Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex

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Polycomb response elements (PREs) are *cis*-acting DNA elements that mediate epigenetic gene silencing by Polycomb group (PcG) proteins. Here, we report that Pleiohomeotic (PHO) and a multiprotein Polycomb core complex (PCC) bind highly cooperatively to PREs. We identified a conserved sequence motif, named PCC-binding element (PBE), which is required for PcG silencing in vivo. PHO sites and PBEs function as an integrated DNA platform for the synergistic assembly of a repressive PHO/PCC complex. We termed this nucleoprotein complex silenceosome to reflect that the molecular principles underpinning its assemblage are surprisingly similar to those that make an enhanceosome.

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Epigenetic regulation refers to effects on eukaryotic gene expression that are inherited through cell divisions (Francis and Kingston 2001; Mahmoudi and Verrijzer 2001). Research over the last decade has established a critical role for covalent chromatin modifications in the perpetuation of gene expression patterns. However, how specialized DNA sequence elements can bring a linked gene under epigenetic control has remained unclear. *Drosophila* Polycomb response elements (PREs) are epigenetic DNA elements defined by three functional properties: (1) PREs maintain segment-specific silencing of linked enhancers in a Polycomb group (PcG) gene-dependent manner (Muller and Bienz 1991; Simon et al. 1993; Chan et al. 1994). (2) PREs can impose pairing-sensitive silencing (PSS) upon a linked reporter gene (Chan et al. 1994; Kassis 1994). In a *white* mutant genetic background, the *mini-white* transgene product is required for the red eye color of transgenic flies. Normally, flies homozygous for the *mini-white* transgene have darker eyes than their heterozygous siblings, whereas *white* mutants are white-eyed. However, the opposite occurs when *mini-white* is under PRE control. Now, silencing is enhanced when a fly is homozygous for the insertion, causing a lighter eye color than that of heterozygotes. Because repression requires pairing of homologous chromosomes, this phenomenon was named PSS. Like silencing of homeotic genes, PSS is dependent on the PcG proteins. (3) PREs are chromosomal binding sites for PcG proteins (Ringrose and Paro 2004). When integrated in a transposable element, PREs create a new chromosomal binding site for PcG protein complexes.

Two functionally distinct classes of PcG repressor complexes (PRCs), referred to as PRC1 and PRC2, have been identified thus far (Levine et al. 2004). PRC1-type complexes harbor the PcG proteins PC, Polyhomeotic (PH), Posterior Sex Combs (PSC), dRING1, and several other proteins. A PRC1 core complex (PCC), comprising PC, PH, PSC, and dRING1, suffices to mediate the formation of transcription-resistant higher-order chromatin (Francis et al. 2001). In addition, dRING1 can create an epigenetic mark through ubiquitylation of histone H2A (H.B. Wang et al. 2004). PRC2-class complexes contain the Enhancer of zeste [E(z)] histone H3 Lys 27 (H3-K27) methyltransferase, creating a mark for PC binding (Levine et al. 2004; L. Wang et al. 2004). However, there may not be a simple one-on-one relationship between a specific histone methyl mark and PC recruitment (Ringrose and Paro 2004).

Because PREs function in transgenes, it follows that there is a DNA sequence code to impose PcG control. The nature of that PRE code has been elusive and controversial. For a comprehensive discussion of this issue, we refer to an excellent review by Ringrose and Paro (2004). However, there is strong evidence for a key role of PHO in PcG targeting. PHO and its paralog, PHOL, are sequence-specific DNA-binding members of the PcG (Brown et al. 1998, 2003). PHO elements are essential for PRE-directed silencing, mutations in PHO cause PcG phenotypes, and PHO interacts genetically with other PcG genes (Brown et al. 1998, 2003; Mihaly et al. 1998; Fritsch et al. 1999; Mishra et al. 2001). Transcription factors typically function through transient interactions with multiple distinct coregulators. PHO can interact with PRC1 through contacting PC and PH (Mohd-Sarip et al. 2002), and PRC2 through binding to E(z) (L. Wang et al. 2004). Possibly, PRC2 is mainly responsible for the epigenetic maintenance of the repressive mark, whereas PRC1 directly blocks transcription.

Deciphering the sequence requirements for PRE function is critical to understanding how DNA elements can direct cellular memory during development. PHO sites in many PREs form part of a larger conserved sequence motif (Mihaly et al. 1998). The functional dissection of these flanking sequences led us to the identification of the PBE, which is required for PcG silencing in vivo. We found that PHO sites and PBEs constitute an integrated platform for highly cooperative DNA binding by PHO and PCC. Based on our results, we propose that the molecular design of an epigenetic silencer is similar to that of enhanceosomes.

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Results and Discussion

PHO and PCC bind cooperatively to the bxd *PRE*

Because PHO can directly bind two subunits of the PCC complex, PC and PH (Mohd-Sarip et al. 2002), we wished to test whether PHO could recruit PCC to DNA. Figure 1 depicts a schematic representation of the DNA templates and the purified proteins used in this study. As representative PREs we used the *bxd* PRE, located ~25 kb upstream of the *Ubx* transcription start site, and the *iab-7* PRE, located ~60 kb downstream of the *Abd-B* promoter. For our initial binding studies, we focused on PHO sites 4 and 5 within the *bxd* PRE (PHO4/5–PRE), which are required for PcG silencing in vivo (Fritsch et al. 1999). PHO, PHO lacking the 22-amino acid PC- and PH-binding domain (ΔPBD), PC, and PCC were expressed in Sf9 cells using the baculovirus expression system and were immunopurified to near homogeneity from cell extracts.

To test DNA binding by PHO and PCC, we performed DNA mobility shift assays (Fig. 2A). Whereas PHO alone bound weakly to the PHO4/5–PRE (Fig. 2A, lane 2), together with PCC, a PHO/PCC/DNA complex

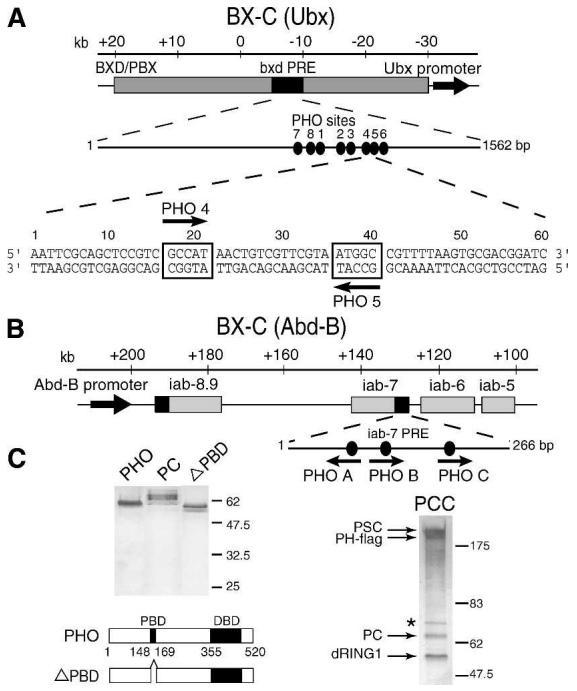

Figure 1. DNA templates and purified proteins. (*A*) Schematic representation of the *Ubx* locus. The BXD/PBX regulatory regions and *bxd* PRE and *Ubx* promoter are indicated. Map positions are according to Bender et al. (1983). The eight PHO sites within the core of the *bxd* PRE are indicated. Numbering is according to Fritsch et al. (1999) and Mahmoudi et al. (2003). For our binding studies, we used a short 50-bp DNA fragment harboring the PHO4 and PHO5 sites, referred to as PHO4/5–PRE. These strong PHO-binding elements are required for *bxd* PRE-directed silencing in vivo (Fritsch et al. 1999). (*B*) Schematic representation of the regulatory region of *Abd-B*. The parasegment-specific regulatory domains *iab-5*, *iab-6*, and *iab-7*, part of the *iab-8*, and the *iab-7* PRE are indicated (Karch et al. 1985). A minimal *iab-7* PRE harboring three PHO sites is used in both reconstituted DNA-binding and in vivo silencing studies (Mihaly et al. 1998). (*C*) Recombinant PHO, PHO lacking the PBD (ΔPBD: amino acids 1–148/169–520), PC, and PCC were immunopurified from extracts of baculovirus-infected Sf9 cells. The eluted proteins were resolved by SDS-PAGE and visualized by silver staining.

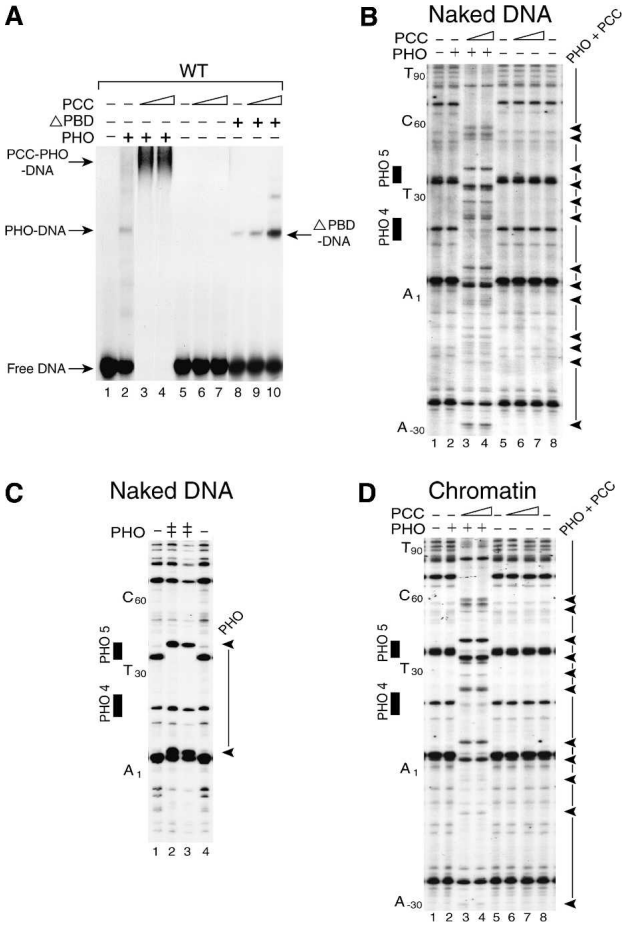

Figure 2. PHO and PCC bind synergistically to the *bxd* PRE. (*A*) PRE binding of PHO, ΔPBD and PCC was studied by bandshift assays. Binding reactions contained ~5 nM PHO and/or ~5–15 nM PCC, and radiolabeled PHO4/5–PRE. PRE binding of PHO and PCC was analyzed by primer extension DNaseI footprinting using either naked DNA (*B*,*C*) or chromatinized PHO4/5–PRE (*D*) templates. Binding reactions on naked DNA contained ~20 nM (+) or 100 nM (‡) PHO and ~20–60 nM PCC. Chromatin binding reactions contained ~50 nM PHO and ~20–60 nM PCC. DNaseI digestion ladders were analyzed by primer extension, resolved on a 6% polyacrylamide gel, and visualized by autoradiography. The positions of the PHO sites and footprinted areas are indicated. Closed arrowheads indicate DNaseI hypersensitive sites induced by protein binding.

was formed very efficiently, resulting in complete saturation of the probe (Fig. 2A, lanes 3,4). In contrast, PCC alone was unable to bind DNA sequence-specifically (Fig. 2A, lanes 6,7). Deletion of the PBD of PHO impaired the synergistic formation of a higher-order PHO/PCC/DNA complex (Fig. 2A, lanes 9,10), revealing the importance of direct protein–protein interactions between PHO and PCC.

To identify the DNA sequences contacted by the PHO/PCC complex, we carried out primer extension DNaseI footprinting assays (Fig. 2B). After addition of PCC to a subsaturating amount of PHO, which by itself does not yield a footprint (Fig. 2B, lane 2), DNA binding was readily detected (Fig. 2B, lanes 3,4). The PHO/PCC footprinted area is very large, comprising ~120 bp, indicative of extensive protein–DNA contacts. As expected, PCC alone is unable to bind DNA sequence-spe-

cifically (Fig. 2B, lanes 6,7). In contrast to PHO/PCC, a saturating amount of PHO generates a small footprinted area of ~40 bp, encompassing the two PHO sites (Fig. 2C, lanes 2,3). Next, we tested whether the cooperation between PHO and PCC also occurred on chromatin templates (Fig. 2D). We used the *Drosophila* embryo-derived S190 assembly system to package the template into a nucleosomal array. PHO alone failed to bind its chromatinized sites (Fig. 2D, lane 2). However, DNA binding was greatly facilitated by the addition of PCC (Fig. 2D, lanes 3,4), which by itself is unable to target the PRE sequence (Fig. 2D, lanes 6,7). We note that we failed to detect PHO binding to chromatin even at the highest amounts we could add (data not shown). Thus, PHO binding to chromatin appears dependent upon PCC. Because nucleosomes are not positioned on these templates, the DNaseI digestion ladder resembles that of naked DNA. Chromatin footprinting requires the use of high amounts of DNaseI, which completely digests any residual naked DNA in the reaction.

To identify specific PCC subunits that directly contact the DNA, we utilized a DNA cross-linking strategy. We generated a radiolabeled PHO4/5–PRE fragment substituted with bromodeoxyuridine (BrdU). After binding of PHO and PCC, the resulting protein–DNA complexes were subjected to ultraviolet (UV) cross-linking. SDS-PAGE analysis, followed by autoradiography, revealed very strong labeling of PHO and PC and weaker labeling of PSC or PH (Fig. 3A, lanes 3,4). We could not resolve the cross-linked PSC and PH well. Because on low percentage gels PSC and PH form a radiolabeled doublet, we assume that both proteins bind DNA. We did not detect labeling of dRING1, suggesting that it does not directly contact DNA. Because PC was strongly cross-linked to DNA and can directly bind PHO (Mohd-Sarip et al. 2002), we tested whether PC can bind DNA together with PHO (Fig. 3B). After addition of PC to a subsaturating amount of PHO (Fig. 3B, lane 3), DNA binding was readily detected (Fig. 3B, lanes 4,5). PC alone is unable to bind DNA sequence-specifically (Fig. 3B, lanes 8–10). Also when PC was added to a saturating amount of PHO, the footprinting pattern changed and was extended (Fig. 3B, lanes 13–15), suggesting additional protein–DNA contacts. Although PC can cooperate with PHO, the level of cooperation and DNA area contacted is modest compared with PHO–PCC (Fig. 3C), emphasizing the contribution of other PCC subunits.

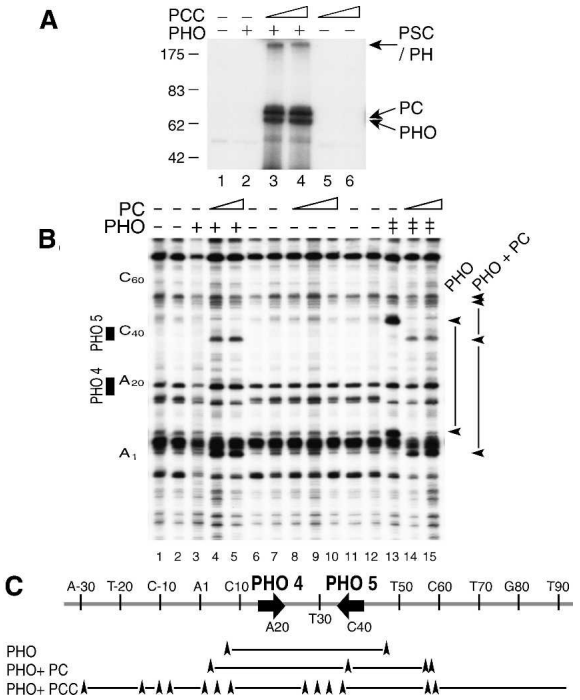

Figure 3. PCC subunits contact DNA. (*A*) UV cross-linking of PHO and PCC subunits to the PRE. Binding reactions contained a ^{32}P-labeled, BrdU-substituted PHO4/5–PRE fragment in the absence or presence of the indicated proteins. After UV cross-linking, proteins were analyzed by SDS-PAGE and visualized by autoradiography. The relative positions of PSC, PH, PC, and PHO are indicated. (*B*) PRE binding of PHO and PC was analyzed by primer extension DNaseI footprinting as described in Figure 2. Reactions contained ~20 or 80 nM PC. (*C*) Summary of the DNaseI footprinting patterns on PHO4/5–PRE.

PHO sites and PBEs constitute an integrated platform for synergistic DNA binding by PHO and PCC

What are the precise DNA sequence requirements for cooperative PRE binding by PHO and PCC? Within many PREs, the PHO core recognition sequence forms part of a larger conserved motif (Mihaly et al. 1998). To determine the functional significance of these sequence constraints, we tested the effect of mutations (Fig. 4A) on PHO binding by DNase footprinting and bandshift analysis (Fig. 4B,C). Whereas the downstream motif (D.mt) had no effect on PHO binding, mutation of the upstream motif (U.mt) reduced PHO affinity. As expected, mutation of the core PHO site (C.mt) abrogated PHO binding. These results suggested that the sequence constraints directly upstream of the PHO core site reflect an extension of the PHO recognition site. The sequence downstream of the PHO site, however, appeared to play no role in PHO binding. Therefore, an attractive possibility was that this motif might mediate docking of PCC and function as a PCC-binding element (PBE). To determine whether synergistic PHO/PCC complex assembly is dependent on each PHO site or the downstream sequence motifs, we mutated individually each PHO site and putative PBEs. Strikingly, each mutation aborted formation of the PHO–PCC–DNA complex (Fig. 4D). Likewise, synergistic binding of PHO and PC was also abrogated by PBE mutations (data not shown). We conclude that cooperative DNA binding of PHO and PCC is strictly dependent on the presence of at least two PHO sites and their juxtaposed PBEs (Fig. 4E).

The PBE is required for PRE silencing in vivo

The conservation of the PBE (Mihaly et al. 1998) and its requirement for cooperative DNA binding by PHO and PCC led us to test if it is also critical for PRE-directed silencing in vivo. We turned to the minimal 260-bp *iab-7* PRE, for which an extensive collection of control lines has already been established (Mishra et al. 2001). The *iab-7* PRE harbors three PHO/PBE elements, but their spacing and phasing is very different from that in the *bxd* PRE. We tested whether PHO and PCC bind cooperatively to the *iab-7* PRE (Fig. 5A). In agreement with our results on the *bxd* PRE, PHO and PCC synergistically recognized the *iab-7* PRE, resulting in a very large DNaseI footprint, including all three PHO and PBE ele-

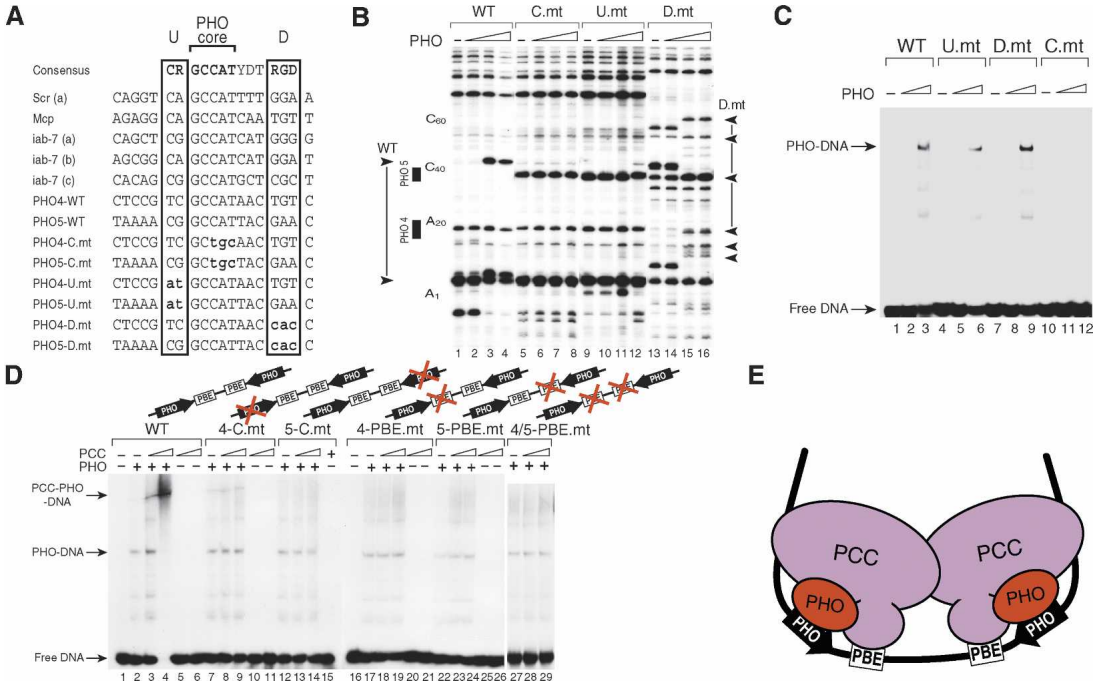

Figure 4. The PBE is required for PCC recruitment to the PRE. (*A*) Alignment of a selection of PHO sites and their flanking motifs from Mihaly et al. (1998), including the *bxd* PRE PHO sites 4 and 5 and *iab-7* PHO sites. The PHO/YY1 consensus core (C) sequence GCCAT and the upstream (U) and downstream (D or PBE) flanking motifs are indicated. The U.mt, C.mt, and D.mt are indicated for PHO4 and PHO5. Corresponding D.mts were induced in the *iab-7* PBEs (Fig. 5). The effects of mutations on PHO binding were tested by DNaseI footprinting on the PHO4/5 PRE (*B*) and by bandshift analysis (*C*). (*D*) Synergistic binding of PHO and PCC requires both PHO4 and PHO5 sites and both accompanying PBEs. Binding was assayed as described above using probes harboring the indicated mutations. (*E*) Model illustrating that cooperative DNA binding by PHO and PCC requires (1) at least two PHO sites, (2) their juxtaposed PBEs, and (3) direct protein–protein interactions between PHO and PCC. PHO sites and PBEs form an integrated platform for the synergistic assembly of a repressive PHO/PCC/PRE nucleoprotein complex. Details are discussed in the text.

ments (Fig. 5A, lanes 1–4). Cooperative binding of PHO and PCC was completely abolished by mutations in the three PBEs juxtaposing the PHO sites (Fig. 5A, lanes 7–10). Thus, the PBEs are required for PHO/PCC complex formation on both the *bxd* and the *iab-7* PRE.

Next, we tested the effects of PBE mutations on in vivo silencing. Because the site of integration within the genome influences silencing, repression does not occur in all transgenic lines. Therefore, PSS is expressed as the percentage of lines that show repression. We established independent lines harboring the *mini-white* transgene under control of either the minimal 260-bp *iab-7* PRE or the PBE mutant PRE (PBE^{mt} iab-7) (Fig. 5B–E). We raised 48 homozygous viable lines with the wild-type PRE in front of the *mini-white* gene. In 46% of these lines, homozygotes (P[w$^+$]/P[w$^+$]) have much lighter eyes than their heterozygous (P[w$^+$]/+) siblings, revealing PSS. In 8% of the lines, the eye color of homozygotes is about the same as that of heterozygotes, reflecting weak PRE-directed silencing. In the remaining 46% of the lines, no PSS was observed and the eyes of homozygotes were darker than that of heterozygotes. In summary, recruitment of a PcG repressing complex is observed in more than half of the generated lines. Strikingly, when the PBEs were mutated, only one line (4.5%) out of a total of 22 analyzed showed strong repression of the *mini-white* gene in homozygotes, and for five lines (23%), homozygotes had an eye color similar to that of their heterozygous siblings. It is worthwhile noting that in the case of the wild-type *iab-7* PRE, the majority of repressed lines showed strong repression (22 of 26). In contrast, the majority of the repressed lines (five of six) harboring the mutant PRE display only weak silencing. Thus, not only is the proportion of repressed lines decreased in the mutant *iab-7* PRE lines but the efficiency of repression is also lowered. These results strongly support the notion that the PBE is a critical PRE element, required for the assembly of a functional repressive PcG complex in vivo.

PcG proteins form a silenceosome on PREs

A central problem in understanding epigenetic gene regulation is how specialized DNA elements recruit silencing complexes to a linked gene. Here, we identified the PBE, a small conserved sequence element required for PcG silencing in vivo. Our results suggest that PHO sites and their juxtaposed PBEs function as an integrated DNA platform for the assembly of a repressive PHO/PCC complex. In a previous study, the failure of PHO sequences fused to a heterologous DNA-binding domain to nucleate the assembly of a silencing complex was interpreted as an argument against its role as a tether of other PcG proteins (Poux et al. 2001). However, in light of the critical role of the PBE in PcG silencing, it is not to be expected that artificially tethered PHO can support PcG complex assembly.

Synergistic PHO/PCC/PRE nucleocomplex formation was strictly dependent on the presence of at least two PHO sites, their accompanying PBEs and protein–protein

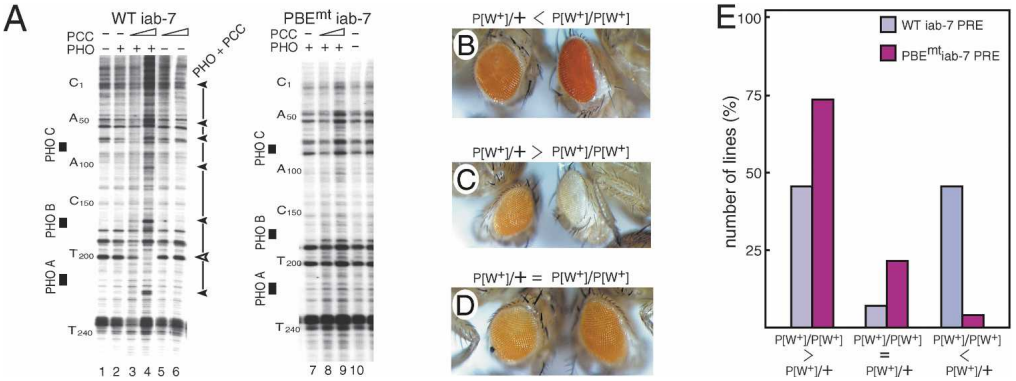

Figure 5. The PBE is required for PRE silencing in vivo. (*A*) Binding of PHO/PCC to the *iab-7* PRE is abrogated when the PBEs are mutated (for sequences, see Fig. 4A), as revealed by DNaseI footprinting. (*B–D*) Mutations in the PBE impair PRE-directed PSS. PSS of *mini-white* by the minimal 260-bp wild-type *iab-7* PRE or a mutant, PBE-less *iab-7* PRE (PBE^{mt} *iab-7* PRE) was compared. Multiple independent lines were established harboring the *mini-white* transgene under control of either the wild-type or PBE^{mt} *iab-7* PRE. For each line, the eye color of homozygotes for the insertion was compared with that of heterozygotes. Representative examples are shown: (*B*) Homozygotes have a much darker eye color than their heterozygous siblings (P[w$^+$]/P[w$^+$]P[w$^+$]/+), revealing the absence of PSS. (*C*) Homozygotes have much lighter eyes than heterozygotes ((P[w$^+$]/P[w$^+$][w$^+$]/+), showing strong PSS. (*D*) The eye color of homozygotes is equal (P[w$^+$]/P[w$^+$] = P[w$^+$]/+) to that of heterozygotes, revealing impaired PSS. (*E*) The number of lines exhibiting no PSS, impaired PSS, or strong PSS were scored and depicted in a graphical representation with the number of lines displaying a PSS phenotype expressed as percentage of the total number of lines analyzed on the *Y*-axis and the phenotype on the *X*-axis. Because the percentages refer to the numbers of independent lines, error bars reflecting the SEM are not applicable. χ^2 test of statistical significance for bivariate tabular analysis confirmed the high significance of the difference in PSS frequency between lines harboring either the wild-type or PBE^{mt} *iab-7* PREs ($\chi^2 = 12.3$, $p < 0.01$).

interactions between PHO and PCC. Our observations revealed a striking similarity in the design of PREs and enhancers. The cooperative assembly of unique transcription factor–enhancer complexes, termed enhanceosomes, is also dependent upon a stereospecific arrangement of binding sites and a reciprocal network of protein–protein interactions (Carey 1998). Thus, the basic principles governing the assembly of distinct higher-order nucleoprotein assemblages with opposing activities are surprisingly similar. To reflect the generality of these rules, we propose to refer to PRE-bound PcG silencing complexes as silenceosomes.

Like enhancers, PREs are complex and their activity involves the combined activity of distinct recognition elements and their cognate factors. In addition to PHO/PBE sites, these modules include the (GA)n-element, recognized by GAGA or Pipsqueak; Zeste sites (Ringrose et al. 2003; Levine et al. 2004; Ringrose and Paro 2004); and the recently identified GAAA motif bound by DSP1, a fly HMGB2 homolog (Dejardin et al. 2005). Finally, histone modifications, including H2A and H2B (de)ubiquitylation, and H3-K27 or H3-K9 methylation, play a critical role in PRE functioning (Levine et al. 2004; Ringrose and Paro 2004; H.B. Wang et al. 2004; van der Knaap et al. 2005). One scenario is that silenceosome formation is nucleated by direct DNA binding and contextual protein–protein and protein–DNA interactions. Next, the silenceosome could be stabilized further through multivalent interactions with the histones guided by selective covalent modifications. The available evidence strongly suggests that a cooperative network of individually weak protein–DNA and protein–protein interactions drive the formation of a PcG silencing complex. We propose that the molecular principles governing silenceosome or enhanceosome formation are very similar.

Materials and methods

DNA constructs and proteins

All cloning and site-directed mutagenesis was performed using standard methods and was verified by sequencing. Details are available upon request. Oligonucleotides harboring wild-type or mutant PHO/PBE sites were cloned into the EcoRI and BamHI sites of pBluescript (PHO4/5-*bxd* PRE). The *iab-7* PRE construct has been described (Mishra et al. 2001). PHO/PBE wild-type and mutant sequences are shown in Figure 3A. DNA sequences encoding PHO or ΔPBD (amino acids 1–148/169–520) were cloned into pVL1392-Flag; PC was cloned in pVL1392-HA (Mohd-Sarip et al. 2002). Recombinant proteins were expressed in Sf9 cells using the baculovirus system and immunopurified as described (Mohd-Sarip et al. 2002). Reconstituted PCC was expressed and purified as described (Francis et al. 2001).

DNA binding assays

Footprinting templates were used either as naked DNA or assembled into chromatin and used in DNaseI primer extension footprinting assays essentially as described (Mahmoudi et al. 2003) with the following modifications. Binding reactions were for 90 min at 25°C. Primer extension was performed using radiolabeled T3 or T7 primers for PHO4/5-bxd PRE or *iab-7* PRE, respectively. Products were resolved on a 6% denaturing polyacrylamide gel. Dideoxy DNA sequencing reactions were run in parallel to map the footprints. DNA bandshift assays were performed essentially as described (Mohd-Sarip et al. 2002). Binding reactions were in 20 µL 0.5× HMG buffer buffer (12.5 mM HEPES-KOH at pH 7.6, 6.25 mM $MgCl_2$, 5% glycerol) containing 70–80 mM NaCl, 50 µg/m BSA, 0.05% NP-40, 1mM DTT, 1 µg poly(dGdC)-poly(dGdC), and ~60 fmol of double-stranded labeled probe. All binding reactions were carried out on ice for 90 min and were analyzed on 4% polyacrylamide gels run overnight (~14 h) at 4°C in 0.5× Tris-glycine buffer, 0.01% NP-40. For UV cross-linking analysis, a ^{32}P-bodylabeled, BrdU-substituted PHO4/5-bxdPRE probe was generated by PCR and used in binding reactions containing 100 ng of poly(dG-dC)-poly(dGdC) but otherwise as described for bandshifts. Processing and analysis was as described (Verrijzer et al. 1995).

PSS analysis

The 260-bp *iab-7* PRE fragment (Mishra et al. 2001) and PBE^{mt} *iab-7* PRE were inserted in the unique EcoRI site upstream of the *mini-white* gene of pCaSpeR. Relevant sequences are shown in Figure 4A. Multiple independent lines harboring either the wild-type or the PBE^{mt} *iab-7* PRE *mini-white* transgene were established. For each line, the eye color of homozygotes for the insertion was compared with that of heterozygotes and grouped in one of three classes: no PSS, homozygotes have a darker eye color than their heterozygous siblings (P[w$^+$]/P[w$^+$] > P[w$^+$]/+); strong PSS, homozygotes have lighter eyes than heterozygotes (P[w$^+$]/P[w$^+$] < P[w$^+$]/+); and impaired PSS, the eye color of homozygotes is equal (P[w$^+$]/P[w$^+$] = P[w$^+$]/+) to that of heterozygotes. Significance analysis was

performed by χ^2 test for bivariate tables. The number of lines displaying a given PSS phenotype was expressed as a percentage of the total number of lines.

Acknowledgments

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