate, pH 7, 2 mM KCl, 0.1 M NaCl). Fixed tissue was then transferred to 45% acetic acid plus 2% formaldehyde for 3 min, a coverslip was added and the glands were squashed. Slides were frozen on dry ice and immersed in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). They were incubated with TBSTB (TBST, 10% goat serum) for 30 min. Primary antibody was diluted (1:500 for mouse anti-β-galactosidase; 1:500 for rabbit anti-HP1 polyclonal; 1:100 for C1A9 mouse anti-HP1 monoclonal, 1:10–1:50 for rabbit anti-Polycomb polyclonal) in TBSTB and added to the slide. HP1 antiserum (for Figure 3 and Figure 6, a polyclonal serum directed against a synthetic peptide based on amino acids 25–47 in the chromo domain sequence; for Figure 5, the monoclonal mouse antibody C1A9 used as cell culture supernatant; both antibodies were gifts of Dr S.C.R.Elgin) or monoclonal mouse anti-β-galactosidase serum (Promega) or Pc antiserum (a polyclonal rabbit serum directed against the C-terminal 199 amino acids of Polycomb; this region excludes the entire Polycomb chromo domain, and has no discernible structural homology to HP1; gift of Dr R.Paro) were used as primary antibodies. Incubation for 1 h in a humid chamber followed. Slides were then rinsed three times for 5 min with TBSTB and secondary antibody was then added. To detect staining of rabbit anti-HP1 and Pc antibodies, FITC-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary stain. To detect β-galactosidase and mouse anti-HP1 (C1A9 monoclonal) staining, Texas red-labeled anti-mouse IgG (Sigma) was used. Secondary sera were diluted 1:50 in TBSTB and incubation was for 1 h, following which slides were rinsed three times for 5 min with TBSTB. Vectashield (Vector) was added before a coverslip was mounted to prevent bleaching. Fluorescence detection was with a Zeiss standard 18 research microscope using a LP590 barrier filter for fluorescein detection and a 515–565 barrier filter for Texas red. No fluorescence bleeding was detected between filters.

Western blot analysis

Ten flies carrying the chimeric HP1–Pc cDNA fused to *lacZ* and 10 flies carrying the V→M mutant HP1 cDNA fused to *lacZ*, were heat-shocked for 30 min. After a 1 h recovery period, flies were homogenized in 200 μl of protein homogenization buffer: 60 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 350 mM β-mercaptoethanol, 0.01% bromophenol blue, containing proteinase inhibitors, 10 μg/ml leupeptin, 1.0 mM PMSF, 10 μM benzamidine, 10 μg/ml pepstatin A, 1 μg/ml phenanthroline and 10 μg/ml aprotinin at the indicated final concentrations. 40 μl of protein were loaded in a 12% SDS–polyacrylamide gel and run for 2 h at 100 V, using the Bio-Rad Mini-Protean II system. Proteins were transferred electrophoretically to a nitrocellulose filter at 50 V for 30 min at 4°C. Following transfer, the filter was blocked for 30 min with 5% casein in TBST. Afterwards, the blocking solution was substituted by TBST containing anti-HP1 antibody at 1:10 000 dilution or an anti-β-galactosidase antibody (Promega) at 1:5000 dilution, and incubated for an additional 30 min. The blot was washed three times with TBST and incubated 30 min with a 1:10 000 dilution of anti rabbit IgG–alkaline phosphatase-conjugated secondary antibody (Promega). After three additional washings in TBST, the immunoreactive bands were detected by adding 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium to the filter and allowing the color reaction to proceed until maximum color was visualized.

Eye pigment measurements

To measure the ability of the different mutants to affect PEV, the following crosses were performed: *In(1)w^{m4}/In(1)w^{m4}; Su(var)205/In(2LR)Cy; +/+ × +/Y; +/+; mutant/TM2*, where 'mutant' refers to a third chromosome containing the transgene with the indicated mutation or the chromo domain swap. Males that were *In(1)w^{m4}/Y; Su(var)205/+; mutant/+*, were collected, aged for 2–3 days, and red eye pigment was extracted and measured spectrophotometrically according to the method of Ephrussi and Herold (1944). As controls, male siblings that were *In(1)w^{m4}/Y; Su(var)205/+; TM2/+* were also assayed. Pigment values are expressed as percent of wild-type (Canton S) red eye pigment. Fold difference is the ratio of the pigment values obtained with controls to those obtained with transgenic flies. Each measurement was made using 50 fly heads.

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