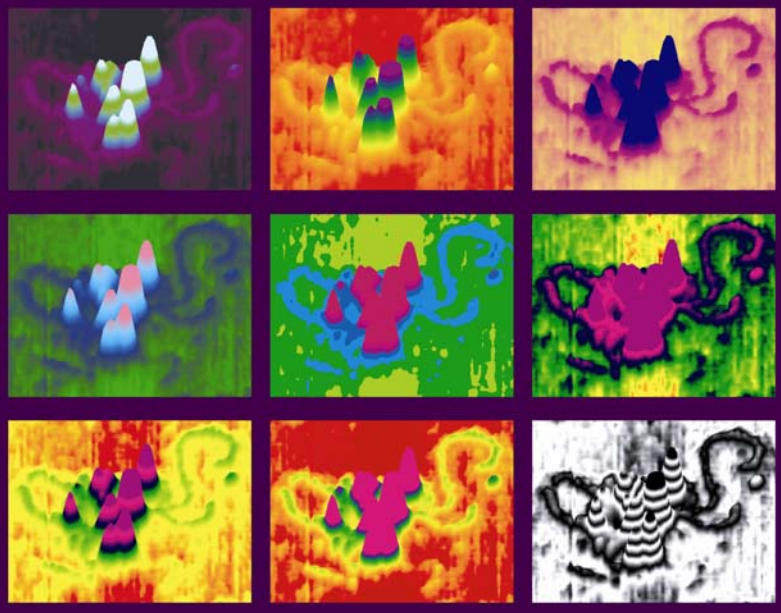
Role of Pleiohomeotic in targeted gene silencing by Polycomb group proteins



Adone Mohd Sarip

Role of Pleiohomeotic in Targeted Gene Silencing by Polycomb Group Proteins

De rol van Pleiohomeotic in selectieve gen-repressie door Polycomb groep eiwitten

Thesis

to obtain the degree of Doctor from the
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Cover: Images of protein complexes on DNA obtained by Scanning force microscopy.
Cover design by Adone Mohd Sarip

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Contents

Chapter 1

Introduction

Drosophila melanogaster

During the development of multicellular organisms, cells become different from one another by changing their genetic program in response to transient stimuli. Long after the stimulus is gone, "cellular memory" mechanisms enable cells to remember their chosen fate over many cell divisions. Epigenetics is the study of reversible heritable changes in gene function that occur without a change in the sequence of DNA. It is also the study of the processes involved in the unfolding development of an organism. In both cases, the object of study includes how gene regulatory information that is not expressed in DNA sequences is transmitted from one generation (of cells or organisms) to the next.

The epigenetic program orchestrated by specialized group of genes has been the topic of interest for current research especially in the fruit fly, *Drosophila melanogaster*. Flies have proved to be an ideal and most valuable model organism in biological research especially in the areas of genetics and developmental biology for nearly a century. Specialized cell clusters present in flies known as the imaginal discs, have been determined for a particular fate during early embryogenesis, and continue to ‘remember’ their cell fate (Hadorn, 1978). Studies of fly genetics reveal an evolutionary conserved class of genes, the homeotic or Hox genes (McGinnis and Krumlauf 1992; Simon 1995). Hox genes are responsible for the correct positioning of the fly body parts along the anterior-posterior axis. Products of hox genes are required continuously once the cell fate is determined and throughout cell division. Two main gene clusters make up the homeotic genes; Antennapedia complex (ANT-C) and the bithorax complex (BX-C) (McGinnis and Krumlauf 1992). ANT-C contains five genes that regulate the head development and first two thoracic segments. The large genomic locus, BX-C contains the three genes, Ultrabithorax (Ubx), abdominal A (abd-A) and Abdominal B (Abd-B), and controls the third thoracic segment and all the abdominal segments. Initial establishment of the homeotic genes are brought about by the combined activities of the transiently expressed early activators and repressors. After the disappearance of these early regulators, the maintenance of the homeotic genes is taken over by the trithorax group (trxG) of activators and Polycomb group (PcG) of repressors. Misregulation of homeotic gene function can lead to transformation of fly body parts (Lewis, 1963). These two antagonistic group of genes were identified from genetic screens of flies with specific segmental transformations. An important question to address is what are the underlying mechanisms that regulate cellular memory. It appears that the maintenance genes, trxG and PcG, play significant roles in forming the molecular mechanisms of epigenetics.

Biochemical properties of PcG proteins

Genetic screens based on homeotic derepression have so far identified 13 Drosophila PcG genes while up to 30 more PcG members may exist (Kennison, 1995). PcG proteins are ubiquitously expressed and effects of mutations in members of PcG proteins frequently result in homeotic transformation, due to misexpression of genes. PcG group proteins are involved in maintaining silenced gene expression patterns in the fly. The

synergistic effects of distinct PcG mutations first suggested that they act in concert to repress target genes (Kennison, 1995). This notion is supported by the co-localization of different PcG proteins on multiple sites in *Drosophila* polytene chromosomes (DeCamillis et al., 1992; Lonie et al., 1994; Orlando et al., 1998; Rastelli et al., 1993; Strutt and Paro, 1997; Zink and Paro, 1989). Direct evidence for the existence of multiprotein PcG complexes was provided by biochemical experiments in flies and mammals. Most PcG proteins contain very well-conserved domains that are often utilized for multimerization and protein-protein interactions to form multiprotein complexes (Figure 1 and Table 1) (Alkema et al., 1997; Franke et al., 1992; Kyba and Brock, 1998; Satijn and Otte, 1999; Shao et al., 1999; Strutt et al., 1997).

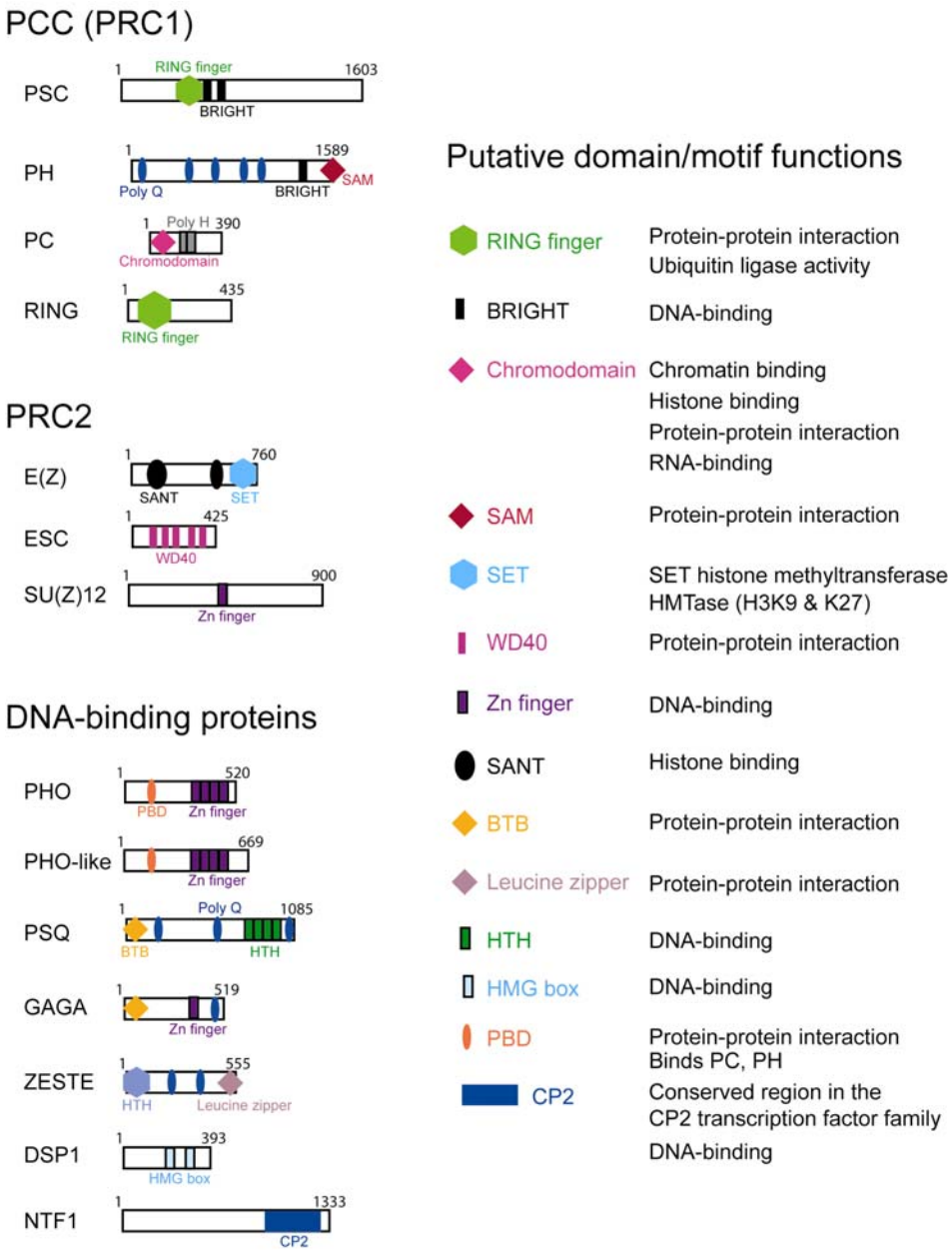


Figure 1. Putative domain motifs of *Drosophila* proteins.

Table 1. Biochemical properties and domain functions of PcG proteins.

PcG proteins	Biochemical Functions
Posterior sex combs (PSC) - PRC1	Chromatin compaction (Francis et al., 2005) Ubiquitin ligase activity
Polyhomeotic (PH) - PRC1	Recruitment of chromatin in trans (with dRING1, but not alone) Oligomerization (SAM domain) (Kim et al., 2002) Sop-2 is sumoylated (Zhang et al., 2002)
Polycomb (PC) - PRC1	Drosophila chromodomain binds H3K27Me3 & H3K9Me3 (Fischle et al., 2003) HPC2 has Sumo E3 activity (Kagey et al., 2003)
dRING/Sex combs extra (Sce) - PRC1	Ubiquitination of H2A (Wang L. et al., 2004) Structural role in PRC1 core
Extra sex combs (Esc) - PRC2	Increase of HMTase activity of E(z) (Nekrasov et al., 2005) Mammalian Eed direct their HKMT activity toward H3K27 & H1K26
Enhancer of zeste (E(z)) - PRC2	HMTase towards H3K9 & H3K27 (Wang L. et al., 2004) H3K27 methylation is associated with PREs
Supressor of zeste 12 (Su(z)12) - PRC2	Nucleosome binding Increase of HMTase activity of E(z) (Nekrasov et al., 2005)
Rpd3	Interacts with HP1 HDAC (Chang et al., 2001)
Nucleosome remodelling factor 55 (NURF55)	Nucleosome binding Increase of HMTase activity of E(z) (Nekrasov et al., 2005)
Polycomb-like (PC-like) - PRC2	Interact with E(z) mediated by Pcl PHD finger PHD finger
Sex comb on midleg (Scm) - PRC1	Copolymerization with PH (SAM domain) (Kim et al., 2005) Self-association (SAM domain)
Ubiquitin-specific protease 7 (usp7)	Deubiquitylation of H2B (van der Knaap et al., 2005) Genetically interacts with PC
Pleiohomeotic (PHO)	Sequence-specific DNA-binding: GCCATHWY (Fritsch et al., 1999) Recruits PcG proteins to PREs (Brown et al., 1998) Can interact with PRC1, PRC2 & Brahma components
Pleiohomeotic-like (PHO-like)	Sequence-specific DNA-binding: GCCATHWY (Brown et al., 2003) Can interact with PRC2 components Recruits PcG proteins to PREs
Pipsqueak (PSQ)	Sequence-specific DNA-binding: GA(n) (Huang et al., 2002) Recruits PcG proteins to chromatin
Dorsal switch protein 1 (DSP1)	DNA-binding: GAAAA (G(A) motif) (Dejardin et al., 2005) Recruits PcG proteins to PREs
Grainyhead/ Neuronal transcription factor 1 (GRH/NTF1)	DNA-binding: KWNYGGTTTTGWA (Johnson et al., 1989) Recruits PcG proteins to PREs

PcG proteins are very well-conserved throughout evolution (Figure 2 and Table 2). Several different complexes have so far been identified and exist at various stages of *Drosophila* development. At least two distinct evolutionary conserved PcG protein complexes exist. One has been broadly categorized as Polycomb repressive complex 1 (PRC1) and consists of the core proteins posterior sex combs (PSC), Polyhomeotic (PH), Polycomb (PC) and dRING1, which is referred to as the Polycomb core complex (PCC). Besides those core subunits, PRC1 components also include transcription factors and several other proteins (Saurin et al., 2001). This multiprotein complex is thought to block transcription by creating a higher order chromatin structure. The PRC2 complex directs trimethylation of histone H3 lysine 27 (H3K27) by Enhancer of zeste (E(z)) (Levine et al., 2004; Wang L. et al., 2004), and harbors other subunits such as extra sex combs (ESC), suppressor of zeste-12 (Su(z)12), histone deacetylase HDAC1 (Rpd3) and nucleosome remodeling factor 55 (Nurf55) (Cao et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Methylation of histones cannot be carried out by E(z) alone, but also requires ESC and SU(z)12-Nurf55 (Nekrasov et al., 2005). The methyl mark generated is also recognized by the chromodomain (chromatin binding

domain) of PC (from the PRC1 complex), and that loss of methylation of histone H3K27 correlates with loss of PC, which clearly indicates that extensive cross-talk exists between these two complexes (Cao et al., 2002; Czermin et al., 2002, Fischle et al., 2003). Histone deacetylase activity has also been linked to both the PRC1 and 2 complexes and PcG silencing (Chang et al., 2001; Saurin et al., 2001; Tie et al., 2001).

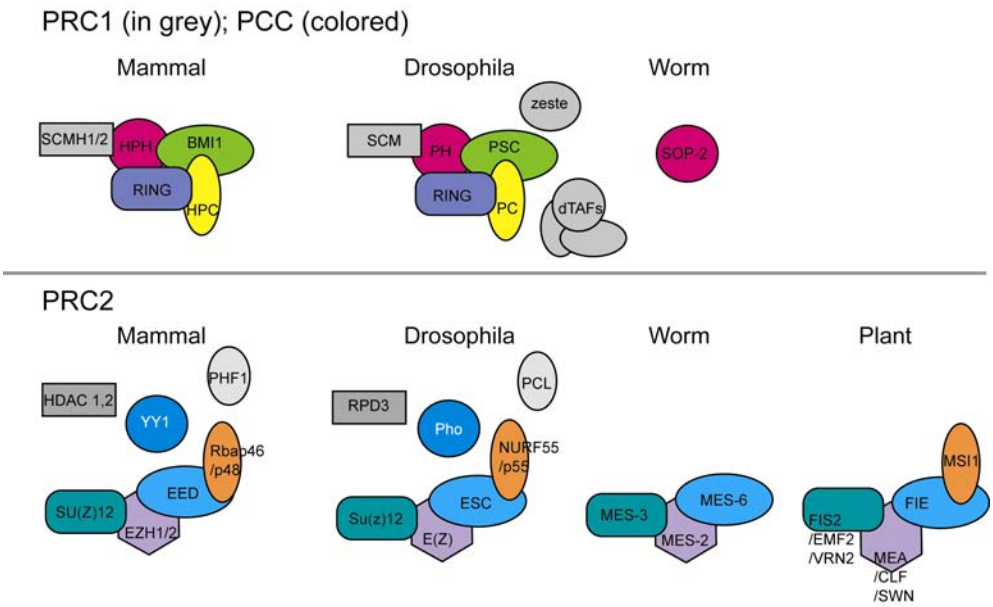


Figure 2. PcG complexes balloon model.
Protein subunits that make up PRC1, PCC and PRC2 are depicted, with homologues in mammal, *Drosophila*, worm and plant shown with the same colors.

Table 2. PcG proteins homologs in mammals and *Drosophla*.

Mammals	Drosophila
PRC1	
HPC1 (M33) HPC2 (Mpc2) HPC3	Pc (Polycomb)
HPH1 (Mph1/Rae28) HPH2 (Mph2) HPH3	Ph (Polyhomeotic)
RING1A (Ring1a) RING1B (Ring1b)	dRING/Sce (Sex combs extra)
BMI1 (Bmi1/Mel18)	Psc (Posterior sex combs)
YY1 (yy1)	Pho (Pleiohomeotic) Pho-like
SCML1 (Scmh1) SCML2 (Scmh2)	Scm (Sex comb on midleg)
PHF1	Pcl (Polycomb-like)
PRC2/3	
EED (Eed)	Esc (Extra sex combs)
EZH1 (Ezh1) EZH2 (Ezh2)	E(z) (Enhancer of zeste)
SUZ12	Su(z)12 (Supressor of zeste 12)
Rbap46/48	NURF55 (Nucleosome remodelling factor 55)
HDAC1,2	Rpd3

In light of recent exhaustive studies on the identification of the biochemical compositions and functions of the PcG proteins in *Drosophila*, the mammalian homologues have also been characterized and are implicated in several diseases (Figure 2). Abnormally expressed PcG genes have been linked to cell cyle regulation. B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) has been implicated in tumourigenesis and overexpressed Bmi1 and PRC2 subunits has been suggested to regulate Bmi1 expression which could lead up to cellular behavioral changes (Bea et al., 2001; Dukers et al., 2004; Dimri et al., 2002; Kim et al., 2004; Raaphorst et al., 2000; Raaphoorst et al., 2004; Sanchez-Beato et al., 2004; van Kemenade et al., 2001; Visser et al., 2001; Vonlanthen et al. 2001). Stem cell fate is also determined by PcG genes where the mouse homologue of PH (PRC1 complex) i.e. Mph1 is involved in stem cell renewal (Kim et al., 2004).

Progress in genetic studies recently carried out in plants, have implicated members of the PRC2 proteins to be involved in the epigenetic control of their development. The developmental processes that the plant homologues (Figure 2) are associated with are repression of floral homeotic genes and vernalisation (Chanvivattana et al., 2004; Goodrich et al., 1997; Grossniklaus et al., 1998; Guitton et al., 2004; Hennig et al., 2003; Katz et al., 2004; Kinoshita et al., 2001; Kohler et al., 2003; Ohad et al., 1999; Yoshida et al., 2001). Homologues of PcG proteins have also been identified in the nematode, *Caenorhabditis elegans* (Figure 2). These involve the regulation of Hox gene expression where they are required for normal anteroposterior patterning during larval development as well as in global gene repression mechanisms (Holdeman et al., 1998; Korf et al., 1998; Pires-daSilva and Sommer, 2003; Ross and Zarkower, 2003; Zhang et al., 2003).

What constitute a Polycomb response element (PRE) and is there a PRE code?

PcG silencing is thought to be mediated by specialized *cis*-acting DNA sequences or epigenetic regulatory elements that are also known as Polycomb response elements (PREs). These maintenance elements (Brock and van Lohuizen, 2001) or cellular memory modules (Cavalli and Paro, 1998) are required for continuously maintaining the silenced state of gene expression (Busturia et al., 1997). Classification of PREs are based on their silencing effect on reporter genes in transgenic flies and position effect variegation (PEV) (Busturia and Bienz, 1993; Chan et al. 1994; Fauvarque and Dura, 1993; Gindhart and Kaufman, 1995; Kassis, 1994; Muller and Bienz, 1991; Pirrotta and Rastelli 1994; Simon, 1995; Simon et al., 1993; Zink et al., 1991; Zink and Paro 1995). Besides that, P-element transpositions with a PRE sequence create new binding sites for PcG proteins at insertional sites (Chan et al., 1994; Chiang et al., 1995; Zink and Paro, 1995). PcG proteins have been shown to be associated to PREs in chromatin immunoprecipitation (ChIP) studies and *Drosophila* polytene chromosomes staining (Orlando and Paro, 1993; Orlando et al, 1998; Strutt et al., 1997). PREs are located in the homeotic loci and can regulate genes over long distances. Several PREs have been shown to act together cooperatively leading to enhanced silencing (Barges

et al., 2000; Lyko and Paro, 1999; Mihaly et al., 1997; Mohd-Sarip et al., 2005, this thesis, Chapter 3; Pirrotta, 1998).

So far, no known PREs have been identified in mammals, even though most of the core PRC1 and PRC2 components are fully conserved from flies to mammals. The question as to whether a PRE code exists has remained elusive and controversial for a long time. PREs can function in transgenes; it appears that a DNA sequence code suffices in imposing PcG control. PREs range from several hundred to a few thousand kilobases of DNA, but the core activity is usually within less than a few hundred base pairs (Mahmoudi et al., 2003; Mohd-Sarip et al., 2005, this thesis Chapter 3). There are usually clusters of sequence-specific DNA-binding sites of PcG proteins that are abundant in several PREs (Americo et al., 2002).

Studies have shown that the presence of more than one DNA-binding site on PREs enhance silencing in flies and that the DNA-binding sequences are clustered and function together as one integrated unit (Francis and Kingston, 2001; Lyko and Paro, 1999; Mahmoudi and Verrijzer, 2001; Ringrose et al., 2003). This points to the direction that cooperativity of these sites plays an important function in the formation of a PcG repressive complex on the PREs. It also appears that several sequence-specific DNA-binding proteins have been implicated in functioning as recruiters for PcG gene-dependent silencing on PREs. Potential recruiters such as Pleiohomeotic (PHO) (Brown et al., 1998), Pleiohomeotic-like (PHOL) (Brown et al., 2003), pipsqueak (PSQ), GAGA (Mahmoudi et al., 2003; Busturia et al., 2001), grainyhead (GRH) (Blastyak et al., 2006) and dorsal switch protein 1 (DSP1) (Dejardin et al., 2005) have been shown to bind to their sites on the PREs, as well as being able to interact with PcG proteins leading to gene silencing. Each of these recruiters do not necessarily work alone, since there could be some redundancy. Possibly, they require multiple and combination of sites for synergistic binding with other PcG proteins. This further proves that a complex combinatorial network is involved in gene repression. At this moment, there is no evidence of how strict these sites are organized in PREs. The spacing between those sites; identical or non-identical, and their orientations have yet to be deciphered.

Further evidences suggest extensive changes occur to compositions of PcG proteins during cell development. In light of the fact that most subunits of the PRCs lack sequence-specific DNA-binding activity, other variants of the PcG complex has been purified, which happens to contain DNA-binding proteins, implicating the essential roles of recruiters for PcG complex formation on PREs. Poux et al. (2001) found that two sequence-specific DNA-binding proteins, PHO and GAGA, together with PH, PC, ESC, EZ and Rpd3, were present in a complex isolated from the pre-blastoderm embryo. This complex appears to be transient, because after blastoderm, this PRE complex split into two separate complexes. One containing ESC, EZ, PHO and Rpd3, while the other containing PC, PH, GAGA factor, Rpd3 and PSC (Poux et al., 2001). This rearrangement is suggestive of different complexes interacting with the PREs and the promoter complex. This promoter complex could be related to the PRC1 complex purified by Saurin et al. (2001), which also contains TFIID. PHO

continues to be associated with the ESC/E(Z) complex even in the later stages of embryo development (Poux et al., 2001), which proves that it still binds to the PREs, however, other studies did not detect PHO in purified ESC/E(z) complex (Tie et al., 2001). Another variant of the PcG protein complex has been reported and contains the sequence-specific DNA-binding protein, PSQ (Huang and Chang, 2004).

Sequence-specific DNA-binding proteins or 'recruiters'

A highly debated issue is how the ubiquitously expressed PcG proteins are recruited to PREs. Several DNA-binding proteins belonging to the PcG family of proteins have been implicated in recruiting PcG proteins to the PREs (Figure 3). One of the essential key players in recruiting PcG proteins to PREs is Pleiohomeotic (PHO) (Brown et al., 1998; Fritsch et al., 1999). PHO is a zinc finger DNA-binding protein and is the homologue of the mammalian transcription factor Yin-Yang 1 (YY1), which also binds to similar core DNA sequence as PHO (Hyde-DeRuyscher et al., 1995; Yant et al., 1995). Core PHO sites are also present in many PREs, clearly suggesting their importance in recruitment (Mihaly et al., 1998). Recently, we identified a conserved sequence motif present in most PREs, named PCC-binding element (PBE), flanking the core PHO binding sites. We found that PHO sites and PBEs constitute an integrated platform for highly cooperative DNA-binding by PHO and PCC (Mohd-Sarip et al., 2005, this thesis Chapter 3). Possibly, there could be more PRE sequences to be identified that are essential in silencing and that PcG proteins could be making much more extensive contacts on DNA than previously anticipated. PHO-like (PHOL), a protein closely related to PHO (Brown et al., 2003) has been reported to recruit PRC2 (which can methylate histone H3K27) followed by the recruitment of PRC1, since PC (which belongs to PRC1) preferentially binds methylated histones (Wang L. et al., 2004). Recruitment of PcG proteins to PREs are not prevented by mutants of PHO and PHOL as seen on polytene chromosomes (Brown et al., 2003) and PHO binding sites are also insufficient for reconstitution of *in vivo* maintenance of gene expression (Strutt et al.,1997). On the other hand, *in vivo* studies have shown that mutations in PHO sites and mutated forms of the PHO protein itself, do affect PcG silencing (Fritsch et al., 1999; Girton and Jeon, 1994; Mohd-Sarip et al., 2002, this thesis Chapter 2; Mohd-Sarip et al., 2005, this thesis Chapter 3). There are possibly other prospective candidate recruiters, which could compensate for the insufficiency of maintaining repression. These candidate recruiters are discussed below.

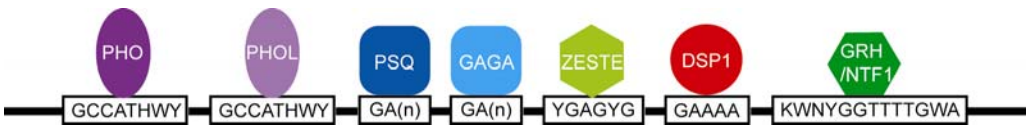


Figure 3. Potential recruiters in targeting PcG proteins to PREs.
Sequence-specific DNA-binding proteins are denoted in varying colored shapes with their corresponding DNA-binding sequence in boxes.

Pipsqueak (PSQ), a sequence-specific DNA-binding protein, belongs to the Chromatin associated silencing complex for homeotics (CHRASCH) (Huang et al., 2004; Hur et al., 2002) and this complex has histone deacetylase activity. PSQ recognizes the (GA)n consensus motif (Huang et al., 2002; Lehman et al., 1998), which is also recognized by GAGA (GAGA factor; GAF). GAGA encodes the trithorax-like gene and belongs to the trxG family of activators. However, GAGA has been shown to act cooperatively with other DNA-binding proteins such as PHO (Mahmoudi et al., 2003; Busturia et al., 2001), in recruiting PcG proteins to PREs. GAGA's true function remains unclear. This brings us to a more attractive candidate, PSQ, since it binds to the same DNA sequence as GAGA, but instead belongs to the PcG of repressors (Huang et al., 2002). On top of that, PSQ is able to target a major PcG complex, CHRASCH to (GA)n sites.

Recently, a protein belonging to the high mobility group (HMG) box family of proteins, the dorsal switch protein 1 (DSP1) protein (Brickman et al., 1999; Decoville et al., 2001; Lehming et al., 1994;), was shown to be able to recruit PcG to chromatin (Dejardin et al., 2005). DSP1 binds GAAAA sites and with artificially made PHO and GAGA sites, these sequences were sufficient to recruit PcG proteins (Dejardin et al., 2005).

A key regulatory gene that is involved in fly development is the transcription factor grainyhead (GRH), also known as neuronal transcription factor 1 (NTF-1). It has been demonstrated that the GRH-dRING complex formation on regulatory DNA elements is involved in GRH-mediated repression (Tuckfield et al., 2001). Besides, GRH is a sequence-specific DNA binding protein that could be a potential recruiter of PcG proteins to PREs. Recently, GRH and PHO was shown to act cooperatively in targeting PcG proteins (Blastyak et al., 2006).

Zeste has dual roles in that it is involved in gene activation and repression (Hur et al., 2002; Laney and Biggin, 1997) which is suggestive of varying ways of Zeste being recruited to the PREs. Zeste binding sites are present in PREs and on polytene chromosomes, it binds to more than 60 sites, where the majority colocalize with PcG proteins (Rastelli et al., 1993). The presence of Zeste binding sites in the Ubx promoter, Zeste binding to its sites (Laney and Biggin, 1997) and its oligomerization properties might link the promoter and distant PREs (Chen and Pirrotta, 1993).

The above-mentioned sequence-specific DNA-binding proteins are clearly intriguing due to their involvement in recruiting members of the PcG proteins to the PREs and thereafter maintaining gene silencing throughout multiple rounds of cell divisions. Remarkably, there appears to be a certain degree of redundancy as well as cooperativity with those proteins.

Drosophila genetics

Three well-established assays have often been used to study the effects of silencing by PcG proteins and PREs (Figure 4A-C). These assays especially have proved to be extremely useful in characterizing whether certain *cis*-regulatory DNA region works as a PRE.

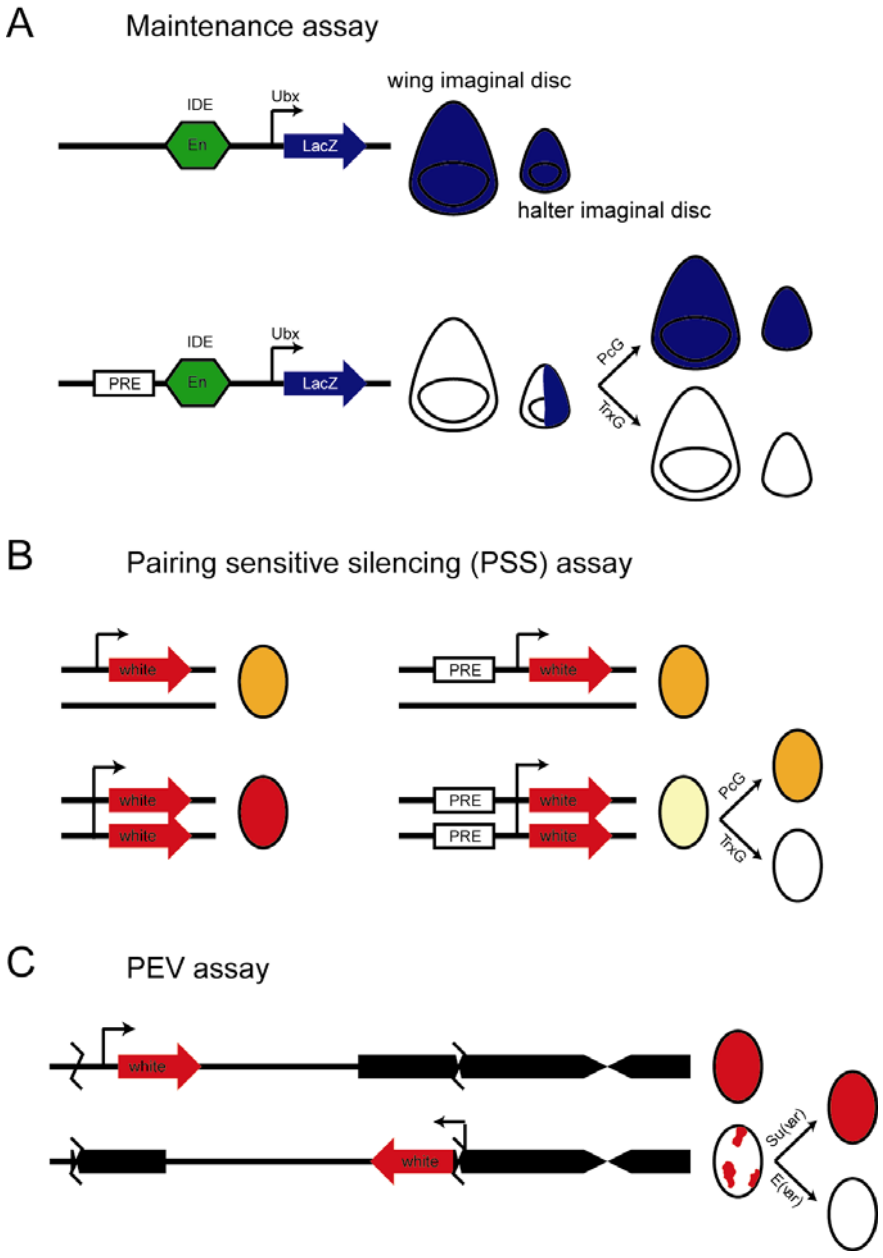


Figure 4. Drosophila genetics assay.
Fly in vivo assays used in PcG silencing studies. (A) Maintenance assay; (B) Pairing sensitive silencing (PSS) assay; (C) Position effect variegation assay (PEV).

In the maintenance assay (Figure 4A), the test construct contains a PRE fragment that flanks a tissue-specific enhancer for e.g. the engrailed gene or Ubx gene (Americo et al., 2002; Horard et al., 2000), followed by the Ubx Promoter and drives the LacZ reporter gene. The correct pattern of LacZ expression that was earlier determined during embryogenesis by

the tissue-specific enhancer should be maintained if the construct has PRE activity. Effects of mutations in PcG and trxG members can also be tested in this assay.

The pairing-sensitive silencing (PSS) assay (Figure 4B) rests on the ability of a PRE to repress the expression of the miniwhite reporter gene. The miniwhite gene is a transporter gene that encodes for the red eye colour in flies, and is often used to select transgenic flies on the basis of their eye colour. Normally, the eye colour of homozygote for a mini-white transgene is about twice as dark as their heterozygous siblings. In contrast, when the transgene carries a PRE, the homozygous flies often have lighter or white eye colour than the heterozygotes, due to the repression of the mini-white gene, and since there is also stronger silencing when the chromosomes are paired (Americo et al., 2002; Dejardin and Cavalli, 2004; Mishra et al., 2001). Because repression requires pairing of the chromosomes, this phenomenon is referred to as the pairing-sensitive (PS) assay. As the PS repression silencing can be released by mutations of PcG genes, it has been proposed that inactivation of the miniwhite gene is due to the assembly of a PcG repressive complex on PREs.

Also with the miniwhite reporter gene, fly eyes can exhibit a variegated effect. In the position effect variegation (PEV) assay (Figure 4C), the miniwhite reporter gene is silenced in some cell lineages and not others, resulting in patches of white and red eyes in the adult flies (Americo et al., 2002; Dejardin and Cavalli, 2004).

Post-translational modifications of histones and histone codes

Post-translational modifications of histones have recently emerged to be involved in transcriptional regulation. Several modifications namely (de)acetylation, (de)ubiquitination, sumoylation and (de)methylation, are associated with the activated or repressive chromatin states (Allfrey et al., 1964; Berger, 2002; Marmostein, 2001; Strahl and Allis, 2000). PcG proteins e.g. E(z) and the chromodomain of PC bind methylated lysines (Levine et al., 2004; Wang L. et al., 2004), while ubiquitin-specific protease 7 (USP7) genetically interacts with PC and deubiquitylate histone H2B to direct repression (van der Knaap et al., 2005). Additionally, dRING1 creates an epigenetic mark by ubiquitylating histone H2A (Wang H.B. et al., 2004). Rpd3, a histone deacetylase, is a constituent of the PRC2 complex, which has enzymatic activity. Rpd3 is likely to be involved in silencing; either directly or indirectly through its effects from other histone modifications such as phosphorylation and methylation (Chang et al., 2001). Histone tail methylation marks (histone H3K9 and/or 27) have been shown to be required for the initiation and establishment of the PRC2 complex in creating a repressive chromatin structure that leads to gene silencing (Czermin et al., 2002). In the early 1990s, the histone code concept was introduced by Turner (1993), which was based on his observations of histone acetylation in the dosage compensation process. This lead to discoveries of histone acetyl- and deacetyl-transferases; components of activator or repressor complexes (Hansen et al., 1998). The histone code was originally based on structural properties, and now is shifted to a structural (Sarraf and Stancheva, 2004) and an information-based code. An example would be the H3K9 methylation, which is regarded as an epigenetic mark since it

provides a docking site for heterochromatin-associated protein 1 (HP1) binding (Jenuwein and Allis, 2001). Although specific modifications do contribute to gene expression in many important ways, it seems doubtful that a combinatorial histone code exists.

Models for maintaining the 'code of silence'

Recent evidence has suggested that the memory for repressed gene expression is the default state and that trxG proteins are antagonists of PcG proteins in order to activate or derepress genes (Klymenko and Muller, 2004; Wang L. et al., 2004;). PcG proteins are important throughout the life cycle of Drosophila, since they are present and required at all points of development. In the past few years, plenty of light has been shed as to how PcG proteins are able to maintain their repression activity as more functional and biochemical activities of the PcG proteins subunits have been unraveled. It is still unclear as to how PcG proteins direct gene silencing. Several models have been proposed as to how PcG proteins mediate transcriptional repression (Figure 5). A unique presence of *cis*-acting DNA elements known as PREs has been proposed in mediating PcG repression. Most PcG protein members interact with one another but they lack sequence-specific DNA-binding activity. One model is that potential recruiters (e.g. PHO) bind DNA sequence-specifically, and at the same time recruit PcG proteins to the PREs. Another model of PcG recruitment is through histone tail modifications such as recognition of methylation marks by PcG subunits (e.g. E(z)).

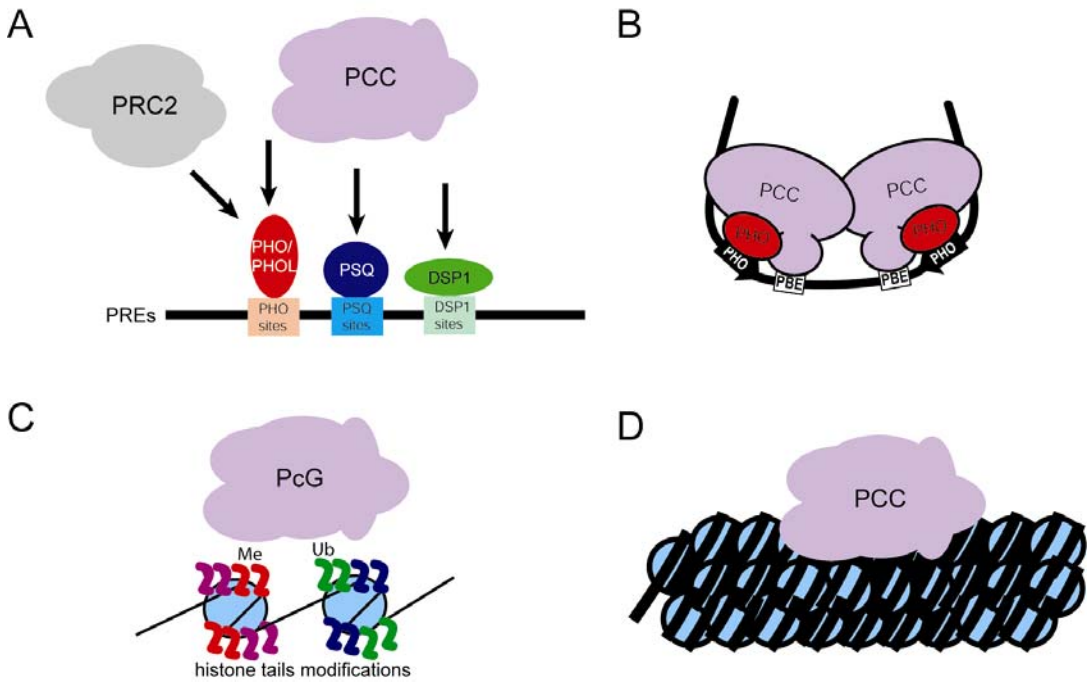


Figure 5. Models of PcG action in silencing.
(A) Putative recruiters of PcG proteins to PREs. (B) Synergistic assembly of a repressive PHO/PCC/PRE nucleoprotein complex. (C) Anchoring of PcG proteins through histone modifications e.g. methylation, ubiquitylation. (D) Chromatin compaction by inducing an inaccessible chromatin structure.

Silencing mechanisms have been suggested to involve modulation of chromatin structure. One basic premise of chromatin regulation is that genes are silenced through compaction of chromatin, which reduces the accessibility of DNA. In contrast, gene expression may require the “opening up” of chromatin. Several PcG proteins have been shown to compact chromatin (e.g. PSC). Repression involves blocking promoter activity. For achieving this, various mechanisms have been proposed for PcG silencing, i.e. blocking the transcription machinery directly or indirectly by establishing a resistant chromatin structure inaccessible to the transcription factors. Another possibility is by blocking and inhibition of transcription machinery formation; either directly or indirectly acting on the transcription factors themselves, which could serve as targets for repression.

Several modes of action as to how PcG proteins could create repressive chromatin structures have been proposed. PcG proteins are able to spread to their neighboring genes from transgenes containing PREs (Paro, 1990). PcG proteins also have the ability to reside in approximately 50 to 100 nuclear foci termed PcG bodies, which have been proposed to be concentrated areas of transcriptional repression possibly containing multiple PcG complexes bound to distinct PREs and form repressive chromatin structures over long distances (Pirrotta, 1998; Simon, 1995). Most *in vivo* studies favor the organizer model because formaldehyde crosslinking studies reveal the clustering of PcG proteins with PREs and promoters (Orlando et al., 1997; Strutt et al., 1997) (in contrast with spreading over chromatin). On the other hand, other studies show that accessibility of DNA is not reduced in genes that are repressed by PcG proteins (Boivin and Dura, 1998; McCall and Bender, 1996; Schossler et al., 1994) and that PREs do play an essential role in creating a repressive complex (Busturia et al., 1997). It should not be ruled out that even though PcG proteins act at discrete and organized sites, protein-protein interactions play important roles in formation of multimeric complexes, which on the other hand supports the spreading model. To support the multiprotein interaction hypothesis, subunits of PCC (especially PSC) have been shown to compact nucleosomes and that neither linker histones nor histone tails are required for this compaction to occur (Francis et al., 2004). This makes it feasible such that even when PcG proteins form within clusters, they will also spread along the DNA, nucleosomes or chromatin, thereby creating an inhibitory chromatin complex as well as blocking the transcription machinery (Bulger and Groudine, 1999; Orlando et al., 1997; Strutt et al., 1997). Due to the presence of TFIID in PRC1 (Saurin et al., 2001), it has been suggested that this complex might be directly involved with the transcription machinery i.e. by blocking RNA polynerase II and inhibiting SWI/SNF activating machinery (Shao et al., 1999), or directly interacting with basal transcription factors and promoters (Bunker and Kingston, 1994; Simon, 1995). Results from chromatin immunoprecipitation (ChIP) studies detected PcG proteins at promoter regions, further supporting the notion that they are in direct contact with the transcription machinery (Orlando et al., 1997; Strutt et al., 1997). Chromatin-independent repression has also been proposed in studies of tethering PcG proteins artificially by transiently introducing

them (Paro, 1990) into tissue culture cells (Bunker and Kingston, 1994; Poux et al., 2001) as well as in Drosophila embryos (Muller J 1995; Poux et al., 1996; Poux S et al. 2001).

Functional dissection of PSC has led to the identification of a specific domain playing a key role in the inhibition of chromatin remodeling and transcription on chromatin templates (King et al., 2005). Electron microscopy studies also reveal that PSC is the core component in nucleosome compaction (Francis et al., 2004). Although it is not clear whether this compaction is sufficient for repression to occur. What seems to be rather interesting is that compaction occurs when nucleosomes (beads on a string) are 'brought closer together' (in close proximity to each other) by bending the loose DNA that is connecting the nucleosomes. Bending of DNA has already been shown to be important in transcriptional activity especially for general transcriptional factors. Several *Drosophila* proteins could play important roles in inducing structural changes to DNA. One of the prospective candidates is DSP1, which is a HMG box protein, since HMG box proteins preferentially bind bent DNA. The other is PHO, the *Drosophila* homologue of the human YY1, since YY1 has been shown to be able to bend DNA in the c-fos promoter (Natesan and Gilman, 1993). Our results presented in Chapter 4 suggest a model whereby the PRE DNA is wrapped around PHO-PCC, and that the DNA architecture is indeed changed.

All these suggested mechanisms mentioned above could be a direct consequence of the recruitment of PcG proteins by sequence-specific DNA-binding proteins and/or anchoring of PcG proteins by recognition of histone tail modification marks. This could then lead to looping of DNA bound with PcG proteins and thereby interacting with the transcription machinery. As a result of that, nucleosomes are compacted into a repressive chromatin structure in order to maintain a stable silenced state. The accumulation of emerging evidences with respect to PcG function being linked to histone modifications, has contributed an interesting twist to its silencing function.

In conclusion, it appears that PcG proteins are involved in epigenetic silencing by recognizing DNA, post-translational modifications of histone tails and modifying chromatin structure. However, the exact mechanisms are far from clear. In the near future, it is probable that extra PREs are yet to be discovered and we might come closer in deciphering the PRE code. Due to the dynamic changes of PcG compositions during *Drosophila* development, mechanisms leading to the maintenance of silence is more complex than imagined. Silencing is, however, a multi-step process while it still remains to be unraveled as to how histone modifications and PRE codes are integrated to bring about epigenetically global changes.

References

Alkema, M.J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L.J., Berns, A., and van Lohuizen, M. (1997). Identification of Bmi1 –interacting proteins as constituents of a multimeric mammalian polycomb complex. Genes Dev. 11: 226-240.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Natl. Acad. Sci. USA. 51: 786-794.

Americo, J., Whiteley, M., Brown, J.L., Fujioka, M., Jaynes, J.B., and Kassis, J.A. (2002). A complex array of DNA-binding proteins required for pairing-sensitive silencing by a Polycomb group response element from the Drosophila engrailed gene. Genetics 160: 1561–71.

Barges, S., Mihaly, J., Galloni, M., Hagstrom, K., Muller, M., Shanower, G., Schedl, P., Gyurkovics, H., and Karch, F. (2000). The Fab-8 boundary defines the distal limit of the bithorax complex iab-7 domain and insulates iab-7 from initiation elements and a PRE in the adjacent iab-8 domain. Development 127: 779-90..

Bea, S., Tort, F., Pinyol, M., Puig, X., Hernandez, L., Hernandez, S., Fernandez, P.L., van Lohuizen, M., Colomer, D., and Campo, E. (2001). BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 61: 2409-2412.

Berger, S.L. (2002). Histone modifications in transcriptional regulation. Curr. Opin. Genet. Development 12: 142-148.

Blastyak, A., Mishra, R.K., Karch, F., and Gyurkovics, H. (2006). Efficient and specific targeting of Polycomb group proteins requires cooperative interaction between grainyhead and pleiohomeotic. Mol. Cell. Biol. 26: 1434-44. Boivin, A., and Dura, J.M. (1998). In vivo chromatin accessibility correlates with gene silencing in Drosophila. Genetics 150: 1539-1549.

Brickman, J. M., Adam, M., and Ptashne, M. (1999). Interactions between an HMG-1 protein and members of the Rel family. Proc. Natl Acad. Sci. USA 96: 10679-10683.

Brock, H.W., and van Lohuizen, M. (2001). The Polycomb group—no longer an exclusive club? Curr. Opin. Genet. Dev. 11: 175-181.

Brown, J.L., Fritsch, C., Mueller, J., and Kassis, J.A. (2003). The Drosophila pho-like gene encodes a YY1-related DNA-binding protein that is redundant with pleiohomeotic in homeotic gene silencing. Development 130: 285-294.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L., and Kassis, J.A. (1998). The Drosophila Polycomb group gene pleiohomeotic encodes a DNA-binding protein with homology to the transcription factor YY1. Mol. Cell 1: 1057-1064.

Bulger, M., and Groudine, M. (1999). Looping versus linking: toward a model for long-distance gene activation. Genes Dev. 13: 2465-2477.

Bunker, C.A., and Kingston, R,E. (1994). Transcriptional repression by Drosophila and mammalian group proteins in transfected mammalian cells. Mol. Cell. Biol. 14: 1721-1732.

Busturia, A., and Bienz, M. (1993). Silencers in abdominal-B, a homeotic Drosophila gene. EMBO J. 12: 1415–1425.

Busturia, A., Wightman, C.D., and Sakonju, S. (1997). A silence is required for maintenance of transcriptional repression throughout Drosophila development. Development 124: 4343-4350.

Busturia, A., Lloyd, A., Bejarano, F., Zavortink, M., Xin, H., and Sakonju, S. (2001). The MCP silencer of the Drosophila Abd-B gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. Development 128: 2163-2173.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of Histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298: 1039-1043.

Cavalli, G., and Paro, R. (1998). The Drosophila Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. Cell 93: 505-518.

Chan, C.S., Rastelli, L., and Pirrotta, V. (1994). A Polycomb response element in the *Ubx* gene that determines an epigenetically inherited state of repression. EMBO J. 13: 2553-2564.

Chang, Y.L., Peng, Y.H., Pan, I.C., Sun, D.S., King, B., and Huang, D.H. (2001). Essential role of Drosophila Hdac1 in homeotic gene silencing. Proc. Natl. Acad. Sci. USA 98: 9730-9735.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development 131: 5263-5276.

Chen, J.D., and Pirrotta, V. (1993). Multimerization of the Drosophila Zeste protein is required for efficient DNA binding. EMBO J. 12: 2075-2083.

Chiang, A., O'Connor, M.B., Paro, R., Simon, J., and Bender W. (1995). Discrete Polycomb-binding sites in each parasegmental domain of the bithorax complex. Development 121: 1681–1689.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111: 185-196.

DeCamillis, M., Cheng, N.S., Pierre, D., and Brock, H.W. (1992). The polyhomeotic gene of Drosophila encodes a chromatin protein that shares polytene chromosome binding sites with Polycomb. Genes Dev, 6: 223-32.

Decoville, M., Giacomello, E., Leng, M., and Locker, D. (2001). DSP1, an HMG-like protein, is involved in the regulation of homeotic genes. Genetics 157: 237-244.

Dejardin, J., Rappaillles, A., Cuvier, O., Grimaud, C., Decoville, M, Locker, D., and Cavalli, G. (2005). Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. Nature 434: 533-538.

Dejardin, J., and Cavalli, G. (2004). Chromatin inheritance upon Zeste-mediated Brahma recruitment at a minimal cellular memory module. EMBO J. 23: 857–868.

Dimri, G.P., Martinez, J.L., Jacobs, J.J., Keblusek, P., Itahana, K., van Lohuizen, M., Campisi, J., Wazer, D.E., and Band, V. (2002). The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. Cancer Res 62: 4736-4745.

Dukers, D.F., van Galen, J.C., Giroth, C., Jansen, P., Sewalt, R.G., Otte, A.P., Kluin-Nelemans, H.C., Meijer, C.J., and Raaphorst, F.M. (2004). Unique Polycomb gene expression pattern in Hodgkin's lymphoma and Hodgkin's lymphoma-derived cell lines. Am. J. Pathol. 164: 873-881.

Fauvarque, M.-O., and Dura, J.M. (1993). Polyhomeotic regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in Drosophila. Genes Dev. 7: 1508-1520.

Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev. 17: 1870-1881.

Francis, N.J., and Kingston, R.E. (2001). Mechanisms of transcriptional memory. Nature Rev. Mol. Cell Biol. 2: 409-421.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science. 306,1574-7.

Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W., and Paro, R. (1992). Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. EMBO J. 11: 2941-2950.

Fritsch, C., Brown, J.L., Kassis, J.A., and Muller, J. (1999). The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a Drosophila homeotic gene. Development 126, 3905-3913.

Gindhart, J.G., and Kaufman, T.C. (1995). Identification of Polycomb and trithorax group responsive elements in the regulatory region of the Drosophila homeotic gene Sex combs reduced. Genetics 139: 797-814.

Girton, J. R., and S. H. Jeon. (1994). Novel embryonic and adult homeotic phenotypes are produced by pleiohomeotic mutations in Drosophila. Dev. Biol. 161: 393-407.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A *Polycomb* group gene regulates homeotic gene expression in Arabidopsis. Nature 386: 44-51.

Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a *Polycomb* group gene in Arabidopsis. Science 280: 446-450.

Guitton, A.E., Page, D.R., Chambrier, P., Lionnet, C., Faure, J.E., Grossniklaus, U., and Berger, F. (2004). Identification of new members of fertilization independent seed polycomb group pathway involved in the control of seed development in Arabidopsis thaliana. Development 131: 2971-2981.

Hadorn E. (1978). Transdetermination in cells. Sci. Am. 219: 110-14.

Hansen, J. C., Tse, C., and Wolffe, A. P. (1998). Structure and function of the core histone N-termini: more than meets the eye. Biochemistry 37: 17637-17641.

Hennig, L., Taranto, P., Walser, M., Schonrock, N., and Gruissem, W. (2003). Arabidopsis MSI1 is required for epigenetic maintenance of reproductive development. Development 130: 2555-2565.

Holdeman, R., Nehrt, S., and Strome, S. (1998). MES-2 maternal protein is essential for viability of the germline in Caenorhabditis elegans, is homologous to a Drosophila Polycomb group protein. Development 125: 2457-2467.

Horard, B., Tatout, C., Poux, S., and Pirrotta, V. (2000). Structure of a Polycomb response element and in vitro binding of Polycomb group complexes containing GAGA factor. Mol. Cell Biol. 20: 3187-97.

Huang, D.H., and Chang Y.L. (2004). Isolation and characterization of CHRASCH, a polycomb-containing silencing complex. Methods Enzymol. 377: 267-282.

Huang, D.H., Chang Y.L., Yang, C.C., Pan, I.C., and King, B. (2002) pipsqueak encodes a factor essential for sequence-specific targeting of a polycomb group protein complex. Mol. Cell. Biol. 22: 6261-6271.

Hur, M.W., Laney, J.D., Jeon, S.H., Ali, J., and Biggin, M.D. (2002). Zeste maintains repression of Ubx transgenes: support for a new model of Polycomb repression. Development, 129: 1339-1343.

Hyde-DeRuyscher, R.P., Jennings, E., and Shenk, T. (1995). DNA binding sites for the transcriptional activator/repressor YY1. Nucleic Acids Res. 23: 4457–4465.

Jenuwein, T., and Allis, C. D. (2001). Translating the histone code. Science 293:1074-1080.

Johnson, W.A., McCormick, C.A., Bray, S.J., and Hirsh, J. (1989). A neuronal specific enhancer of the Drosophila dopa decarboxylase gene. Genes Dev. 3: 676-686.

Kagey, M.H., Melhuish, T.A., and Wotton, D. (2003). The Polycomb protein Pc2 is a SUMO E3. Cell 113: 127-137.

Kassis, J.A. (1994). Unusual properties of regulatory DNAfrom the *Drosophila engrailed* gene: three "pairing-sensitive" sites within a 1.6-kb region. Genetics 136: 1025-38.

Katz, A., Olivia, M., Mosquna, A., Hakim, O., and Ohad, N. (2004). FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression durinf sporophyte development. Plant J. 37: 707-746.

Kennison, J.A. (1995). The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function.Annu. Rev. Genet. 29: 289-303.

Kim, C.A., Gingery, M., Pilpa, R.M., and Bowie, J.U. (2002). The SAM domain of Polyhomeotic forms a helical polymer. Nat. Struct. Biol. 9: 453-457.

Kim, C.A., Sawaya, M.R., Cascio, D., Kim, W., and Bowie, J.U. (2005). Structural organization of a Sex-comb-on-midleg/polyhomeotic copolymer. J Biol Chem. 280: 27769-27775.

Kim, J.H., Yoon, S.Y., Kim, C.N., Joo, J.H., Moon, S.K., Choe, I.S., Choe, Y.K., and Kin, J.W. (2004). The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. Cancer Lett. 203: 217-224.

King, I.F.G., Emmons, R., Francis, N.J., Wild, B., Muller, J., Kingston, R.E., and Wu, C-T. (2005). Analysis of a Polycomb group protein defines regions that link repressive activity on nucleosomal templates to in vivo function. Mol. Cell. Biol. 25: 6578-6591.

Kinoshita, T., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (2001). Polycomb repression of flowering during early plant development. Proc. Natl. Acad. Sci. USA 98: 14156-14161.
1592-1605.

Klymenko, T., and Muller, J. (2004). The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. EMBO Rep. 5: 1-5.

Köhler, C., and Grossniklaus, U. (2002). Epigenetic inheritance of expression states in plant development: the role of Polycomb group proteins. Curr. Opin. Cell Biol. 14: 773–779.

Korf, I., Fan, Y., and Strome, S. (1998). The Polycomb group gene in Caenorhabditis elegans and maternal control of germline development. Development 125: 2469-2478.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferases activity associated with a human multiprotein complex containing the enhancer of Zeste protein. Genes Dev. 16: 2893-2905.

Kyba, M., and Brock, H.W. (1998). The Drosophila polycomb group protein Psc contacts ph and Pc through specific conserved domains. Mol. Cell Biol. 18: 2712-2720.

Laney, J.D., and Biggin, M.D. (1997). Zeste-mediated activation by an enhancer is independent of cooperative DNA-binding in vivo. Proc. Natl. Acad. Sci. USA 94: 3602-3604.
Lehming, N., Thanos, D., Brickman, J.M., Ma, J., Maniatis, T., and Ptashne, M. (1994). An HMG-like protein that can switch a transcriptional activator to a repressor. Nature 371: 175-179.
Levine, S.S., King, I.F.G., and Kingston, R.E. (2004). Division of labour in Polycomb group repression. Trends Biochem. Sci. 29: 478-485.
Lewis, E.B. (1963). Genes and developmental pathways. Am. Zool. 3: 33-56.
Lonie, A., D'Andrea, R., Paro, R., and Saint, R. (1994). The Polycomb and trithorax group proteins of Drosophila: trans-regulators of homeotic gene function.Development 120: 2629-2636.
Lyko, F. and Paro, R. (1999). Chromosomal elements conferring epigenetic inheritance. Bioessays 21, 824-832.
Mahmoudi, T., and Verrijzer, C.P. (2001). Chromatin silencing and activation by Polycomb and trithorax group proteins. Oncogene 20: 3055-3066.
Mahmoudi, T., Zuijderduijn, L.M.P., Mohd-Sarip, A., and Verrijzer, C.P. (2003). GAGA facilitates binding of Pleiohomeotic to a chromatinized Polycomb response elements. Nucleic Acids Res. 31: 4147–4156.
Marmostein, R. (2001). Protein modules that modulate histone tails for chromatin regulation. Mol. Cell. Biol. 2: 422-432.
McCall, K., and Bender, W. (1996). Probes for chromatin accessibility in the Drosophila bithorax complex respond differently to Polycomb-mediated repression. EMBO J. 15: 569-580.
McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell 68: 283-302.
Mihaly, M., Hogga, I., Gausz, J., Gyurkovics, H., and Karch, F. (1997). In situ dissection of the bithorax complex into a chromatin domain boundary and a Polycomb-response element. Development 124: 1809-1820.
Mihaly, J., Mishra, R.K., and Karch, F. (1998). A conserved sequence motif in Polycomb-response element. Mol. Cell 1: 1065-1066.
Mishra, R.K., Mihaly, J., Barges, S., Spierer, A., Karch, F., Hagstrom, K., Schweinsberg, S.E., and Schedl, P. (2001). The iab-7 polycomb response element maps to a nucleosome-free region of chromatin and requires both GAGA and Pleiohomeotic for silencing activity. Mol. Cell. Biol. 21: 1311-1318.
Mohd-Sarip, A., Cleard, F., Mishra, R.K., Karch, F., and Verrijzer C.P. (2005) Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex. Genes Dev. 19, 1755-1760.
Mohd-Sarip, A., Venturini, F., Chalkley, G.E., and Verrijzer, C.P. (2002). Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. Mol. Cell Biol. 22, 7473-7483.
Muller, J. (1995). Transcriptional silencing by the Polycomb protein in Drosophila embryos. EMBO J. 14: 1209-1220.
Muller, J., and Bienz, M. (1991). Long range repression conferring boundaries of Ultrabithorax expression in the Drosophila embryo. EMBO J. 10: 3147-3155.
Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferases activity of a Drosophila Polycomb repressor complex. Cell 111: 197-208.
Natesan, S., and Gilman, M.Z. (1993). DNA bending and orientation-dependent function of YY1 in the c-fos promoter. Genes Dev. 7: 2497-2509.
Nekrasov, M., Wild., and Muller, J. (2005). Nucleosome binding and histone methyltransferases activity of Drosophila PRC2. EMBO Rep. 6: 348-353.
Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in *FIE*, a WD *Polycomb* group gene, allow endosperm development without fertilization. Plant Cell 11: 407-416.
Orlando, V., Jane, E.P., Chinwalla, V., Harte, P., and Paro, R. (1998). Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early Drosophila embryogenesis. EMBO J. 17: 5141-5150.
Orlando, V., and Paro. R. (1993). Mapping of Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. Cell 75, 1187-1198.
Paro R. (1990). Imprinting a determined state into the chromatin of Drosophila. Trends Genet 6: 416-421.
Pires-daSilva, A, and Sommer, R.J. (2003). Finally, worm polycomb-like genes meet Hox regulation. Dev. Cell. 4: 770-772.
Pirrotta V. (1998). Polycombing the genome: PcG, trxG and chromatin silencing. Cell 93: 333-336.
Pirrotta, V., and Rastelli, L. (1994). White gene expression, repressive chromatin domains and homeotic gene regulation in Drosophila. Bioessays 16: 549–556.
Poux, S., Kostic, C., and Pirrotta, V. (1996) Hunchback-independent silencing of late Ubx enhancers by polycomb group response element. EMBO J. 15:n4713-4722.
Poux, S., McCabe, D., and Pirrotta, V. (2001). Recruitment of components of Polycomb group chromatin complexes in Drosophila. Development 128: 75-85.
Raaphoorst FM, van Kemenade, F.J., Fieret, E., Hamer, K., Satijn, A.P., Otte AP, and Meiher, C.J.L.M. (2000). Cutting edge: Polycomb gene expression reflect distinct B cell differentiation stages in human germinal centers. J. Immunol. 164: 1-4.
Raaphoorst, F.M., Vermeer, M., Fieret, E., Blokzijl, T., Dukers, D., Sewalt, R.G., Otte, A.P., Willemze, R., and Meijer, C.J. (2004). Site-specific expression of Polycomb-group genes encoding the HPC-HPH/PRC1 complex in clinically defined primary nodal and cutaneous large B-cell lymphomas. Am. J. Pathol. 164:.533-542.
Rastelli, L., Chan, C.S., and Pirrotta, V. (1993). Related chromosome binding sites for Zeste, Suppressors of Zeste and Polycomb group proteins in *Drosophila* and their dependence on Enhancer of Zeste function. EMBO J. 12: 1513-1522.
Ringrose, L., Rehmsmeier, M., Dura, J.M., and Paro, R. (2003). Genome-wide prediction of Polycomb/Trithorax response elements in Drosophila melanogaster. Dev. Cell 5: 759-771.
Ross, J.M., and Zarkower, D. (2003). Polycomb group regulation of Hox gene expression in C. elegans. Dev. Cell 4: 891-901.
Sanchez-Beato, M., Sanchez, E., Garcia, J.F., Perez-Rosado, A., Montoya, M.C., Fraga, M., Artiga, M.J., Navarrete, M., Abraira, V., Morente, M., Esteller, M., Koseki, H., Vidal, M., and Piris, M.A. (2004). Abnormal PcG protein expression in Hodgkin's lymphoma. Relation with E2F and NFkappaB transcription factors. J Pathol 204: 528-537.

Sarraf, S. A., and Stancheva, I. (2004). Methyl-CpG inding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. Mol. Cell 15: 595–605.
Satijn, D.P., and Otte, A.P. (1999). Polycomb group protein complexes: Do different complexes regulate distinct target genes? Biochim. Biophys. Acta 1447: 1-16.
Saurin, A. J., Z. Shao, H. Erdjument-Bromage, P. Tempst, and R. E. Kingston. (2001). A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. Nature 412: 655–660.
Schossler, J., Eggert, H., Paro, R., Cremer, S., Jack, R.S. (1994). Gene inactivation in Drosophila mediated by the Polycomb gene product or by position-effect variegation does not involve in major changes in the accessibility of the chromatin fibre. Mol. Gen. Genet. 243: 453-462.
Shao, Z., Raible, F., Mollaaghababa, R.,. Guyon, J. R, Wu, C.T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 98: 37-46.
Simon, J. (1995). Locking in stable states of gene expression: transcriptional control during Drosophila development. Curr. Opin. Cell Biology 7: 376-385.
Simon, J., Chiang, A., Bender, W., Shimell, M.J., Oçonnor, M. (1993). Elements of the Drosophila bithorax complex that mediate repression by Polycomb group products. Dev. Biol. 158: 131-144.
Strahl, B.D., and Allis, C.D. (2000). The language of histone modifications. Nature 403: 41-45.
Strutt, H., Cavalli, G., and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. EMBO J. 16: 3621-3632
Strutt, H., and Paro, R. (1997). The polycomb group protein complex of Drosophila melanogaster has different compositions at different target genes. Mol. Cell. Biol. 17: 6773-6783.
Tie, F., Furuyama, T., Prasad-Sinha, J., Hane, E., and Harte, P.J. (2001). The Drosophila Polycomb group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. Development 128: 275-286.
Tuckfield, A., Clouston, D.R., Wilanowski, T.M., Zhao, L-L., Cunningham, J.M., and Jane, S.M. (2002). Binding of the RING Polycomb proteins to specific target genes in complex with the grainyhead-like family of developmental transcription factors. Mol. Cell. Biol. 22: 1936-1946.
Turner, B. M. (1993). Decoding the nucleosome. Cell 75: 5–8.
van der Knaap, J.A., Kumar, B.R.P., Moshkin, Y.M., Langenberg, K., Krijgsveld, J., Heck, A.J.R., Karch, F., and Verrijzer, C.P. (2005). GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. Mol. Cell 17: 695-707.
van Kemenade, F.J., Raaphorst, F.M., Blokzij, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P., and Meijer, C.J. (2001). Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood 97: 3896-3901.
Visser, H.P., Gunster, M.J., Kluin-Nelemans, H.C., Manders, E.M., Raaphorst, F.M., Meijer, C.J., Willemze, R., and Otte, A.P. (2001). The Polycomb group protein EZH2 is upregulated in proliferating, cultured, mantle cell lymphoma. Br. J. Haematol. 112: 950-958.
Vonlanthen, S., Heighway, J., Altermatt, H.J., Gugger, M., Kappeler, A., Borner, M.M., van Lohuizen, M., and Betticher, D.C. (2001). The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br. J. Cancer 84: 1372-1376.
Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004). Hierarchical recruitment of Polyomb group silencing complexes. Mol. Cell 14: 637-646.
Wang, H.B., Wang, L.J., Erdjument-Bromage, H., Vidal, M., Tempst, P, Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in polycomb silencing. Nature 431: 873-878.
Yant SR, Zhu W, Millinoff D, Slightom JL, Goodman M, Gumucio DL. 1995. High affinity YY1 binding motifs: identification of two core types (ACAT and CCAT) and distribution of potential binding sites within the human beta globin cluster. Nucleic Acids Res. 23: 4353-4362.
Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., and Takahashi, S. (2001). EMBRYONIC FLOWER2, a novel *Polycomb* group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell 13: 2471-2481.
Zhang, H., Azevedo, R.B.R., Lints, R., Doyle, C., Teng, Y., Haber, D., and Emmons, S.W. (2003). Global regulation of Hox gene expression in C. elegans by a SAM domain protein. Dev. Cell 4: 903-915.
Zink, B., Engstrom, Y., Gehring, W., and Paro, R. (1991). Direct interaction of the Polycomb protein with Antennapedia regulatory sequences in Drosophila melanogaster. EMBO J. 10: 153-162.
Zink, D., and Paro, R. (1995). Drosophila polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. EMBO J. 14: 5660-5671.
Zink, D., and Paro, R. (1989). In vivo binding pattern of a trans-regulator of homeotic genes in Drosophila melanogaster. Nature 337: 468-471.

Chapter 1

Introduction

A higher order of silence.

A. Mohd-Sarip and C.P. Verrijzer
(2005) Science 306, 1484-1485

MOLECULAR BIOLOGY

A Higher Order of Silence

Adone Mohd-Sarip and C. Peter Verrijzer

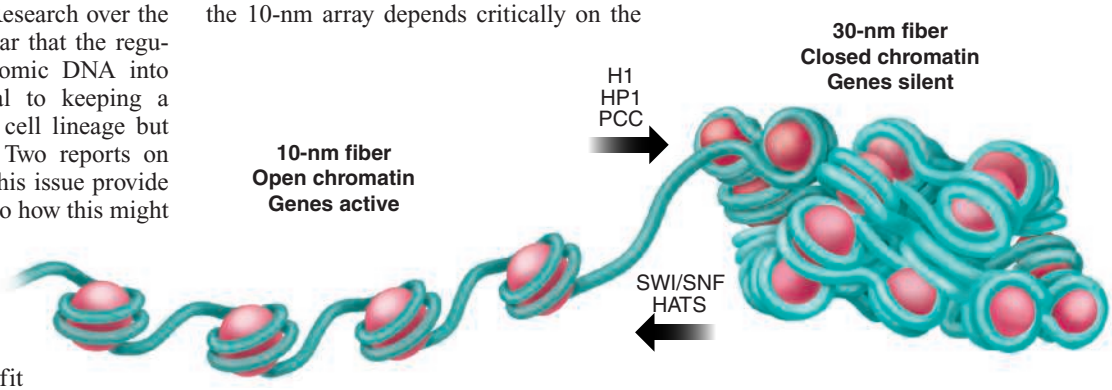
During the development of multicellular organisms, a single fertilized egg gives rise to a plethora of specialized cell types, which are the building blocks of distinct tissues. Because virtually all the cells in our body contain an identical genome, it is the discriminative reading of the genetic information that determines whether a cell is a muscle, skin, or nerve cell. In order to have the "right cell" at the "right place," it is essential that a chosen cellular gene expression program be maintained throughout cell division. Failures in cellular memory or epigenetic control can lead to serious developmental defects and diseases such as cancer. Research over the past decade has made clear that the regulated compaction of genomic DNA into chromatin is fundamental to keeping a gene turned "on" in one cell lineage but turned "off" in another. Two reports on pages 1571 and 1574 of this issue provide intriguing new insights into how this might be achieved (*1*, *2*).

The packaging of DNA into chromatin allows the DNA of human cells (about 2 m in length if stretched out) to fit into a nucleus with a diameter of only 10 μm. The basic repeat element of chromatin is the nucleosome, which consists of 147 base pairs (bp) of DNA wrapped 1.7 times around an octamer of histone proteins (two copies each of core histones H2A, H2B, H3, and H4). Core histones contain a trihelical histone fold domain that mediates histone-histone and histone-DNA binding, as well as unstructured amino-terminal tail domains that are subjected to extensive covalent modifications. Nucleosomes, connected by about 20 to 60 bp of linker DNA, form a 10-nm "beads-on-a-string" array, which can be compacted further into a "30-nm" chromatin fiber (see the figure) (*3*, *4*). Whereas the three-dimensional structure of the nucleosome is known in exquisite detail (*5*), the structure of the higher order 30-nm chromatin fiber is poorly understood.

One basic issue is the arrangement of the nucleosomes within the 30-nm fiber. Two classes of model have been proposed: (i) the "one-start helix" in which nucleosomes, connected by bent linker DNA, are arranged linearly in a higher order helix; and (ii) the "two-start helix" in which nucleosomes, connected by straight linker DNA, zigzag back and forth between two adjacent helical stacks. To distinguish between these two competing models of higher order chromatin folding, Dorigo and co-workers (*1*) developed an ingenious experimental approach using a fully defined in vitro system to generate regular nucleosomal arrays. Further compaction of the 10-nm array depends critically on the base of the histone H4 amino-terminal tails, believed to contact the histone H2A/H2B dimer of the neighboring nucleosome. Indeed, disulfide cross-links between a pair of cysteine residues that replaced selected amino acids in histone H4 and H2A stabilized the higher order chromatin structure. Next, Dorigo *et al*. digested the linker DNA connecting adjacent nucleosomes within the cross-linked compacted chromatin. Analysis of the length of the nucleosome stacks, now solely connected by internucleosomal cross-links, revealed a two-start rather than a one-start organization. This conclusion was corroborated by electron microscopy. In addition to important structural insights, this study shows that local interactions between nucleosomes can drive self-organization into a higher order chromatin fiber.

But what is the physiological relevance of higher order chromatin? Notably, the buffer conditions promoting formation of a 30-nm chromatin fiber reflect the in vivo environment better than do those that yield a 10-nm fiber. One basic premise of chromatin regulation is that genes are silenced through compaction of chromatin, which reduces the accessibility of DNA. In contrast, gene expression may require the "opening up" of chromatin. The Polycomb group (PcG) of gene repressors and the trithorax group (trxG) of gene activators are two antagonistic classes of proteins that may act through modulation of chromatin structure (*6*–*8*). Together, these factors maintain the gene expression patterns of key developmental regulators and hence are crucial players in cellular differentiation, stem cell renewal, and cancer. The trxG group includes members of the SWI/SNF family of adenosine triphosphate (ATP)–dependent chromatin remodeling factors, which use energy derived from ATP hydrolysis to open up chromatin. Conversely, in vivo studies suggest that PcG repression reduces DNA accessibility, but how this is achieved remains unclear (*6*–*9*).

In their study, Francis *et al*. (*2*) used electron microscopy to visualize the compaction of a nucleosomal array promoted by a core polycomb complex, named PCC. It will be of interest to determine whether PCC-induced compacted chromatin forms a bona fide two-start 30-nm fiber. One



CREDIT: KATHARINE SUTLIFF/*SCIENCE*

Regulated chromatin folding directs gene expression. A parsimonious model illustrating the transition from a 10-nm "beads-on-a-string" open chromatin formation to the next level of chromatin organization: the compacted 30-nm chromatin fiber. Depicted is one possible form of the chromatin fiber produced by a "two-start helix." Folding or unfolding of the chromatin fiber affects the accessibility of DNA to regulatory factors, which control gene expression. Whereas gene silencing factors such as the PCC complex, HP1, and H1 stabilize higher order chromatin folding, gene activators such as the SWI/SNF remodeling complexes and histone acetyl transferases (HATS) initiate chromatin unfolding.

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PCC complex compacts about three nucleosomes, which suggests that each complex might contact multiple nucleosomes and bring them closer together. Removal of the unstructured histone tails by the protease trypsin did not affect chromatin compaction by PCC; hence, these tails may not be required. Histone tail modifications may, however, contribute to the recruitment of PCC in vivo (*10*). Furthermore, it remains possible that the base of the H4 tail, which is important for internucleosome association, was not completely removed by trypsin treatment. One subunit of PCC, named PSC, appears to be particularly critical; a region of PSC that is essential in vivo is also important for chromatin compaction in vitro.

The term "higher order chromatin" is frequently used, or abused, to explain epigenetic effects on gene expression, but what it refers to in molecular terms has not been well defined. The Dorigo *et al*. study provides a first glimpse of chromatin folding at the next level beyond the nucleosomal array. Meanwhile, the Francis *et al*. findings support the notion that PCC creates compacted chromatin domains that silence genes. These studies emphasize that higher order folding is an intrinsic attribute of the nucleosomal array used by gene regulatory factors. Silencing factors such as PCC, HP1, or the linker histone H1 appear to act, albeit through different mechanisms, by stabilizing the internucleosome interactions that drive higher order folding. Conversely, gene activation by SWI/SNF chromatin remodelers and histone acetyl transferases is likely to involve destabilization of the 30-nm fiber. The dissection of the diverse mechanisms by which chromatin folding is regulated will be central to understanding the molecular basis of cellular memory.

References

1. B. Dorigo *et al.*, *Science* **306**, 1571 (2004).
2. N. J. Francis *et al.*, *Science* **306**, 1574 (2004).
3. T. J. Richmond, J. Widom, in *Chromatin Structure and Gene Expression*, S. C. R. Elgin, J. L. Workman, Eds. (Oxford Univ. Press, Oxford, 2000), pp. 1–23.
4. P. J. Horn, C. L. Peterson, *Science* **297**, 1824 (2002).
5. K. Luger *et al.*, *Nature* **389**, 251 (1997).
6. T. Mahmoudi, C. P. Verrijzer, *Oncogene* **20**, 3055 (2001).
7. L. Ringrose, R. Paro, *Annu. Rev. Genet.* **38**, 413 (2004).
8. J. A. Simon, J. W. Tamkun, *Curr. Opin. Genet. Dev.* **12**, 210 (2002).
9. D. P. Fitzgerald, W. Bender, *Mol. Cell. Biol.* **21**, 6585 (2001).
10. L. Wang *et al.*, *Mol. Cell* **14**, 637 (2004).

PHYSICS

What Is Dark Matter Made Of?

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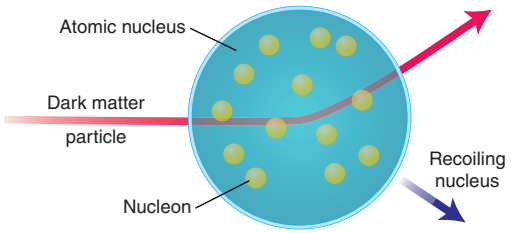
Astrophysical observations reveal that galaxies and clusters of galaxies are gravitationally held together by vast halos of dark (that is, nonluminous) matter. Theoretical reasoning points to two leading candidates for the particles that may make up this mysterious form of matter: weakly interacting massive particles (WIMPs) and axions. Particle accelerators have not yet detected either of the two particles, but recent astrophysical observations provide hints that both particles may exist in the universe, although definitive data are still lacking. Dark matter need not consist exclusively of only one of these two types of particles.

Precise measurements of the cosmic microwave background have shown that dark matter makes up about 25% of the energy budget of the universe; visible matter in the form of stars, gas, and dust only contributes about 4%. However, the nature of dark matter remains a mystery. To explain it, we must go beyond the standard model of elementary particles and look toward more exotic types of particles.

One such particle is the neutralino, a WIMP that probably weighs as much as 1000 hydrogen atoms (henceforth, we refer to the neutralino as a generic WIMP). Neutralinos are postulated by supersymmetric models, which extend the standard model to higher energies. To date, no neutralinos have been created in particle accelerators, but in the future they may be produced in the world's most powerful particle accelerator, the Large Hadron Collider currently being built at CERN. A recent precise measurement of the magnetic dipole moment of the muon favors the existence of new particles such as neutralinos.



Detection of neutralinos. Neutralinos can be detected directly with underground detectors through their elastic scattering on nuclei. The energy deposited by the recoiling nucleus (large circle) is expected to provide the direct signature.

Another possibility for the direct detection of neutralinos is to seek evidence for the tiny nuclear recoils produced by interactions between neutralinos (created when the universe was very young and very hot) and atomic nuclei (see the first figure). Because such interactions are rare and the effects small, they can only be detected in experiments that are conducted underground, where the high-energy cosmic radiation is suppressed by several orders of magnitude.

Astrophysical observations could provide indirect evidence for neutralinos. On astrophysical scales, collisions of neutralinos with ordinary matter are believed to slow them down. The scattered neutralinos, whose velocity is degraded after each collision, may then be gravitationally trapped by objects such as the Sun, Earth, and the black hole at the center of the Milky Way galaxy, where they can accumulate over cosmic time scales. Such dense agglomerates could therefore yield an enhanced signal for the postulated neutralinos of cosmic origin.

Another possible signal may come from collisions between two neutralinos, which are believed to result in pairwise annihilation of the neutralinos in dense condensates of such particles. This process would be highly energetic, with energies of billions of electron volts (eV)—much higher than the energy of solar neutrinos, which does not exceed tens of millions of eV. The neutrinos resulting from neutralino annihilation should carry a distinct signature that could be observed with neutrino telescopes designed to search for dark matter of this kind. Neutrinos (for example, from annihilating neutralinos deep within the solar core) are the only particles associated with neutralino annihilation or decay that are likely to escape from their place of birth.

Recently, the gamma-ray spectrometer on the European Space Agency's INTEGRAL satellite has provided evidence for a "fountain" of antimatter electrons (that is, positrons) that are being ejected from some object near the galactic center, presumably a black hole. The data indicate that some

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Chapter 2

Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression.

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Pleiohomeotic Can Link Polycomb to DNA and Mediate Transcriptional Repression

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Polycomb group (PcG) proteins function through *cis*-acting DNA elements called PcG response elements (PREs) to stably silence developmental regulators, including the homeotic genes. However, the mechanism by which they are targeted to PREs remains largely unclear. Pleiohomeotic (PHO) is a sequence-specific DNA-binding PcG protein and therefore may function to tether other PcG proteins to the DNA. Here, we show that PHO can directly bind to a Polycomb (PC)-containing complex as well as the Brahma (BRM) chromatin-remodeling complex. PHO contacts the BRM complex through its zinc finger DNA-binding domain and a short N-terminal region. A distinct domain of PHO containing a conserved motif contacts the PcG proteins PC and Polyhomeotic (PH). With mobility shift assays and DNA pulldown experiments, we demonstrated that PHO is able to link PC, which lacks sequence-specific DNA-binding activity, to the DNA. Importantly, we found that the PC-binding domain of PHO can mediate transcriptional repression in transfected *Drosophila* Schneider cells. Concomitant overexpression of PC resulted in stronger PHO-directed repression that was dependent on its PC-binding domain. Together, these results suggest that PHO can contribute to PRE-mediated silencing by direct recruitment of a PC complex to repress transcription.

Cellular differentiation and development of multicellular organisms is the result of the temporal and spatial orchestration of gene expression patterns. The Polycomb group (PcG) of repressors and trithorax group (trxG) of activators are required for maintenance of the determined gene expression patterns of several genes, including the homeotic genes (4, 5, 9, 22, 44, 47, 49, 61). Early in *Drosophila* development, the transiently expressed gap and pair-rule proteins establish the expression patterns of the homeotic genes that determine the identity of the body parts. These early regulators disappear later during development, and their function is taken over by the PcG and trxG proteins. The PcG proteins act to perpetuate silencing of the homeotic genes outside their expression domains, whereas the trxG proteins are necessary for the maintenance of transcriptional activity. Since PcG and trxG proteins conserve a state of gene expression over multiple rounds of cell division, this process is often referred to as epigenetic regulation.

PcG proteins act in concert as components of defined multiprotein complexes that are believed to silence gene transcription by inducing a higher-order chromatin structure (1, 9, 22, 24, 46, 49, 64, 66, 67, 68). Currently, two functionally distinct classes of PcG protein complexes have been identified. First, biochemical analysis uncovered a 3-MDa PRC1 complex that harbors PcG proteins Polycomb (PC), Polyhomeotic (PH), Sex combs on midleg, Posterior sex combs (PSC), and several other proteins, including components of the basal transcription factor TFIID and Zeste, a sequence-specific DNA-binding protein (22, 67, 68). Coimmunoprecipitation experiments and protein-protein interaction studies suggested that the mammalian homologues of the PcG proteins in PRC1 also form a complex (1, 66).

A second type of PcG complex contains the PcG proteins Enhancer of Zeste [E(z)], extra sex combs (Esc), the histone deacetylase Rpd3, and the histone-binding protein p55 that is also part of the chromatin assembly factor CAF1 and the chromatin remodeling factor NURF (56, 75). The association of E(z) and ESC is conserved in mammals, and repression by the mammalian ESC/E(z) complex involves histone deacetylation (66, 79). In contrast, repression by vertebrate PC homologues is resistant to inhibitors of histone deacetylation, suggesting that PC repression occurs through a distinct molecular mechanism (72, 79). Instead, PRC1 may act by inhibition of chromatin remodeling by the SWI/SNF complex in a process that does not require the histone tails (23, 68). Thus, there are at least two distinct PcG complexes, each of which represses transcription by a different mechanism. The ESC/E(z) complex appears to direct deacetylation of the histone tails, whereas PRC1 may induce a stabilized SWI/SNF-resistant chromatin structure. Recent coimmunoprecipitation experiments have indicated that there might be a transient interaction between these two distinct PcG complexes during early development (64).

An outstanding question is how PcG proteins act in a gene-specific manner. In *Drosophila melanogaster*, PcG-dependent silencing is mediated by large, rather poorly defined DNA sequences that are named Polycomb response elements (PREs) or cellular memory modules (5, 22, 47, 49, 61). PREs were identified by their PcG protein-dependent silencing effect on linked reporter genes in transgenic flies (12, 15, 21, 28, 41, 54, 62, 71, 82). Indeed, chromatin immunoprecipitation exper-

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iments revealed that PREs are bound by PcG proteins (57, 58, 73, 74), and immunostaining of polytene chromosomes showed that the insertion of a P-element containing a PRE creates a new PcG protein-binding site (15, 16, 82). Collectively, these results demonstrate that PcG proteins associate with PREs to mediate transcriptional silencing.

The majority of PcG proteins appear to lack sequence-specific DNA-binding activity, suggesting that protein-protein interactions play an important role in PcG complex formation on PREs. The exception so far is the PcG protein Pleiohomeotic (PHO), which contains a zinc finger DNA-binding domain (DBD), which is related to that of the mammalian transcription factor YY1 (9). There is a second region of about 25 residues that shows similarity, corresponding to a small portion of the so-called spacer region in YY1 (69).

The ability of PHO to bind DNA makes it an attractive candidate for a PcG tethering factor. Indeed, PHO has been shown to bind to PREs from the *engrailed* (*en*) gene (9), the *Ultrabithorax* (*Ubx*) gene (24), and the *Abdominal-B* (*Abd-B*) region (13, 52). Sequence inspection has revealed that most PREs contain potential PHO binding sites, suggesting that PHO might be involved in the targeting of PcG silencing (51). The PHO binding sites in the *en* PRE are essential for its function as a pairing-sensitive silencer of a miniwhite reporter gene, and silencing is partially impaired in *pho* mutants (10). Point mutations in PHO sites in the *Ubx* PRE abolish PcG silencing in imaginal disks, and PHO was also shown to synergize with PC to repress the *Ubx* gene in vivo (25, 76). Likewise, both the PHO and GAGA sites in the MCP silencer and *iab-7* are required for the maintenance of repression (12, 51). However, although PHO sites are necessary in these studies, by themselves they are not sufficient to reconstitute PRE activity in vivo. Instead, the activity of additional DNA-binding proteins, such as the trxG protein GAGA, appears to be required for PcG silencing (13, 30, 35, 52, 63, 70).

Recent studies have indicated that PHO might associate with the ESC/E(z) complex (64). Moreover, the related YY1 protein has been reported to interact with the mammalian ESC/E(z) complex (65). However, these results do not exclude the possibility of a transient interaction with components of a PC-containing PRC1-related complex. Indeed, YY1 has been found to bind RYBP, a component of the vertebrate PC complex (26). Furthermore, as discussed above, PHO and PC appear to cooperate in vivo during fly development. A simple explanation for this cooperation would be a direct interaction between PHO and a PC-containing repressive complex.

Because transcription factors in general are not stably associated with their coactivators or corepressors (55), we set out to identify a putative repression domain in PHO and test whether it interacted with a PC complex. We found that PHO can directly bind a PC complex as well as the Brahma (BRM) chromatin remodeling complex in *Drosophila* embryo nuclear extracts. Distinct protein domains of PHO are involved in targeting either the PC or the BRM complex. PHO specifically targets PC and PH. We used mobility shift assays and DNA pulldown experiments to assess the ability of PHO to link PC to the DNA. Finally, we tested the ability of PHO to direct PC-mediated transcriptional repression in transfected *Drosophila* Schneider cells. Our results suggest that PHO contributes to PcG repression by connecting PC to gene-regulatory DNA elements.

MATERIALS AND METHODS

DNA constructs. Details of cloning procedures are available upon request. The glutathione *S*-transferase (GST) fusion constructs were generated by a PCR-based strategy. PC-, PH-, and PHO-encoding DNA fragments were cloned in pGEX-2TKN, a derivative of pGEX-2TK (Pharmacia). The GST-BRM fusion construct has been described (18). Similarly, templates for in vitro translation were generated by cloning of the corresponding coding sequences into pTβSTOP (40). The coding sequence of full-length PHO was cloned into modified versions of the shuttle vector pVL1392 (Pharmingen) expressing either an in-frame amino-terminal Flag or GST tag, and the PHO DNA-binding domain (DBD; amino acids 355 to 520) was cloned into pVL1392-Flag. The luciferase reporter contains five Gal4 binding sites located upstream of the herpes simplex virus thymidine kinase promoter in front of luciferase (pGL3; Promega). The Gal4 DNA-binding domain chimeras were constructed by subcloning the indicated cDNAs in-frame in a modified pCDNA3. The full-length coding sequence of PC was cloned in pSuper-CATCH containing an N-terminal Flag tag. The reporter used for the long-distance repression experiment (Fig. 7C) has been described (48).

Protein procedures. Recombinant PHO and DBD containing an N-terminal Flag epitope was expressed in Sf9 cells in the baculovirus expression system and immunopurified with anti-Flag M2 beads (Sigma) or glutathione-Sepharose (Pharmacia) essentially as described previously (14, 40).

The *Drosophila* nuclear extracts and protein fractions were prepared essentially as described previously (3, 33). Briefly, all protein procedures were carried out at 4°C or on ice with HEMG buffer [25 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol (DTT), 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 μM pepstatin, 0.01% Nonidet P-40] containing various amounts of KCl. Nuclear extracts of dechorionated *Drosophila* embryos (0 to 12 h) were prepared as described previously by Kadonaga (38). The nuclear extracts were either used directly or concentrated by Poros-Heparin (Perseptive Biosystems) chromatography essentially as described previously (3, 33). The heparin–400 mM KCl fractions (H0.4) contained the vast majority of BRM, initiation switch (ISWI), and general transcription factors. The H0.4 pool was further purified by Sephacryl S-300 column chromatography guided by Western blot analysis with antibodies directed against BRM, PC, and PH. Fractions containing the bulk of the above factors were pooled and further purified on a BioScale Q10 column (Bio-Rad). It should be noted that essentially all the PC, PH, and BRM present in nuclear extracts was retained in this fraction, as judged by Western blotting analysis with the appropriate antibodies (data not shown). Most pulldown experiments were performed with crude nuclear extracts as well as with a fraction from the Q10 column with essentially similar results. The results shown were obtained with the nuclear extracts (Fig. 1) or with the partially purified Q10 fraction (Fig. 2, 3, and 6).

Recombinant GST fusion proteins were expressed in *Escherichia coli* BL21 and purified by glutathione-Sepharose chromatography by standard procedures. ^{35}S-labeled proteins were expressed with the TNT rabbit reticulocyte lysate (Promega). The GST pulldown experiments were performed as described previously (36) with the following modifications. The lysis buffer contained 25 mM HEPES-KOH (pH 7.6), 10% glycerol, 0.5 M NaCl, 0.01% NP-40, 5 mM DTT, 2.5 mM $MgCl_2$, 50 μM $ZnCl_2$, and protease inhibitors. Binding reactions (Fig. 2 and 4) were carried out in binding buffer [20 mM HEPES-KOH (pH 7.6), 2.5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT] containing 70 mM KCl and 0.02% NP-40. Unbound proteins were removed with a series of washes with wash buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% NP-40, 100 mM NaCl]. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Western blotting.

For pulldowns with GST fused to full-length PHO, GST-PHO was purified from baculovirus-infected Sf9 cells. Binding assays (Fig. 1B) were performed as described above with the following modifications: binding was performed in HEMG containing 100 mM NaCl, and washes were performed with HEMG containing 150 mM NaCl. Far-Western analysis was carried out as described previously (39) with ^{35}S-labeled reticulocyte-expressed protein derivatives.

Following autoradiography to detect bound proteins, blots were reprobed with antibodies against BRM, PH, and PC. All immunological procedures were performed essentially as described previously (31, 32). Rabbit antisera directed against PHO (SN842), PH (SN964), PC (SN965), Groucho (GRO) (PV1 and PV2, pooled), and Moira (MOR) (SN670 and SN671, pooled) were raised by immunization with GST fusion proteins corresponding to PHO amino acids 1 to 49, 42 to 119, and 118 to 172; PH amino acids 1 to 595, 557 to 855, 817 to 1096,

and 1077 to 1590; PC amino acids 1 to 215, 196 to 390, and 1 to 390, PV1 and -2, full-length GRO, SN670 and SN671, and full-length MOR. Additional antisera (used in Fig. 2 and 3) were generated by immunization of rabbits with peptides coupled to keyhole limpet hemocyanin essentially as described previously (31, 32). The following peptides were used: PV69 anti-PC, RERDMKGDSSPVA; PV86 anti-PH, KEVPPPGEAKDPGAQ; and PV35 anti-polymerase II 140-kDa subunit (DmRP140), MSVQRIVEDSPAIELQ. The antibodies directed against BRM and ISWI (40), OSA (77) and PSC (50) have been described before.

Antibodies were affinity purified as described by Hancock and Evan (31). When appropriate, all critical immunoprecipitations and Western immunoblot experiments were repeated with different antisera. For coimmunoprecipitation experiments (Fig. 1A), 300 μl of *Drosophila* embryo nuclear extract was incubated overnight at 4°C on a spinning wheel with 30 μl of antiserum directed against PHO. Next, 50 μl of protein A-Sepharose beads (Pharmacia) was added and incubated for another hour, and following a series of extensive washes with HEMG buffer containing 250 mM NaCl, bound proteins were eluted with HEMG–1 M NaCl, resolved by SDS-PAGE, and analyzed by Western immunoblotting.

DNA-binding assays. The DNA band shift assays were essentially performed as described previously (14). Double-stranded oligonucleotides harboring a PHO site (5′-AATTCCGGCGCAGCCATTATGGTGG-3′) (51) were end labeled with T4 polynucleotide kinase. Binding reactions were carried out in a reaction volume of 20 μl of 0.5× HEMG buffer containing 70 mM NaCl, 50 μg of bovine serum albumin per ml, 0.05% NP-40, 1 mM DTT, ≈60 fmol of double-stranded labeled probe, 1 μg of poly(dGdC)-poly(dGdC), and the indicated polypeptide. All binding reactions were carried out on ice for 90 min and were analyzed on 5% polyacrylamide gels run in 0.5× Tris–glycine–0.01% NP-40 buffer at room temperature. For supershift experiments, recombinant GST-PC or GST alone was added to the binding reaction. In the antibody supershift experiments, either affinity-purified anti-PC antiserum or preimmune antiserum was added to the reaction directly before the addition of the labeled probe.

For recruitment assays, PC or BRM complexes were immunopurified from Mono Q fraction 26 with affinity-purified antibodies directed against each of these proteins that were cross-linked to protein A beads with dimethylpimelimidate as described previously (32). Affinity resins were incubated with protein fractions for 2 h at 4°C in HEMG containing 75 mM KCl, followed by extensive washes with excess HEMG containing 150 mM KCl. Next, these beads were incubated in either the presence or absence of recombinant PHO or PHO DBD, and radiolabeled double-stranded oligonucleotides harboring five PHO sites present in natural PREs (5′-CTAGACGGCGCAGCCATTATGGTGCAGTC GGCCATGAGTGATAAAGGCAGCCATTTTCCTGTGCTGCCGCCATAT TATTTTGCGGCAGCCATGTTGGATG-3′) (51) as well as an unrelated control DNA fragment that lacks PHO sites. The binding reaction was carried out in binding buffer [25 mM HEPES (pH 7.6), 12.5 mM $MgCl_2$, 10% glycerol, 0.1 mg of bovine serum albumin per ml, 100 ng of poly(dGdC)-poly(dGdC) per μl, and 70 mM KCl] at room temperature for 30 min. After several washes with binding buffer containing 100 mM KCl, bound DNA was resolved on a 1.75% agarose gel and visualized by autoradiography.

Transient-transfection assays. Plasmids for transfection studies in *Drosophila* Schneider L2 cells were isolated with Qiagen columns according to the manufacturer's instructions. SL2 cells were propagated in Ultimate Insect serum-free medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and fungizone (amphotericin B; 250 μg/ml; Gibco-BRL). All transfections were performed with Fugene (Roche), according to the manufacturer's instructions. Empty vector was added for each transfection to a total amount of 250 ng or 1 μg of DNA (for 24- and 6-well plates, respectively).

For repression assays, SL2 cells were plated at 60 to 80% confluency in 24- or 6-well plates, fresh medium was added the following day, and the plasmids described above were transfected. The next day, the medium was replaced, and 48 h after transfection, the cells were harvested, washed in phosphate-buffered saline, and resuspended in 100 μl or 500 μl (for 24- and 6-well plates, respectively) of lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100). Luciferase activity was determined according to the manufacturer's protocol (Promega).

RESULTS

PHO binds the BRM complex and a PC complex. To assess whether PHO might be involved in the recruitment of other PcG proteins, we tested its ability to bind endogenous PC, PH, and PSC present in *Drosophila* embryo extracts. We also investigated whether PHO could bind the multisubunit chromatin-remodeling BRM complex, containing at least three subunits encoded by trxG genes: BRM itself, Osa (OSA), and Moira (MOR) (17, 18, 40). First, we used either preimmune serum or antiserum directed against PHO for immunoprecipitation experiments with *Drosophila* embryo nuclear extracts (Fig. 1A). Following extensive washes with a buffer containing 250 mM NaCl and detergent, PHO-associated proteins were eluted with a buffer containing 1 M NaCl. Western immunoblot analysis revealed the presence of PcG proteins PC, PH, and PSC (all components of PRC1) as well as three subunits of the BRM complex, BRM, MOR, and OSA, suggesting that a PC complex as well as the BRM complex can associate with PHO in embryo extracts. In contrast, the corepressor Groucho (GRO) and the RNA polymerase II complex detected by an antibody directed against the 140-kDa subunit did not associate with PHO. As expected, none of these proteins were immunoprecipitated with beads coupled to preimmune serum.

Because we suspected a dynamic rather than a stable association of PHO with the PC or BRM complex, we next tested

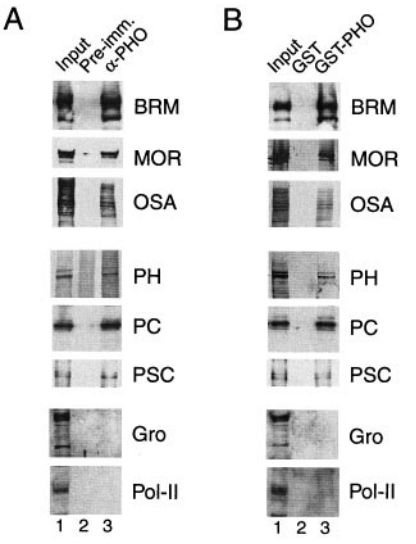

FIG. 1. PHO interacts with PcG proteins and the BRM complex in *Drosophila* embryo nuclear extracts. (A) Coimmunoprecipitation experiments with antiserum directed against PHO with *Drosophila* embryo nuclear extracts. Nuclear extracts were incubated with either preimmune serum (lane 2) or antiserum directed against PHO (lane 3), followed by the addition of protein A beads. Following extensive washes with a buffer containing 250 mM NaCl and detergent, associated proteins were eluted with a buffer containing 1 M NaCl, resolved by SDS-PAGE, and analyzed by Western immunoblotting with antibodies directed against BRM (39), MOR (SN670 and SN671, pooled), OSA (74), PH (SN964), PC (SN965), PSC (49), GRO (PV1 and PV2, pooled), and the 140-kDa subunit of RNA polymerase II (Pol-II; DmRP140) (PV35). Lane 1 represents 10% of the input material used in the binding reactions. (B) The ability of a GST-tagged full-length PHO to recruit PcG proteins or the BRM complex within an embryo nuclear extract was tested by GST pulldown assays. GST alone (lane 2) or GST-PHO was immobilized on glutathione-Sepharose beads and incubated with *Drosophila* embryo nuclear extracts. Following a series of extensive washes with a buffer containing 150 mM NaCl, bound proteins were resolved by SDS-PAGE and analyzed by Western immunoblotting. Lane 1 represents 10% of the input material used in the binding reactions.

whether full-length recombinant PHO was able to bind either of these complexes present in embryo nuclear extracts. For these experiments, we purified GST-tagged PHO from extracts of insect Sf9 cells infected with recombinant baculoviruses. An affinity resin was generated by immobilization of GST-PHO on glutathione-Sepharose beads and incubated with *Drosophila* nuclear embryo extracts. Following a series of extensive washes, bound proteins were resolved by SDS-PAGE and analyzed by Western immunoblotting (Fig. 1B). In agreement with the immunoprecipitation experiments, recombinant PHO was found to efficiently bind the BRM complex as well as a PC complex. Neither GRO nor RNA polymerase II was bound by PHO, and none of the PHO-binding proteins were retained on GST beads.

To map the PHO domains involved in binding the BRM or PC complex, we expressed and purified distinct PHO deletions as GST fusion proteins and immobilized these polypeptides on glutathione-Sepharose beads. The various PHO affinity resins were incubated with a partially purified fly embryo nuclear extract (see Materials and Methods). PHO-associated proteins were resolved by SDS-PAGE and analyzed by Western immunoblotting (Fig. 2). The zinc finger DBD (Fig. 2, lane 3) and the first 49 residues of PHO (Fig. 2, lane 9) efficiently bound the BRM complex, as revealed by the presence of its BRM, MOR, and OSA subunits. Neither GST alone (Fig. 2, lane 2) nor other regions of PHO (Fig. 2, lanes 4 to 8) were able to bind the BRM complex. Conversely, the two BRM-binding domains of PHO did not bind PC or PH. However, a distinct region, comprising amino acids 118 to 172, efficiently retained both PC and PH but not the BRM complex (Fig. 2, lane 7). This domain harbors a stretch of residues conserved between PHO and YY1 (indicated with a black box in Fig. 2). None of the remaining regions of PHO or GST alone interacted with either PC or PH, indicating that the protein-protein interactions are selective.

It is well established that PC and PH are part of a large multiprotein complex (24, 65, 66). Indeed, coimmunoprecipitation and size exclusion chromatography experiments confirmed that PC and PH were stably associated in our extracts (data not shown). This PcG protein complex is likely to be similar or related to the previously described PRC1 (65, 66). However, since we have not characterized it further, we will refer to it as the PC complex. In summary, these experiments established that distinct regions of PHO can mediate binding to either the BRM or PC complex. The PHO N-terminal domain (amino acids 1 to 49) and its DBD can bind independently to the BRM complex. A separate domain of 55 residues (amino acids 118 to 172) mediates PC binding.

Identification of targets of PHO within the PC and BRM complexes. To identify the molecular weights of potential PHO targets, we performed a far-Western experiment with a partially purified column fraction containing both the BRM and PC complexes (Mono Q fraction 26; Fig. 3A). Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Following denaturation and renaturation, the membrane was probed with radiolabeled full-length PHO or various deletion constructs (Fig. 3B and C). Autoradiography of the membrane suggested direct binding of PHO to proteins that precisely comigrated with BRM, PH, or PC (Fig. 3C, lane 1).

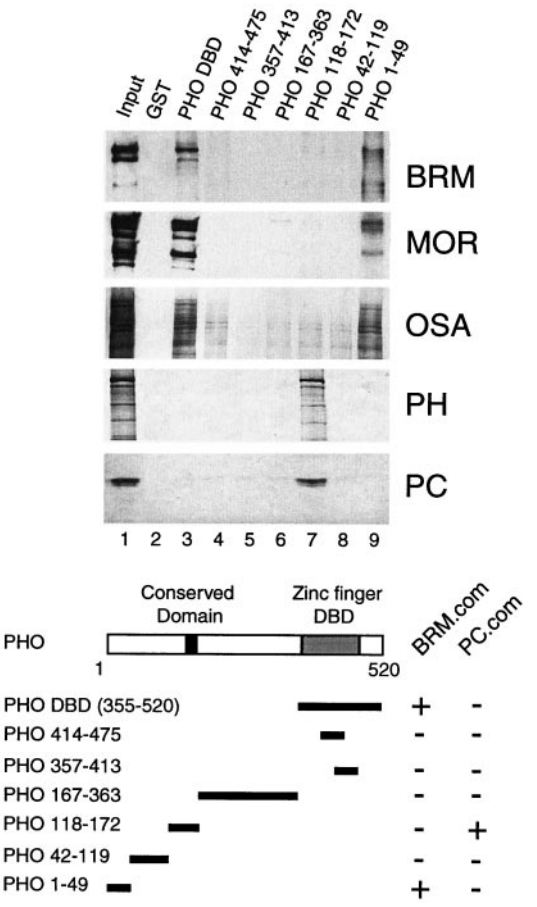

FIG. 2. PHO interacts with PcG proteins and the BRM complex. The ability of various PHO polypeptides to recruit PC, PH, and the BRM complex from *Drosophila* embryo nuclear extracts was tested by GST pulldown assays. GST alone (lane 2), GST-PHO DBD (amino acids 355 to 520; lane 3), GST-PHO(414-475) (lane 4), GST-PHO(357-413) (lane 5), GST-PHO(167-363) (lane 6), GST-PHO(118-172) (lane 7), GST-PHO(42-119) (lane 8), and GST-PHO(1-49) (lane 9) were immobilized on glutathione-Sepharose beads and incubated with a partially purified column fraction containing the PC and BRM complexes. Protein complexes were washed, resolved by SDS-PAGE, and transferred to nitrocellulose. The blots were probed with antibodies directed against BRM (39), MOR (SN670 and SN671, pooled), OSA (74), PH (PV86), or PC (PV69). Lane 1 represents 5% of the input material used in the binding reactions. The domain structure of PHO, including the zinc finger DNA-binding domain (DBD), a conserved region present in YY1, and the amino acid residues present in the various derivatives are indicated. The binding of the PHO deletion constructs to either the BRM complex (BRM.com) or the PC complex (PC.com) is summarized.

The presence and position of BRM, PH, and PC were established by reprobing the far-Western blots with antibodies directed against these proteins (Fig. 3C, lanes 5 to 6). In addition, we observed binding to a protein of around 110 kDa. None of the other proteins present in the protein fraction used (see Fig. 3A) were significantly bound by PHO, indicating that the interactions detected in the far-Western analysis were selective. PHO(1-172) (Fig. 3C, lane 2), which contains the putative PC-binding domain and the N-terminal BRM complex-binding region, efficiently bound to both BRM and PC, while weak binding to PH was observed. Although PHO(167-363)

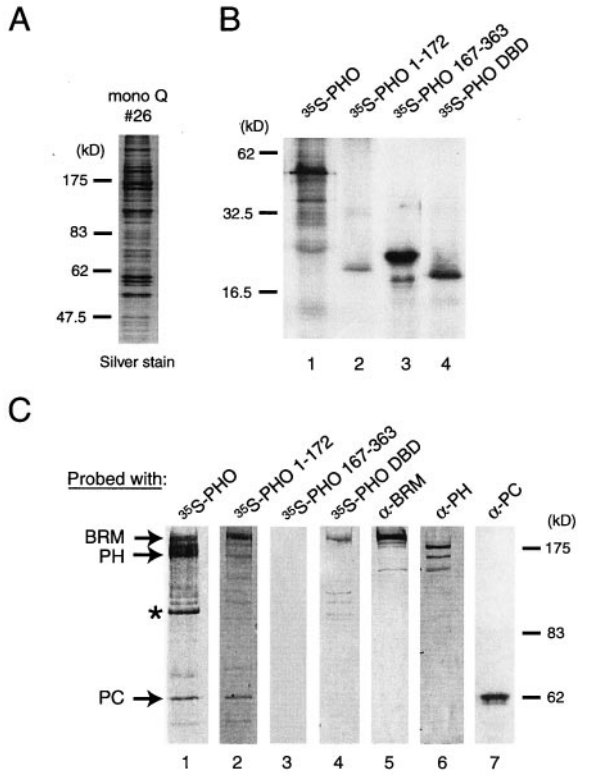

FIG. 3. Identification of PHO targets within the PC and BRM complexes. (A) Polypeptide composition of a partially purified fly embryo nuclear extract containing BRM and PC (Mono Q10 fraction 26) used in the far-Western analysis. Most of the PC, PH, and BRM present in nuclear extracts is retained in this fraction, as judged by Western blotting analysis with the appropriate antibodies (data not shown). Proteins were resolved by SDS-PAGE and visualized by silver staining. The molecular masses of protein standards are indicated. (B) In vitro-translated proteins used as probes in the far-Western experiments: ^{35}S-labeled full-length PHO (lane 1), PHO(1-172) (lane 2), PHO(167-363) (lane 3), and PHO DBD(355-520) (lane 4). (C) Far-Western blotting analysis of PHO-binding proteins. The purified BRM and PC complexes (Mono Q10 fraction 26) was resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was treated with 6 M guanidine-HCl, renatured, washed, and incubated with ^{35}S-labeled reticulocyte-expressed full-length PHO(1-520) (lane 1), PHO(1-172) (lane 2), PHO(167-363) (lane 3), and PHO DBD(355-520) (lane 4). After extensive washing, the filter was exposed to film. Filters were reprobed with antibodies directed against BRM (lane 5), PH (lane 6), or PC (lane 7). The positions of BRM (39), PH (PV86), and PC (PV69) which coincide with the immunoreactive band are indicated. An unidentified protein with an estimated molecular mass of about 110 kDa that was bound by PHO is indicated with an asterisk.

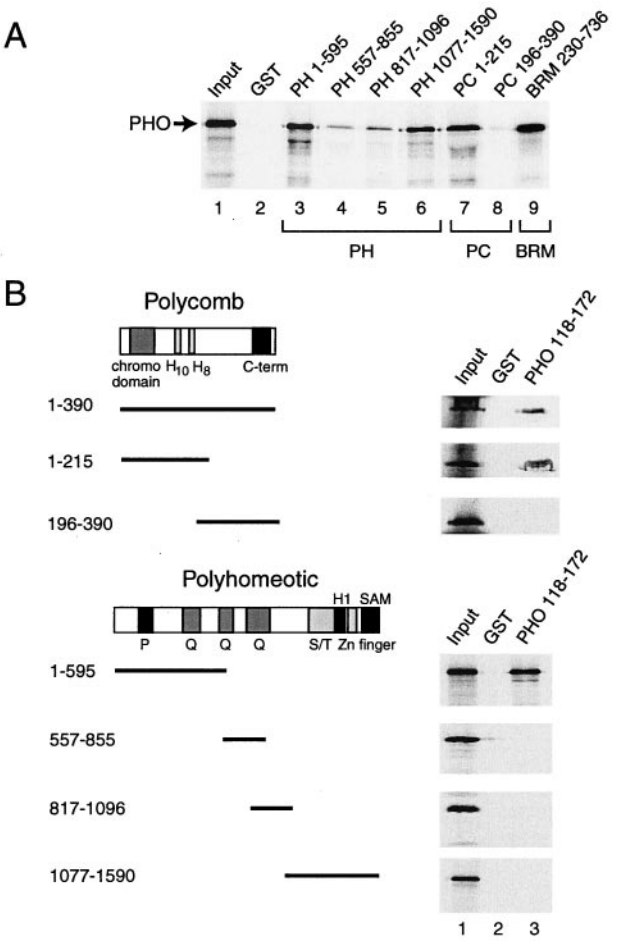

FIG. 4. PHO interacts directly with PC, PH, and BRM. (A) ^{35}S-labeled full-length PHO was incubated with GST alone (lane 2), GST-PH(1-595) (lane 3), GST-PH(557-855) (lane 4), GST-PH(817-1096) (lane 5), GST-PH(1077-1590) (lane 6), GST-PC(1-215) (lane 7), GST-PC(196-390) (lane 8), or GST-BRM(230-736) (lane 9) immobilized on glutathione-Sepharose beads. Protein complexes were washed and resolved by SDS-PAGE, and bound proteins were detected by autoradiography. Lane 1 represent 5% of the input material used in the binding reactions. (B) Mapping of the PC- and PH-binding domain of PHO by GST pulldown assays. GST alone (lane 2) or GST-PHO(118-172) (lane 3) was immobilized on glutathione-Sepharose beads and incubated with ^{35}S-labeled full-length PC (residues 1 to 390), PC(1-215), PC(196-390), PH(1-595), PH(557-855), PH(817-1096), or PH(1077-1590). Protein complexes were washed and resolved by SDS-PAGE, and bound proteins were detected by autoradiography. Lane 1 represents 5% of the input material used in the binding reactions. The domain structure of PC (as described by Breiling et al. [7]) and PH (as described by Kyba and Brock [46]) and the residues present in the various GST fusion constructs are indicated.

was efficiently expressed and labeled, it failed to recognize any protein present on the membrane (Fig. 3C, lane 3). The far-Western analysis suggested that the PHO DBD can bind directly to BRM (lane 4) but not to PC or PH. Thus, in agreement with the pulldown assays with embryo extracts (Fig. 1), these experiments suggest that separate PHO domains mediate association with the BRM and PC complexes.

The far-Western analysis indicates that BRM, PC, and PH are the most likely targets contacted by PHO. To obtain direct evidence for binding to PHO, we expressed and purified various polypeptides corresponding to PC, PH, and BRM as GST fusion proteins. These fusion proteins were immobilized on glutathione-Sepharose beads and tested for their ability to bind radiolabeled full-length PHO. As shown in Fig. 4A, PHO bound efficiently to the N-terminal half of PC but not to its C-terminal half. Moreover, PHO associated with a central portion (amino acids 230 to 736) of BRM and the N-terminal and C-terminal domains of PH but not with the central regions of PH or with GST alone. Thus, these experiments with recom-

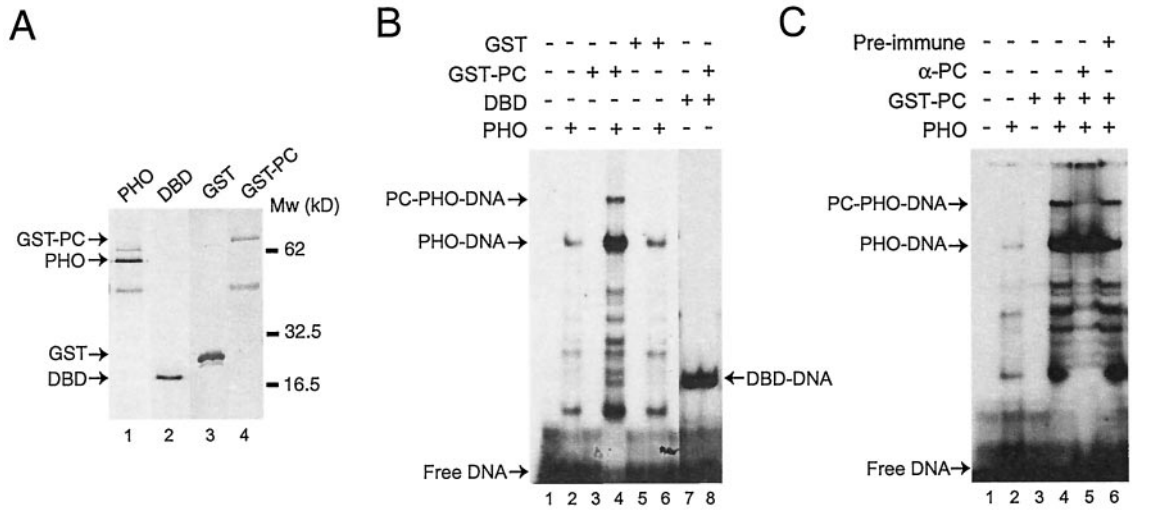

FIG. 5. PHO can link PC to the DNA. (A) Recombinant Flag-tagged PHO and PHO DBD (residues 355 to 520) were immunopurified from extracts of baculovirus-infected Sf9 cells with an anti-Flag column and eluted under native conditions with a peptide corresponding to the Flag epitope (lanes 1 and 2). Recombinant GST and GST-PC were expressed in *E. coli* BL21, purified by glutathione-Sepharose chromatography, and eluted with reduced glutathione (lanes 3 and 4). Proteins were resolved by SDS-PAGE and visualized by silver staining. (B) The DNA-binding activity of recombinant PHO and PHO DBD was tested in the absence and presence of GST-PC or GST by electrophoretic mobility shift assays with a radiolabeled double-stranded oligonucleotide containing a single PHO site. Binding reactions were done either in the absence of protein (lane 1) or in the presence of recombinant PHO alone (lane 2), GST-PC (lane 3), both PHO and GST-PC (lane 4), GST (lane 5), both GST and PHO (lane 6), DBD alone (lane 7), or DBD and PC (lane 8). (C) Mobility shift experiment similar to that in B. Binding reactions were done either in the absence of protein (lane 1) or in the presence of recombinant PHO (lane 2), GST-PC (lane 3), or PHO and GST-PC (lanes 4 to 6). Incubations were done either in the absence of antibodies (lane 4) or in the presence of affinity-purified anti-PC (PV69) (lane 5) or preimmune serum (lane 6). The positions of free DNA, PHO-DNA, DBD-DNA, and the ternary PC-PHO-DNA complex are indicated.

binant polypeptides provide further evidence for the notion that PHO interacts specifically with BRM, PC, and PH.

Since our experiments with embryo extracts (Fig. 2) suggested that PHO residues 118 to 172 could recruit an endogenous PC complex, we tested whether this domain could directly recognize recombinant PC or PH in a pulldown assay (Fig. 4B). Indeed, the polypeptide PHO(118-172) efficiently retained full-length PC or its N-terminal half but not its C-terminal half. Moreover, this region of PHO bound the N-terminal portion of PH(1-595) but not to the remainder of the protein. We conclude that PHO(118-172) constitutes a PC-binding domain that associates with both PC and PH.

PHO can link PC to the DNA. Since PHO but not PC possesses sequence-specific DNA-binding ability, we wondered whether PHO could tether PC to a PHO recognition DNA sequence. Full-length PHO and the C-terminal portion harboring the DBD were expressed in Sf9 insect cells infected with recombinant baculoviruses. PHO polypeptides were immunopurified from Sf9 cell extracts to near homogeneity with their N-terminal Flag epitopes (Fig. 5A). PC was expressed as a GST fusion protein and purified from *E. coli* extracts (Fig. 5, lane 4). Next, we performed electrophoretic mobility shift assays with the PHO polypeptides either alone or in the presence of GST-PC (Fig. 5B). As expected, full-length PHO (Fig. 5B, lane 2) as well as the DBD (Fig. 5B, lane 7) could bind efficiently a DNA fragment bearing a PHO-binding site. Addition of GST-PC to the binding reaction containing PHO led to the appearance of a novel slower-migrating species, which we interpret as a PC-PHO-DNA complex. Indeed, GST-PC by itself failed to bind DNA (Fig. 5B, lane 3), and GST alone did not induce a PHO supershift (lane 6). Thus, the appearance of the supershifted species depends on the presence of both PHO and PC. Moreover, PHO DBD, which lacks the PC-binding domain, was not supershifted by GST-PC (Fig. 5B, lane 8).

Following incubation with affinity-purified antibodies directed against PC, the PC-PHO-DNA complex but not the PHO-DNA complex disappeared (Fig. 5C, lane 5). The appearance of label just below the well may indicate the presence of an antibody-PC-PHO-DNA complex that has difficulty entering the gel. As expected, formation of the ternary PC-PHO-DNA complex was not blocked by the addition of preimmune serum (Fig. 5C, lane 6).

To test whether PHO could link an endogenous PC complex to the DNA, we used beads coated with affinity-purified antibodies directed against either PC or BRM to purify the complexes from embryo nuclear extracts. Next, we assessed the ability of the immobilized PC and BRM complexes to associate with specific DNA sequences in either the presence or absence of PHO. The affinity resins were incubated with a radiolabeled DNA fragment containing five PHO-binding sites and an unrelated control fragment in the presence of an excess of poly(dGdC)-poly(dGdC) competitor DNA. After a series of washes, bound DNA was recovered and analyzed by agarose gel electrophoresis, followed by autoradiography (Fig. 6).

Although the PC complex alone was unable to bind DNA (Fig. 6, lane 5, upper and lower panels), selective association with the PHO sites but not with the control DNA was observed in the presence of PHO (Fig. 6, upper panel, lane 6). An unrelated transcription factor (NTF-1) was unable to link the PC complex to the DNA (Fig. 6, lane 7). Furthermore, PHO was able to tether the BRM complex to the PHO elements but not to the control DNA (Fig. 6, upper panel, lane 13). The

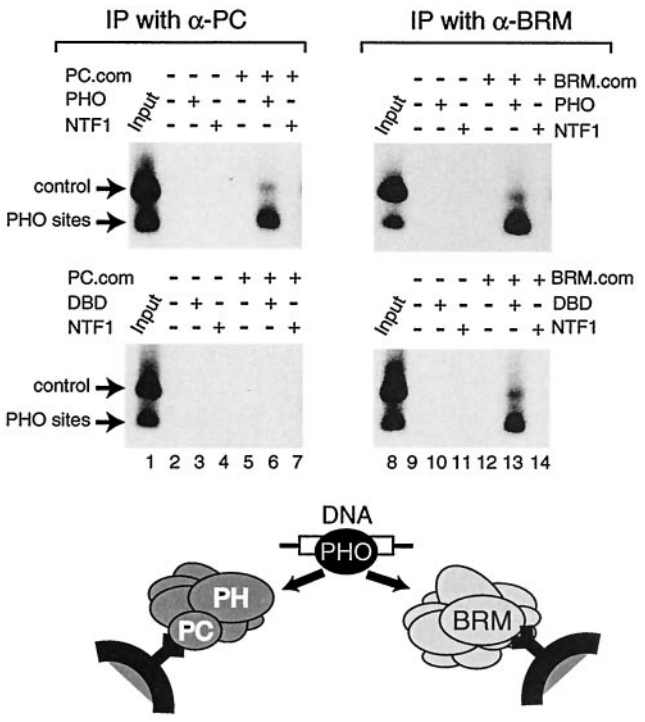

FIG. 6. PHO can tether an endogenous PC complex to a PRE. PHO links PC and BRM complexes to DNA. The PC or BRM complex was purified with beads coated with affinity-purified antibodies directed against either PC or BRM. The immobilized PC complex (left-hand panels) or BRM complex (right-hand panels) was incubated with a radiolabeled DNA fragment containing PHO-binding sites and an unrelated control fragment in the presence of an excess of poly-(dGdC)-poly(dGdC) competitor DNA. Binding reactions were done, as indicated, in the presence of either no additional protein, PHO, or the unrelated transcription factor NTF-1. Following a series of washes, bound DNA was recovered and analyzed by agarose gel electrophoresis, followed by autoradiography. Lanes 1 and 8 represent 5% of the input material used in the binding reactions.

PHO DBD, lacking the PC-binding domain, failed to connect the PC complex to the DNA, whereas it still efficiently recruited the BRM complex (Fig. 6, compare lanes 6 and 13, bottom panel). We conclude that PHO can act as a tethering factor that uses distinct domains to link the BRM complex as well as the PC complex to a regulatory DNA element.

PC binding domain of PHO mediates transcriptional repression. So far, our results have shown that PHO is able to recruit a PC complex to the DNA. Next, we wished to investigate the functional consequences of the PHO-PC interaction in *Drosophila* cells. Previously, it was demonstrated that PcG proteins tethered to the DNA by fusion to the Gal4 DBD act as transcriptional repressors in transiently transfected cells (11). We took a similar approach and replaced the PHO DBD with that of Gal4 and tested the effect of the fusion protein on gene expression in transfected *Drosophila* Schneider L2 cells. As a reporter, we used a plasmid containing five Gal4-binding sites located upstream of a strong basal promoter (herpes simplex virus thymidine kinase) driving expression of the luciferase gene.

Cotransfection of the reporter plasmid with a vector expressing Gal4-PHO(1-356) resulted in a clear dose-dependent transcriptional repression (Fig. 7A). As expected, expression of the Gal4-DBD alone did not significantly influence reporter activity. Importantly, the PC-binding domain [Gal4-PHO(118-172)] was sufficient to mediate repression. While the PHO polypeptide containing both the PC- and BRM-binding regions [Gal4-PHO(1-356)] repressed transcription, the isolated BRM-binding domain of PHO (residues 1 to 49) functioned as an activation domain. Thus, the repressive function mediated by the PC-binding domain was dominant in the longer PHO polypeptide. Finally, Gal4-PHO(170–356), which binds neither PC nor BRM, did not affect transcription.

These results show that the PC-binding domain of PHO can repress transcription, possibly through recruitment of an endogenous PC complex. In order to obtain additional support for this notion, we tested whether the overexpression of PC could enhance PHO-directed repression. Indeed, transcriptional repression by Gal4 fusions to PHO polypeptides that could bind PC [PHO(1-356) and PHO(118-172)] was markedly enhanced by overexpression of PC (Fig. 7B). In contrast, neither the Gal4 DBD nor Gal4-PHO(170–356) was able to mediate PC repression.

Next, we tested whether Gal4-PHO was able to mediate repression from a distal position. For these experiments, we used a reporter containing Gal4 binding sites flanked by GAGA sites separated by over 2 kb of intervening DNA from a promoter containing GAGA sites (48). This reporter was cotransfected in the absence or presence of various combinations of expression vectors for the Gal4 DBD, Gal4-PHO, or PC (Fig. 7C). In this setting, Gal4-PHO was again able to mediate transcriptional repression, which was dependent on the presence of the PC-binding domain. Moreover, concomitant expression of PC led to stronger repression. From these results, we conclude that the ability of PHO polypeptides to bind PC in vitro correlates well with their capacity to mediate PC repression in *Drosophila* cells.

DISCUSSION

PcG-mediated gene silencing is accomplished via the *cis*-acting PREs, which are the DNA targets for the PcG proteins. However, it is not yet understood how PC and other PcG proteins that lack any apparent sequence-specific DNA-binding ability are directed towards PREs. In this study, we show that PHO can link PC and the BRM chromatin-remodeling complex to the DNA. A small domain in PHO mediates the recruitment of the PC complex by binding to PC and PH. This PC-binding domain directs transcriptional repression in transfected cells, which is enhanced by concomitant overexpression of PC. These results support the notion that PHO contributes to PRE-mediated silencing by tethering a PC complex to repress transcription.

Consistent with a direct role for PHO in PcG silencing, the phenotypes of *pho* mutants show similarity to those of mutants with changes in other PcG genes (6, 27, 29). Due to a large maternal contribution, animals homozygous for *pho* null alleles survive up to the pupal stage but display homeotic transformations. In the absence of maternal *pho* mRNA, embryos die early during development and exhibit segmentation defects as well as severe homeotic transformations. Previous in vivo studies have shown that mutations in PHO DNA-binding sites or in

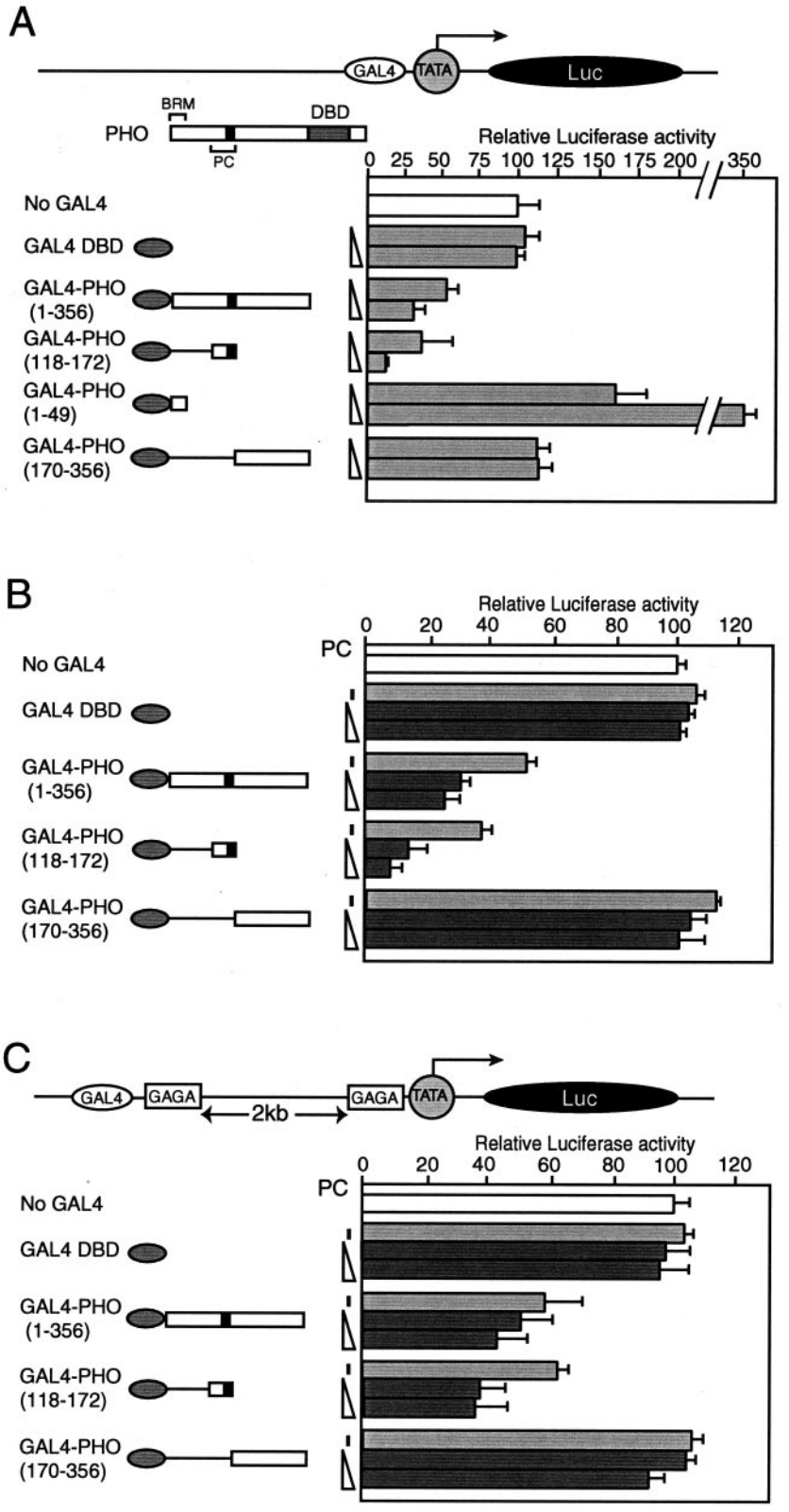

FIG. 7. PHO represses transcription in transiently transfected *Drosophila* Schneider L2 cells. (A) The PC-binding domain of PHO mediates transcriptional repression. Schneider L2 cells were transfected with either 75 ng of reporter plasmid alone (no Gal4) or together with increasing amounts of the plasmid expressing the Gal4 DNA-binding domain (Gal4 DBD) alone or with various Gal4-PHO fusion constructs (50 ng and 150 ng), as indicated by the schematic representation on the left. The structure of the reporter plasmid is indicated schematically. (B) PC enhances transcriptional repression by Gal4-PHO fusions containing the PC-binding domain. L2 cells were cotransfected with 50 ng of reporter plasmid in

the PHO protein itself compromise PcG silencing (10, 13, 25, 29, 52, 70), and PHO silencing has been observed to be PC dependent in in vivo genetic experiments (25, 76). These observations suggest that PHO DNA-binding elements are important components of at least a subclass of PREs.

In the work presented here, we provide a biochemical and functional link between PHO and PC. We found that PHO can bind both PC and PH through a small 55-amino-acid domain. PHO contacts the N-terminal portion of PC but not its C-terminal repression domain that interacts with nucleosomes and, possibly, other PcG proteins (7, 46). In agreement with the inhibitory function of PC, the PC-binding domain but not other portions of PHO mediates transcriptional repression in transfected *Drosophila* cells. Significantly, concomitant overexpression of PC leads to stronger repression, supporting the notion that PHO acts through recruitment of PC.

These experiments established a correlation between the ability of PHO polypeptides to bind PC in vitro and their capacity to mediate PC-dependent repression in cells. It should be noted that, because we have not used a purified defined PC complex in our experiments, additional factors might be involved. Furthermore, we have not addressed the role of chromatin in PcG silencing, and it is not clear whether the repression we detected in our transfection assay involves any modulation of chromatin structure. Alternatively, PC may directly block the functioning of the general transcription machinery. This possibility is of interest in light of the recently described interaction between PcG proteins and components of the basal machinery (8, 67). The various potential mechanisms of PcG repression (reviewed in reference 21) are not mutually exclusive, and stable silencing might be the result of multiple blocks to transcription, each acting at a different level.

In addition to PC recruitment, we found that PHO interacts with the BRM complex. PHO contains two BRM complex-binding domains, its N-terminal 49 amino acids and the zinc finger DBD. Interestingly, similar to the zinc finger DBDs of Sp1, EKLF, and GATA-1 (2, 37), the PHO DBD recruits the BRM complex via binding to BRM itself. Although the structural determinants are not yet clear, it appears that a class of zinc finger DBDs has evolved that can simultaneously bind DNA and target a chromatin-remodeling complex (37). Previously, we found that the trxG protein Zeste selectively recruits the BRM complex to activate transcription on chromatin templates (40). In contrast to PHO, Zeste does not contact BRM itself but rather interacts with other BRM-associated proteins, including the trxG proteins Moira and Osa. Thus, different regulators target the BRM complex by binding to distinct subunits. Although we did not directly address the role of BRM in transcriptional repression by PHO, there is evidence to indicate that ATP-dependent remodelers are involved in repression as well as activation (34, 45, 53, 80, 81). The role of the BRM complex may simply be to remodel chromatin and facilitate PHO-DNA binding. Alternatively, BRM may play a more direct role in silencing and cooperate with PcG proteins in the formation of repressive higher-order chromatin structures.

In summary, PHO has been implicated in binding the ESC/E(z) complex (64), the PC complex, and the BRM complex (this paper). Likewise, YY1 has been reported to bind the mammalian homologues of the ESC/E(z) complex (65) and the PC complex (26). Moreover, in light of the high conservation of the Kruppel-like zinc finger DBD of YY1, it seems probable that it will also interact with BRM. Most of these associations appear to be relatively weak and of a transient nature, which is typical of a transcription factor-coregulator interaction (55). Thus, the association of PHO with multiple distinct complexes does not necessarily have to occur simultaneously. For instance, one might speculate that the BRM complex helps PHO to gain access to a chromatinized PRE. Subsequent recruitment of the ESC/E(z) complex may lead to histone deacetylation, followed by recruitment of a PC-containing PRC1-related complex.

Although it has been well established that PHO contributes to PRE function, the presence of a series of PHO sites by itself does not suffice to reconstitute a PRE (10). This indicates that PHO may act in a combinatorial fashion with other tethering factors such as GAGA and Zeste. It has become clear that PREs are composed of a multitude of distinct binding elements which, depending on their context, can be redundant with, cooperate with, or antagonize each other (5, 47, 49, 61). For example, the trxG protein GAGA, generally thought of as an activator that induces chromatin remodeling (19, 20, 42, 78), has been implicated in PcG repression (13, 30, 35, 52).

Interestingly, we recently found that GAGA is required for PHO binding to a chromatinized PRE, suggesting that PHO and GAGA elements together may form a functional module (T. Mahmoudi, L. M. P. Zuijderduijn, A. Mohd-Sarip, and C. P. Verrijzer, submitted for publication). Moreover, evidence has been presented indicating that GAGA may be more directly involved in PC recruitment. In coimmunoprecipitation experiments, GAGA has been found associated with a complex containing PC, PH, PHO, E(z), ESC, and RPD3 (but not PSC) in early embryonic extracts (64). In extracts from older embryos, GAGA was found to be associated with a complex including PC, PH, PSC, and RPD3, whereas PHO coimmunoprecipitates with ESC, E(z), and RPD3. However, in other studies, PHO was not found in the purified ESC/E(z) complex (75), and GAGA was absent from the purified PRC1 complex containing PC, PSC, PH, dRING1, and many of the TATA-binding protein-associated factor components of the general transcription factor TFIID (67, 68).

Interestingly, the sequence-specific DNA-binding protein Zeste was identified as an approximately stoichiometric com-

either the absence or presence of plasmids (100 ng) expressing the Gal4 DNA-binding domain (Gal4 DBD) alone or various Gal4-PHO fusion constructs. The various Gal4 constructs were cotransfected either with empty vector or together with increasing amounts of a plasmid expressing full-length PC (50 ng and 100 ng), represented by the dark grey bars. (C) The experiment described in B was repeated with a reporter harboring Gal4-binding sites flanked by GAGA sites separated by over 2 kb of intervening DNA from a promoter containing GAGA sites [pGL3-Prom(GAGA)Enh(GAGA/Gal4] (as described by Mahmoudi et al. [48]). This reporter was cotransfected in the absence or presence of various combinations of expression vectors for Gal4 DBD, Gal4-PHO, or PC as indicated. The luciferase activities were normalized so that the reporter plasmid alone averaged at 100%. The structure of the reporter plasmid is indicated schematically.

ponent of PRC1, raising the possibility that Zeste may contribute to DNA targeting (67). Although Zeste can activate transcription in a BRM-dependent manner (40), it also displays genetic interactions with PcG repressors (59, 60). Thus, similar to our results with PHO, it appears that Zeste can interact with the BRM complex as well as with a PC complex. Since neither LexA-PHO nor LexA-GAGA suffices to mediate stable PcG silencing (63) and since by themselves the binding elements for Zeste, PHO, and GAGA do not constitute a PRE, it seems clear that PRE silencing is not achieved by a single recruiter.

Finally, it should be noted that in addition to setting up the expression pattern of the homeotic genes, the Gap proteins may very well play a role in the initial recruitment of PcG complexes (5, 43). For instance, the early repressor HB binds the dMi2 chromatin remodeling and histone deacetylase complex that genetically participates in PcG repression (43). Although dMi-2 might interact directly with PcG proteins, an alternative scenario would be that the deacetylation by dMi-2 creates a chromatin structure conducive to the subsequent assembly of a silencing PcG complex.

In conclusion, current evidence suggests that PcG-mediated silencing is not achieved by a one-step mechanism. While the underlying mechanisms remain enigmatic, it has become clear that PRE function involves a highly elaborate interplay of protein-DNA and protein-protein interactions that direct the formation of a specialized higher-order chromatin structure. At least three distinct steps appear to be distinguishable: targeting to a specific gene, transcriptional repression, and heritable maintenance of the silenced state (4). In this study, we have investigated the role of one of the PRE-binding proteins, PHO, in the recruitment of a PC complex. Our results demonstrate a direct biochemical and functional link between PHO and PC-mediated transcriptional repression.

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REFERENCES

1. **Alkema, M. J., M. Bronk, E. Verhoeven, A. Otte, L. J. van't Veer, A. Berns, and M. van Lohuizen.** 1997. Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian Polycomb complex. Genes Dev. **11:**226–240.
2. **Armstrong, J. A., J. J. Bieker, and B. M. Emerson.** 1998. A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF in vitro. Cell **95:**93–104.
3. **Austin, R. J., and M. D. Biggin.** 1996. Purification of the *Drosophila* RNA polymerase II general transcription factors. Proc. Natl. Acad. Sci. USA **93:**5788–5792.
4. **Beuchle, D., G. Struhl, and J. Muller.** 2001. Polycomb group proteins and heritable silencing of *Drosophila* Hox genes. Development **128:**993–1004.
5. **Bienz, M., and J. Muller.** 1995. Transcriptional silencing of homeotic genes in *Drosophila*. Bioessays **17:**775–784.
6. **Breen, T. R., and I. M. Duncan.** 1986. Maternal expression of genes that regulate the bithorax complex of *Drosophila melanogaster*. Dev. Biol. **118:**442–456.
7. **Breiling, A., E. Bonte, S. Ferrari, P. B. Becker, and R. Paro.** 1999. The *Drosophila* Polycomb protein interacts with nucleosomal core particles in vitro via its repression domain. Mol. Cell. Biol. **19:**8451–8460.
8. **Breiling, A., B. M. Turner, M. E. Bianchi, and V. Orlando.** 2001. General transcription factors bind promoters repressed by Polycomb group proteins. Nature **412:**651–655.
9. **Brock, H. W., and M. van Lohuizen.** 2001. The Polycomb group—no longer an exclusive club? Curr. Opin. Genet. Dev. **11:**175–181.
10. **Brown, J. L., D. Mucci, M. Whiteley, M. L. Dirksen, and J. A. Kassis.** 1998. The *Drosophila* Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol. Cell **1:**1057–1064.
11. **Bunker, C. A., and R. E. Kingston.** 1994. Transcriptional repression by *Drosophila* and mammalian Polycomb group proteins in transfected mammalian cells. Mol. Cell. Biol. **14:**1721–1732.
12. **Busturia, A., and M. Bienz.** 1993. Silencers in abdominal-B, a homeotic *Drosophila* gene. EMBO J. **12:**1415–1425.
13. **Busturia, A., A. Lloyd, F. Bejarano, M. Zavortink, H. Xin, and S. Sakonju.** 2001. The MCP silencer of the *Drosophila* Abd-B gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. Development **128:**2163–2173.
14. **Chalkley, G. E., and C. P. Verrijzer.** 1999. DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250-TAF(II)150 complex recognizes the initiator. EMBO J. **18:**4835–4845.
15. **Chan, C. S., L. Rastelli, and V. Pirrotta.** 1994. A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. EMBO J. **13:**2553–2564.
16. **Chiang, A., M. B. O'Connor, R. Paro, J. Simon, and W. Bender.** 1995. Discrete Polycomb-binding sites in each parasegmental domain of the bithorax complex. Development **121:**1681–1689.
17. **Collins, R. T., T. Furukawa, N. Tanese, and J. E. Treisman.** 1999. Osa associates with the Brahma chromatin remodeling complex and promotes the activation of some target genes. EMBO J. **18:**7029–7040.
18. **Crosby, M. A., C. Miller, T. Alon, K. L. Watson, C. P. Verrijzer, R. Goldman-Levi, and N. B. Zak.** 1999. The *trithorax* group gene *moira* encodes a Brahma-associated putative chromatin-remodeling factor in *Drosophila melanogaster*. Mol. Cell. Biol. **19:**1159–1170.
19. **Croston, G. E., L. A. Kerrigan, L. M. Lira, D. R. Marshak, and J. T. Kadonaga.** 1991. Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription. Science **251:**643–649.
20. **Espinas, M. L., E. Jimenez-Garcia, A. Vaquero, S. Canudas, J. Bernues, and F. Azorin.** 1999. The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. J. Biol. Chem. **274:**16461–16469.
21. **Fauvarque, M. O., and J. M. Dura.** 1993. polyhomeotic regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in *Drosophila*. Genes Dev. **7:**1508–1520.
22. **Francis, N. J., and R. E. Kingston.** 2001. Mechanisms of transcriptional memory. Nat. Rev. Mol. Cell. Biol. **2:**409–421.
23. **Francis, N. J., A. J. Saurin, Z. Shao, and R. E. Kingston.** 2001. Reconstitution of a functional core polycomb repressive complex. Mol. Cell **8:**545–556.
24. **Franke, A., M. DeCamillis, D. Zink, N. Cheng, H. W. Brock, and R. Paro.** 1992. Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*. EMBO J. **11:**2941–2950.
25. **Fritsch, C., J. L. Brown, J. A. Kassis, and J. Muller.** 1999. The DNA-binding Polycomb group protein Pleiohomeotic mediates silencing of a *Drosophila* homeotic gene. Development **126:**3905–3913.
26. **Garcia, E., C. Marcos-Gutierrez, L. M. del Mar, J. C. Moreno, and M. Vidal.** 1999. RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. EMBO J. **18:**3404–3418.
27. **Gehring, W. J.** 1970. A recessive lethal *(l(4)29)* with a homeotic effect in *D. melanogaster*. *Drosophila* Information Service **45:**103.
28. **Gindhart, J. G., Jr., and T. C. Kaufman.** 1995. Identification of Polycomb and trithorax group responsive elements in the regulatory region of the *Drosophila* homeotic gene Sex combs reduced. Genetics **139:**797–814.
29. **Girton, J. R., and S. H. Jeon.** 1994. Novel embryonic and adult homeotic phenotypes are produced by pleiohomeotic mutations in *Drosophila*. Dev. Biol. **161:**393–407.
30. **Hagstrom, K., M. Muller, and P. Schedl.** 1997. A Polycomb and GAGA dependent silencer adjoins the Fab-7 boundary in the *Drosophila* bithorax complex. Genetics **146:**1365–1380.
31. **Hancock, D. C., and G. I. Evan.** 1998. Production and characterization of antibodies against synthetic peptides. Methods Mol. Biol. **80:**15–22.
32. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Heberlein, U., and R. Tjian.** 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by *Drosophila* stage-specific embryonic extracts. Nature **33:**410–415.
34. **Holstege, F. C., E. G. Jennings, J. J. Wyrick, T. I. Lee, C. J. Hengartner, M. R. Green, T. R. Golub, E. S. Lander, and R. A. Young.** 1998. Dissecting the regulatory circuitry of a eukaryotic genome. Cell **95:**717–728.
35. **Horard, B., C. Tatout, S. Poux, and V. Pirrotta.** 2000. Structure of a polycomb response element and in vitro binding of polycomb group complexes containing GAGA factor. Mol. Cell. Biol. **20:**3187–3197.
36. **Jimenez, G., C. P. Verrijzer, and D. Ish-Horowicz.** 1999. A conserved motif in goosecoid mediates groucho-dependent repression in *Drosophila* embryos. Mol. Cell. Biol. **19:**2080–2087.
37. **Kadam, S., G. S. McAlpine, M. L. Phelan, R. E. Kingston, K. A. Jones, and**

B. M. Emerson. 2000. Functional selectivity of recombinant mammalian SWI/SNF subunits. Genes Dev. **14:**2441–2451.
38. **Kadonaga, J. T.** 1990. Assembly and disassembly of the *Drosophila* RNA polymerase II complex during transcription. J. Biol. Chem. **265:**2624–2631.
39. **Kaelin, W. G. Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, and M. A. Blanar.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
40. **Kal, A. J., T. Mahmoudi, N. B. Zak, and C. P. Verrijzer.** 2000. The *Drosophila* brahma complex is an essential coactivator for the trithorax group protein zeste. Genes Dev. **14:**1058–1071.
41. **Kassis, J. A.** 1994. Unusual properties of regulatory DNA from the *Drosophila* engrailed gene: three "pairing-sensitive" sites within a 1.6-kb region. Genetics **136:**1025–1038.
42. **Katsani, K. R., M. A. Hajibagheri, and C. P. Verrijzer.** 1999. Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. EMBO J. **18:**698–708.
43. **Kehle, J., D. Beuchle, S. Treuheit, B. Christen, J. A. Kennison, M. Bienz, and J. Muller.** 1998. dMi-2, a hunchback-interacting protein that functions in Polycomb repression. Science **282:**1897–1900.
44. **Kennison, J. A.** 1995. The Polycomb and trithorax group proteins of *Drosophila*: trans-regulators of homeotic gene function. Annu. Rev. Genet. **29:**289–303.
45. **Kingston, R. E., and G. J. Narlikar.** 1999. ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. Genes Dev. **13:**2339–2352.
46. **Kyba, M., and H. W. Brock.** 1998. The *Drosophila* Polycomb group protein Psc contacts ph and Pc through specific conserved domains. Mol. Cell. Biol. **18:**2712–2720.
47. **Lyko, F., and R. Paro.** 1999. Chromosomal elements conferring epigenetic inheritance. Bioessays **21:**824–832.
48. **Mahmoudi, T., K. R. Katsani, and C. P. Verrijzer.** 2002. GAGA can mediate enhancer function in *trans* by linking two separate DNA molecules. EMBO J. **21:**1775–1781.
49. **Mahmoudi, T., and C. P. Verrijzer.** 2001. Chromatin silencing and activation by Polycomb and trithorax group proteins. Oncogene **20:**3055–3066.
50. **Martin, E. C., and P. N. Adler.** 1993. The Polycomb group gene Posterior Sex Combs encodes a chromosomal protein. Development **117:**641–655.
51. **Mihaly, J., R. K. Mishra, and F. Karch.** 1998. A conserved sequence motif in Polycomb-response elements. Mol. Cell **1:**1065–1066.
52. **Mishra, R. K., J. Mihaly, S. Barges, A. Spierer, F. Karch, K. Hagstrom, S. E. Schweinsberg, and P. Schedl.** 2001. The *iab-7* Polycomb response element maps to a nucleosome-free region of chromatin and requires both GAGA and pleiohomeotic for silencing activity. Mol. Cell. Biol. **21:**1311–1318.
53. **Moreira, J. M., and S. Holmberg.** 1999. Transcriptional repression of the yeast CHA1 gene requires the chromatin-remodeling complex RSC. EMBO J. **18:**2836–2844.
54. **Muller, J., and M. Bienz.** 1991. Long range repression conferring boundaries of Ultrabithorax expression in the *Drosophila* embryo. EMBO J. **10:**3147–3155.
55. **Naar, A. M., B. D. Lemon, and R. Tjian.** 2001. Transcriptional coactivator complexes. Annu. Rev. Biochem. **70:**475–501.
56. **Ng, J., C. M. Hart, K. Morgan, and J. Simon.** 2000. A *Drosophila* ESC-E(Z) protein complex is distinct from other polycomb group complexes and contains covalently modified ESC. Mol. Cell. Biol. **20:**3069–3078.
57. **Orlando, V., E. P. Jane, V. Chinwalla, P. J. Harte, and R. Paro.** 1998. Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early *Drosophila* embryogenesis. EMBO J. **17:**5141–5150.
58. **Orlando, V., and R. Paro.** 1993. Mapping Polycomb-repressed domains in the bithorax complex with in vivo formaldehyde cross-linked chromatin. Cell **75:**1187–1198.
59. **Pelegri, F., and R. Lehmann.** 1994. A role of polycomb group genes in the regulation of gap gene expression in *Drosophila*. Genetics **136:**1341–1353.
60. **Phillips, M. D., and A. Shearn.** 1990. Mutations in polycombeotic, a *Drosophila* polycomb-group gene, cause a wide range of maternal and zygotic phenotypes. Genetics **125:**91–101.
61. **Pirrotta, V.** 1998. Polycombing the genome: PcG, trxG, and chromatin silencing. Cell **93:**333–336.
62. **Pirrotta, V., and L. Rastelli.** 1994. White gene expression, repressive chromatin domains and homeotic gene regulation in *Drosophila*. Bioessays **16:**549–556.
63. **Poux, S., D. McCabe, and V. Pirrotta.** 2001. Recruitment of components of Polycomb Group chromatin complexes in *Drosophila*. Development **128:**75–85.
64. **Poux, S., R. Melfi, and V. Pirrotta.** 2001. Establishment of Polycomb silencing requires a transient interaction between PC and ESC. Genes Dev. **15:**2509–2514.
65. **Satijn, D. P., K. M. Hamer, J. den Blaauwen, and A. P. Otte.** 2001. The Polycomb group protein EED interacts with YY1, and both proteins induce neural tissue in *Xenopus* embryos. Mol. Cell. Biol. **21:**1360–1369.
66. **Satijn, D. P., and A. P. Otte.** 1999. Polycomb group protein complexes: do different complexes regulate distinct target genes? Biochim. Biophys. Acta **1447:**1–16.
67. **Saurin, A. J., Z. Shao, H. Erdjument-Bromage, P. Tempst, and R. E. Kingston.** 2001. A *Drosophila* Polycomb group complex includes Zeste and dTAFII proteins. Nature **412:**655–660.
68. **Shao, Z., F. Raible, R. Mollaaghababa, J. R. Guyon, C. T. Wu, W. Bender, and R. E. Kingston.** 1999. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell **98:**37–46.
69. **Shi, Y., J. S. Lee, and K. M. Galvin.** 1997. Everything you have ever wanted to know about Yin Yang 1. Biochim. Biophys. Acta **1332:**F49–F66.
70. **Shimell, M. J., A. J. Peterson, J. Burr, J. A. Simon, and M. B. O'Connor.** 2000. Functional analysis of repressor binding sites in the iab-2 regulatory region of the abdominal-A homeotic gene. Dev. Biol. **218:**38–52.
71. **Simon, J., A. Chiang, W. Bender, M. J. Shimell, and M. O'Connor.** 1993. Elements of the *Drosophila* bithorax complex that mediate repression by Polycomb group products. Dev. Biol. **158:**131–144.
72. **Strouboulis, J., S. Damjanovski, D. Vermaak, F. Meric, and A. P. Wolffe.** 1999. Transcriptional repression by XPc1, a new Polycomb homolog in *Xenopus laevis* embryos, is independent of histone deacetylase. Mol. Cell. Biol. **19:**3958–3968.
73. **Strutt, H., G. Cavalli, and R. Paro.** 1997. Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. EMBO J **16:**3621–3632.
74. **Strutt, H., and R. Paro.** 1997. The Polycomb group protein complex of *Drosophila melanogaster* has different compositions at different target genes. Mol. Cell. Biol. **17:**6773–6783.
75. **Tie, F., T. Furuyama, J. Prasad-Sinha, E. Jane, and P. J. Harte.** 2001. The *Drosophila* Polycomb group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. Development **128:**275–286.
76. **Tillib, S., S. Petruk, Y. Sedkov, A. Kuzin, M. Fujioka, T. Goto, and A. Mazo.** 1999. Trithorax- and Polycomb-group response elements within an Ultrabithorax transcription maintenance unit consist of closely situated but separable sequences. Mol. Cell. Biol. **19:**5189–5202.
77. **Treisman, J. E., A. Luk, G. M. Rubin, and U. Heberlein.** 1997. *eyelid* antagonizes *wingless* signaling during *Drosophila* development and has homology to the Bright family of DNA-binding proteins. Genes Dev. **11:**1949–1962.
78. **Tsukiyama, T., P. B. Becker, and C. Wu.** 1994. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature **367:**525–532.
79. **van der Vlag, J., and A. P. Otte.** 1999. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat. Genet. **23:**474–478.
80. **Vignali, M., A. H. Hassan, K. E. Neely, and J. L. Workman.** 2000. ATP-dependent chromatin-remodeling complexes. Mol. Cell. Biol. **20:**1899–1910.
81. **Workman, J. L., and R. E. Kingston.** 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. **67:**545–579.
82. **Zink, D., and R. Paro.** 1995. *Drosophila* Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. EMBO J. **14:**5660–5671.

Chapter 3

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

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RESEARCH COMMUNICATION

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

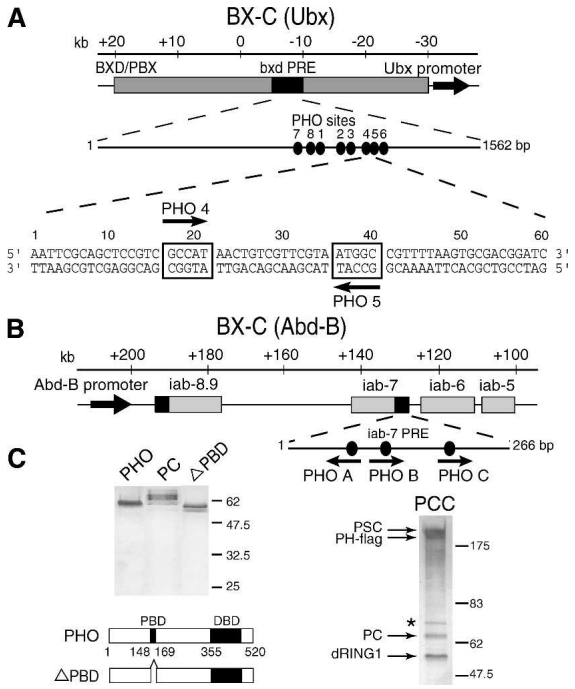

Figure 1. DNA templates and purified proteins. (*A*) Schematic representation of the *Ubx* locus. The BXD/PBX regulatory regions and *bxd* PRE and *Ubx* promoter are indicated. Map positions are according to Bender et al. (1983). The eight PHO sites within the core of the *bxd* PRE are indicated. Numbering 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.

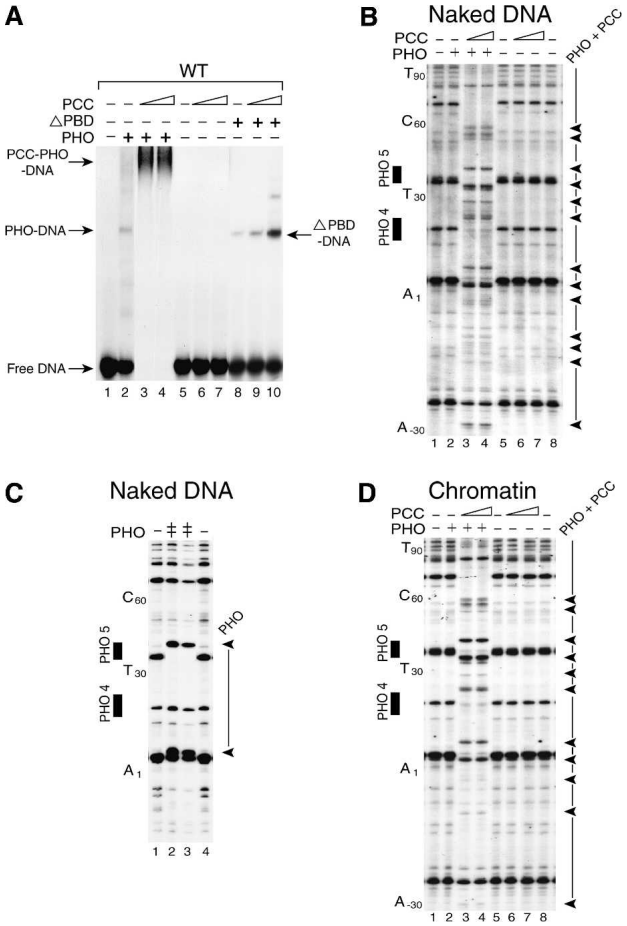

Figure 2. PHO and PCC bind synergistically to the *bxd* PRE. (*A*) PRE binding of PHO, ΔPBD and PCC was studied by bandshift assays. Binding reactions contained ~5 nM PHO and/or ~5–15 nM PCC, and radiolabeled PHO4/5–PRE. PRE binding of PHO and PCC was analyzed by primer extension DNaseI footprinting 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.

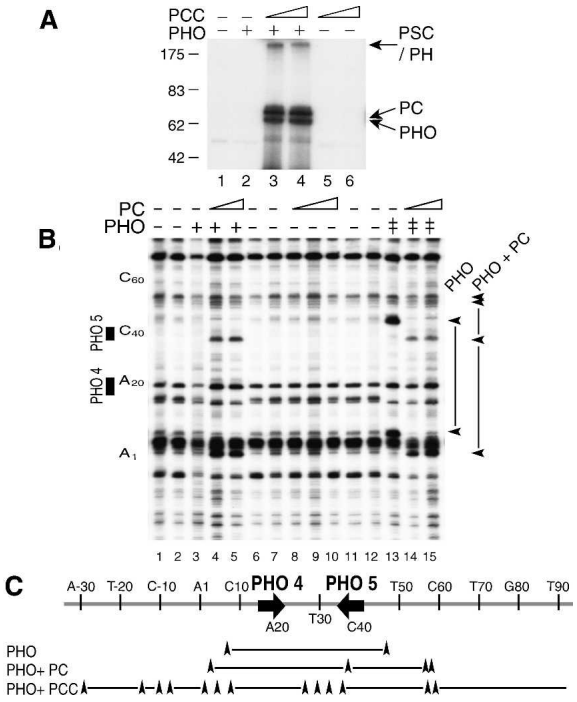

Figure 3. PCC subunits contact DNA. (*A*) UV cross-linking of PHO and PCC subunits to the PRE. Binding reactions contained a ^{32}P-labeled, BrdU-substituted PHO4/5–PRE fragment in the absence or presence of the indicated proteins. After UV cross-linking, proteins were analyzed by SDS-PAGE and visualized 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-

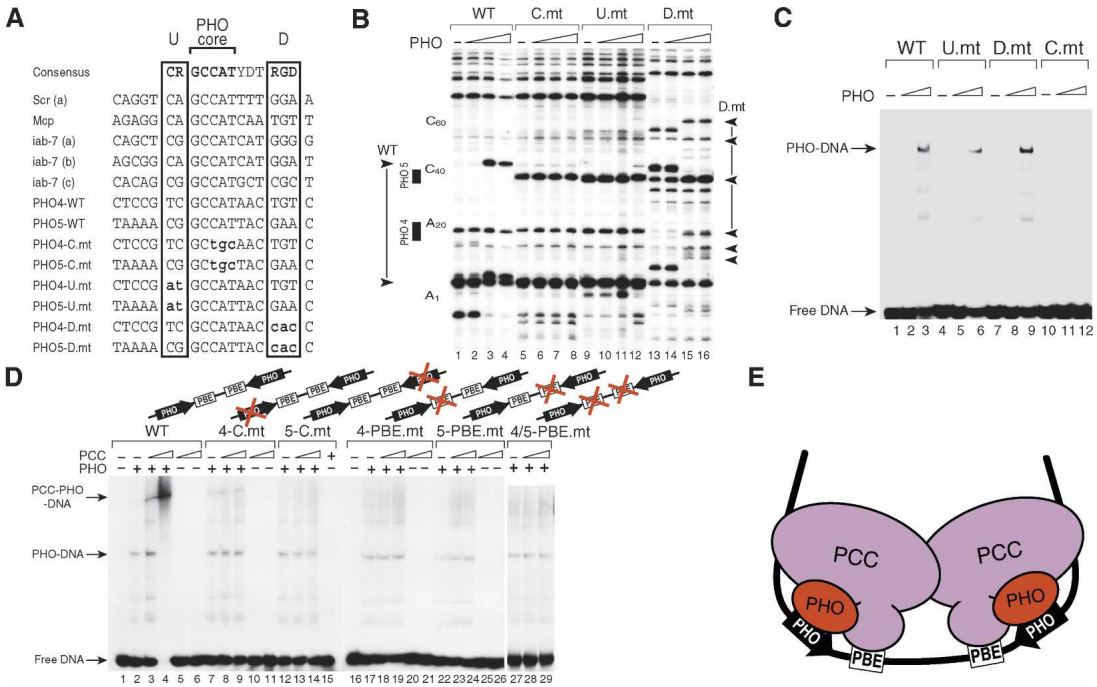

Figure 4. The PBE is required for PCC recruitment to the PRE. (*A*) Alignment of a selection of PHO sites and their flanking motifs from Mihaly et al. (1998), including the *bxd* PRE PHO sites 4 and 5 and *iab-7* PHO sites. The PHO/YY1 consensus core (C) sequence GCCAT and the upstream (U) and downstream (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

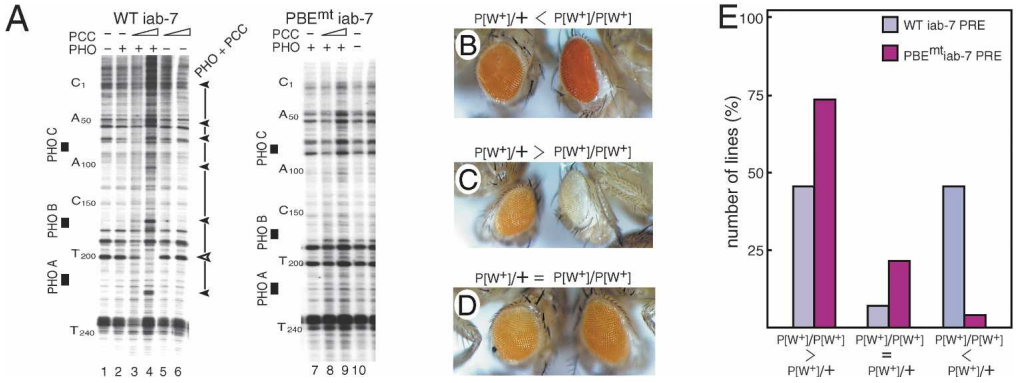

Figure 5. The PBE is required for PRE silencing in vivo. (*A*) Binding of PHO/PCC to the *iab-7* PRE is abrogated when the PBEs are mutated (for sequences, see Fig. 4A), as revealed by DNaseI footprinting. (*B–D*) Mutations in the PBE impair PRE-directed PSS. PSS of *mini-white* by the minimal 260-bp 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.

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References

Bender, W., Akam, M., Karch, F., Beachy, P., Peifer, M., Spierer, P., Lewis, E., and Hogness, D. 1983. Molecular genetics of the bithorax complex in *Drosophila melanogaster*. *Science* **221:** 23–29.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L., and Kassis, J.A. 1998. The *Drosophila* Polycomb group gene pleiohomeotic encodes a DNA-binding protein with homology to the transcription factor YY1. *Mol. Cell* **1:** 1057–1064.

Brown, J.L., Fritsch, C., Mueller, J., and Kassis, J.A. 2003. The *Drosophila* pho-like gene encodes a YY1-related DNA-binding protein that is redundant with pleiohomeotic in homeotic gene silencing. *Development* **130:** 285–294.

Carey, M. 1998. The enhanceosome and transcriptional synergy. *Cell* **92:** 5–8.

Chan, C.S., Rastelli, L., and Pirrotta, V. 1994. A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. *EMBO J.* **13:** 2553–2564.

Dejardin, J., Rappaillles, A., Cuvier, O., Grimaud, C., Decoville, M., Locker, D., and Cavalli, G. 2005. Recruitment of *Drosophila* Polycomb group proteins to chromatin by DSP1. *Nature* **434:** 533–538.

Francis, N.J. and Kingston, R.E. 2001. Mechanisms of transcriptional memory. *Nat. Rev. Mol. Cell Biol.* **2:** 409–421.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. 2001. Reconstitution of a functional core polycomb repressive complex. *Mol. Cell* **8:** 545–556.

Fritsch, C., Brown, J.L., Kassis, J.A., and Muller, J. 1999. The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a *Drosophila* homeotic gene. *Development* **126:** 3905–3913.

Karch, F., Weiffenbach, B., Peifer, M., Bender, W., Duncan, I., Celniker, S., Crosby, M., and Lewis, E.B. 1985. The abdominal region of the bithorax complex. *Cell* **43:** 81–96.

Kassis, J.A. 1994. Unusual properties of regulatory DNA from the *Drosophila* engrailed gene: Three 'pairing-sensitive' sites within a 1.6-kb region. *Genetics* **136:** 1025–1038.

Levine, S.S., King, I.F.G., and Kingston, R.E. 2004. Division of labour in Polycomb group repression. *Trends Biochem. Sci.* **29:** 478–485.

Mahmoudi, T. and Verrijzer, C.P. 2001. Chromatin silencing and activation by Polycomb and trithorax group proteins. *Oncogene* **20:** 3055–3066.

Mahmoudi, T., Zuijderduijn, L.M.P., Mohd-Sarip, A., and Verrijzer, C.P. 2003. GAGA facilitates binding of Pleiohomeotic to a chromatinized Polycomb response elements. *Nucleic Acids Res.* **31:** 4147–4156.

Mihaly, J., Mishra, R.K., and Karch, F. 1998. A conserved sequence motif in Polycomb-response element. *Mol. Cell* **1:** 1065–1066.

Mishra, R.K., Mihaly, J., Barges, S., Spierer, A., Karch, F., Hagstrom, K., Schweinsberg, S.E., and Schedl, P. 2001. The iab-7 polycomb response element maps to a nucleosome-free region of chromatin and requires both GAGA and pleiohomeotic for silencing activity. *Mol. Cell Biol.* **21:** 1311–1318.

Mohd-Sarip, A., Venturini, F., Chalkley, G.E., and Verrijzer, C.P. 2002. Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. *Mol. Cell Biol.* **22:** 7473–7483.

Muller, J. and Bienz, M. 1991. Long range repression conferring boundaries of Ultrabithorax expression in the *Drosophila* embryo. *EMBO J.* **10:** 3147–3155.

Poux, S., McCabe, D., and Pirrotta, V. 2001. Recruitment of components of Polycomb group chromatin complexes in *Drosophila*. *Development* **128:** 75–85.

Ringrose, L. and Paro, R. 2004. Epigenetic regulation of cellular memory by the polycomb and trithorax group proteins. *Annu. Rev. Genet.* **38:** 413–443.

Ringrose, L., Rehmsmeier, M., Dura, J.M., and Paro, R. 2003. Genome-wide prediction of Polycomb/Trithorax response elements in *Drosophila melanogaster*. *Dev. Cell* **5:** 759–771.

Simon, J., Chiang, A., Bender, W., Shimell, M.J., and O'Connor, M.B. 1993. Elements of the *Drosophila* bithorax complex that mediate repression by polycomb group products. *Dev. Biol.* **158:** 131–144.

van der Knaap, J.A., Kumar, B.R.P., Moshkin, Y.M., Langenberg, K., Krijgsveld, J., Heck, A.J.R., Karch, F., and Verrijzer, C.P. 2005. GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. *Mol. Cell* **17:** 695–707.

Verrijzer, C.P., Chen, J.L., Yokomori, K., and Tjian, R. 1995. Binding of TAFs to core elements directs promoter selectivity by RNA-polymerase II. *Cell* **81:** 1115–1125.

Wang, H.B., Wang, L.J., Erdjument-Bromage, H., Vidal, M., Tempst, P, Jones, R.S., and Zhang, Y. 2004. Role of histone H2A ubiquitination in polycomb silencing. *Nature* **431:** 873–878.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. 2004. Hierarchical recruitment of Polyomb group silencing complexes. *Mol. Cell* **14:** 637–646.

Chapter 4

Architecture of a Polycomb Silenceosome.

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Submitted

Architecture of a Polycomb Silenceosome

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Summary

Polycomb group (PcG) epigenetic silencing proteins act through cis-acting DNA sequences, named Polycomb response elements (PREs). Within PREs, Pleiohomeotic (PHO) binding sites and juxtaposed Pc-binding elements (PBEs) function as an integrated DNA platform for the synergistic binding of PHO and the multi-subunit Polycomb core complex (PCC). Here, we analyzed the architecture of the PHO/PCC/PRE nucleoprotein silenceosome. DNaseI footprinting revealed extensive contacts between PHO/PCC and the PRE. Scanning force microscopy (SFM) in combination with DNA topological assays suggested that PHO/PCC wraps the PRE DNA around its surface in a constrained negative supercoil. These features of the silenceosome architecture are difficult to reconcile with the simultaneous presence of nucleosomes. Indeed, nuclease mapping in cells and embryos demonstrated that PREs are nucleosome-free in vivo. We discuss the implications of these findings for models explaining PRE function.

Introduction

Epigenetic gene regulation refers to effects on gene expression that are maintained through cell divisions (Francis and Kingston, 2001; Mahmoudi and Verrijzer, 2001). A fundamental gap in our understanding of epigenetic gene regulation is the mechanism by which specialized DNA elements can bring a linked gene under epigenetic control. One example of such an epigenetic DNA element is the Polycomb response element (PRE). During fly development, PREs initiate and maintain parasegment-specific silencing of linked enhancers in a PcG gene-dependent manner and function as chromosomal tethers for PcG proteins (Levine et al., 2004; Ringrose and Paro, 2004).

PRE function involves a cooperative network of protein-DNA and protein-protein interactions, which drive the formation of a PcG silencing complex. There are two major classes of PcG repressor complexes (PRCs), referred to as PRC1 and PRC2 (Levine et al., 2004; Ringrose and Paro, 2004). PRC1-type complexes harbor a core, comprising PcG proteins PC, Polyhomeotic (PH), Posterior Sex Combs (PSC), dRING1 and several other proteins. The PRC1 core complex (PCC) can mediate the formation of transcription-resistant higher order chromatin (Francis et al., 2001). In addition, the enzymatic activity of dRING1 contributes to PcG silencing through ubiquitylation of histone H2A (Wang H. et al., 2004). PRC2 directs trimethylation of histone H3 lysine 27 (H3K27) by Enhancer of zeste [E(z)], which, in turn, promotes H3 binding by PC, providing a mechanism for the spreading of PcG silencing (Levine et al., 2004; Ringrose and Paro, 2004; Wang L. et al., 2004).

The critical property of PREs is that they act as cis-acting DNA elements that function in transgenes, outside their native location. Therefore, there must be a DNA sequence code that suffices to dictate gene control by PcG proteins. The precise nature of this “PRE-code” is still not understood completely, but recent studies have started to establish key components. Several sequence-specific DNA-binding proteins have been proposed to be involved in the recruitment of PcG silencing factors, to at least some PREs. These include GAGA and Pipsqueak, which recognize the same DNA element, Zeste, DSP1, Grainyhead, Sp1/KLF and PHO (reviewed in Ringrose and Paro, 2004; see also Dejardin et al., 2005; Blastyak et al., 2006; Brown et al., 2005).

Several lines of evidence have established PHO as a key player in PcG targeting. (1) PHO binding sites are essential for PRE-mediated silencing (Brown et al., 1998, 2003; Busturia et al., 2001; Fritsch et al., 1999; Mishra et al., 2001). (2) PHO, and its paralog PHO-like (PHOL), are bona fide PcG proteins, and mutations in PHO cause PcG phenotypes (Brown et al., 1998, 2003). (3) PHO interacts genetically with other PcG genes (Brown et al., 1998, 2003; Fritsch et al., 1999; Kwon et al., 2003). (4) PHO can interact biochemically with both PRC1 and PRC2 (Mohd-Sarip et al., 2002, 2005; Wang L. et al., 2004). The latter activity mediates the targeting of the H3K27 trimethylation by the E(z) subunit of PRC2 (Wang L. et al., 2004). Importantly, a novel PcG silencing complex comprising PHO and dSfmbt has been described recently (Klymenko et al., 2006). (5) We have shown that PHO and PCC bind highly cooperatively to PREs (Mohd-Sarip et al., 2005). Synergism requires two or more PHO

sites, their downstream PCC-binding elements (PBEs) and protein-protein interactions between PHO and PCC. The PBE, juxtaposed to PHO sites in PREs is a small, conserved sequence element that directs docking of PCC and which is required for PRE-directed silencing in vivo.

We named the PHO/PCC/PRE nucleoprotein complex silenceosome to stress the striking similarity in design between PREs and enhancers (Mohd-Sarip et al., 2005). Both involve a stereo-specific arrangement of binding sites and a reciprocal network of protein-protein interactions (Carey, 1998). Moreover, the cooperative interactions between DNA-binding regulators can overcome DNA-binding site occlusion by nucleosomes, allowing efficient chromatin binding (Mohd-Sarip et al., 2005). Here, we used scanning force microscopy in combination with a variety of biochemical approaches to investigate the architecture of the PHO/PCC/PRE silenceosome. Our results suggest a model in which the PRE DNA is wrapped around a PHO/PCC multimer in a negatively supercoiled conformation. Such a reorganization of PRE DNA by PHO/PCC is predicted to preclude normal nucleosome formation. Indeed, nuclease mapping in cells and embryos demonstrated that PREs are nucleosome-free in vivo. Collectively, our findings support the silenceosome hypothesis and have important implications for models explaining PRE function.

Results

Architecture of the PHO/PCC/PRE complex

To study the effects of PHO/PCC binding on the PRE DNA conformation we focused on the well-characterized *bxd* PRE, located ~25 kb upstream of the *Ubx* transcription start site (Figure 1A). The functional core of the *bxd* PRE harbors 6 PHO binding elements (blue arrow) and their juxtaposed PBEs (yellow box; Figure 1B, C). For our initial binding studies we focused on PHO sites 4 and 5 within the *bxd* PRE (PHO4/5-PRE), which are critical for PcG silencing in vivo (Fritsch et al., 1999). Recombinant PHO and PCC were expressed in Sf9 cells using the baculovirus expression system and immunopurified to near homogeneity from cell extracts (Figure 1D). As we demonstrated previously (Mohd-Sarip et al., 2005), binding of PHO/PCC to PHO4/5-PRE creates dramatic changes in the DNaseI digestion pattern over a ~190 bp region (Figure 2A). We considered it likely that such an extended mode of DNA-binding would have consequences for the DNA trajectory or conformation.

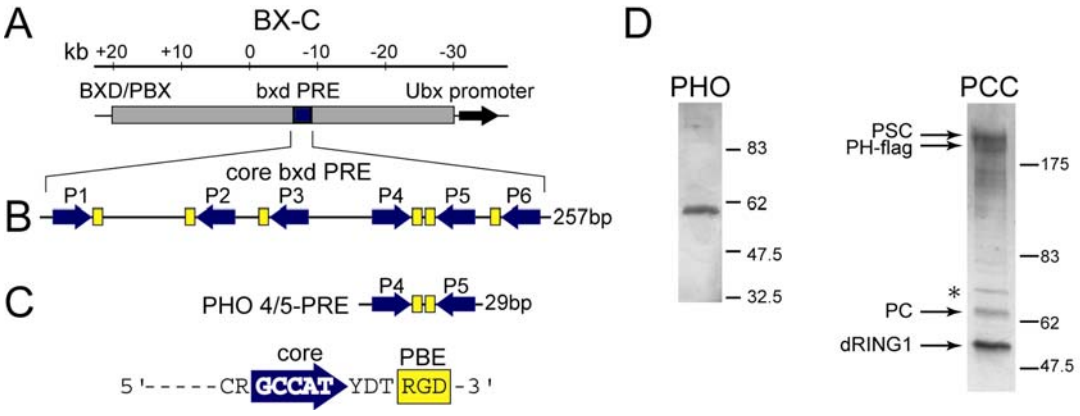

Figure 1. DNA templates and purified proteins
(A) Schematic representation of the *Ubx* locus. The BXD/PBX regulatory regions and *bxd* PRE and *Ubx* promoter are indicated. Map positions are according to Bender et al. (1983). The six PHO/PBE modules within the core of the *bxd* PRE are indicated. The PHO binding sites are depicted as blue arrows and the PBE as a yellow box. Numbering of the elements is according to Fritsch et al. (1999). (B) For binding studies, a 400 bp core fragment harboring all PHO/PBE modules, spread out over 257 bp was used that had been subcloned into the pBluescript vector (PRE-C, Mahmoudi et al., 2003). (C) We also used a short 50 bp DNA fragment harboring the PHO4 and PHO5 sites (covering 29 bp). The fragment, subcloned into pBluescript is referred to as PHO4/5–PRE (Mohd-Sarip et al., 2005). These 2 strong PHO-binding elements are required for *bxd* PRE-directed silencing in vivo (Fritsch et al., 1999). (D) Recombinant PHO and PCC were immunopurified from extracts of baculovirus-infected Sf9 cells. The eluted proteins were resolved by SDS-PAGE and visualized by silver staining.

To obtain detailed architectural information on the PHO/PCC/PRE complex, we employed direct imaging by SFM. For these experiments we used a 1210 bp DNA fragment harboring the 50 bp PHO4/5-PRE, located a little off-center (Figure 2B). PHO and PCC were incubated with PHO4/5-PRE linear DNA fragments and visualized by SFM. As illustrated by Figure 2C, PHO/PCC binding to the PHO4/5-PRE was readily detected. Under these conditions, ~11 % of the DNA molecules were bound by PHO/PCC. No specific complex formation could be observed in the presence of either PHO or PCC alone, confirming the dependence on co-operative DNA-binding that we reported previously (not shown). Likewise, complex formation strictly required the presence of PHO/PBE sites, demonstrating the requirement for sequence-specific DNA-binding (not shown).

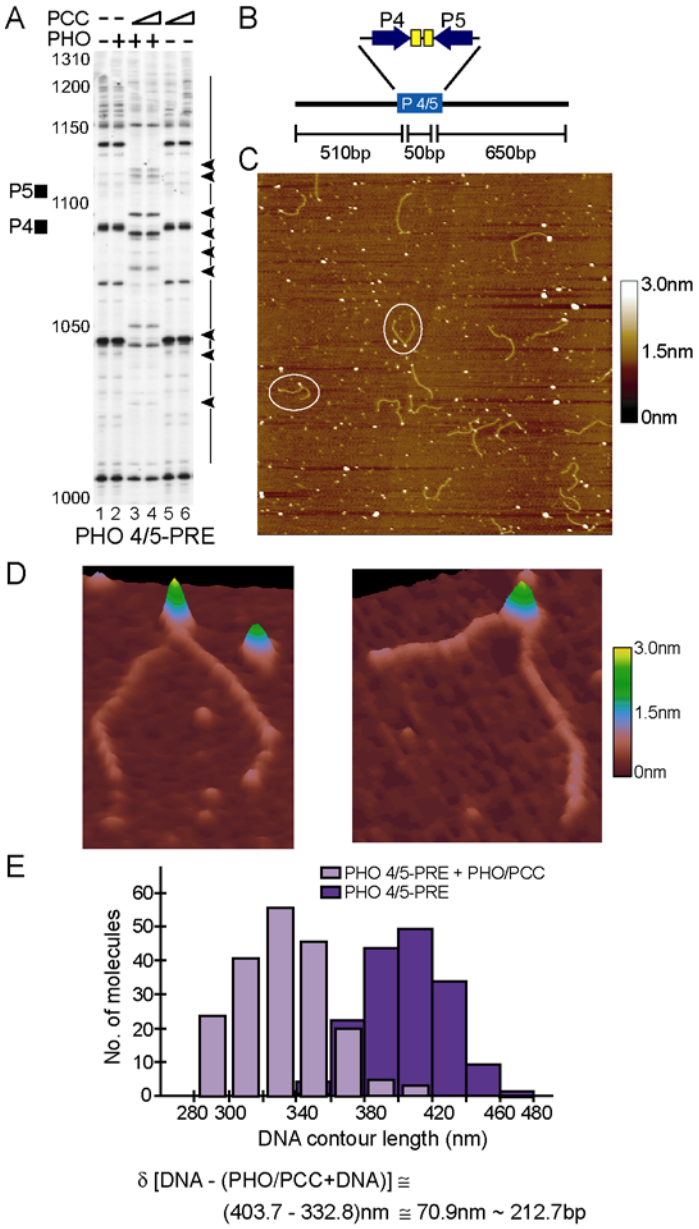

Figure 2. SFM analysis of PHO/PCC bound to the PHO4/5-PRE DNA

(A) Cooperative binding of PHO and PCC to the PHO4/5–PRE was analyzed by primer extension DNaseI footprinting. Binding reactions contained ~20 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 two PHO sites and footprinted areas are indicated. Closed arrowheads indicate DNaseI hypersensitive sites induced by protein binding. (B) A schematic representation of the position of the two PHO/PBE modules within the 1210 bp linear DNA fragment used in the SFM studies. (C) SFM images of the formation of PHO/PCC complexes on linear PHO 4/5-PRE in a 2 X 2 µm scan. The circles indicate DNA-bound PHO/PCC. (D) Tilted view of two zooms of the PHO/PCC/DNA complexes presented as a line plot to emphasize topography. The left panel displays a DNA-bound PHO/PCC complex and a free PCC. The images were processed by flattening only, to remove background slope. Height (*z* dimension) is indicated by color as shown on the scale bar. (E) Histogram displaying the contour length distribution of protein-free and PHO/PCC-bound DNA molecules. For DNA-protein complexes, the contour length was traced as the shortest possible DNA path through the bound protein. Dark purple bars represent free DNA molecules, while lighter purple represent DNA bound by PHO/PCC. We measured 162 free DNA molecules and 164 PHO/PCC/PRE complexes. The average contour length of the naked 1210 bp fragment was ~ 404 (± 2.5) nm whereas that of PHO/PCC-bound DNA was ~ 333 (± 2.5) nm. The significance of the difference was determined by the Mann-Whitney U test ($p < 0.001$). The apparent 71 nm shortening of the DNA contour length upon PHO/PCC binding corresponds to ~ 213 bp.

Representative higher magnification 3-D images illustrate the salient structural features of the PHO/PCC/PHO4/5-PRE complex (Figure 2D). Firstly, we observed a well-defined protein mass at precisely the location of PHO4/5-PRE. We found no evidence for

protein-coated DNA filaments. Secondly, the architecture of the PHO/PCC/DNA complexes was indicative of DNA wrapping. The DNA molecules did not pass straight through the center of the protein mass, but rather the DNA trajectory appeared to be dramatically changed due to PHO/PCC binding. Typically, the DNA entered and exited the protein-DNA complex from the same side of the complex in a way highly suggestive of DNA wrapping around the surface of the PHO/PCC complex.

If indeed PHO/PCC binding to the PHO4/5-PRE DNA changed DNA conformation by wrapping, the contour length of protein-DNA complexes should be decreased compared to naked DNA. A comparison between the contour lengths of free- and PHO/PCC-bound DNA molecules in the same deposition revealed a significant reduction upon PHO/PCC binding (Figure 2E). The average contour length of the naked 1210 bp fragment was ~ 404 ($\pm$ 2.5) nm whereas the PHO/PCC-bound DNA had an average contour length of ~ 333 ($\pm$ 2.5) nm. This difference was statistically significant as determined by the Mann-Whitney U test ($p < 0.001$). The apparent 71 nm shortening of the DNA contour length upon PHO/PCC binding corresponds to ~ 213 bp. These findings fit well with the estimated ~ 190 bp of DNA contacted by PHO/PCC, as determined by DNaseI footprinting. Collectively, these results suggest that PHO/PCC forms a single large multimeric complex that binds the PHO4/5-PRE. Rather than spreading along the DNA, PHO/PCC forms a centered protein mass, contacting about 190 bp of DNA, which appears to wrap around the PHO/PCC surface.

Binding and wrapping of the *bxd* PRE by PHO/PCC is highly cooperative

In the previous experiments we used two closely spaced natural PHO/PBE elements from the *bxd* PRE (Figure 1A-C). Next, we wondered what the effect would be of PHO/PCC binding to all 6 PHO/PBE modules spread over 257 bp within the functional core of the *bxd* PRE. DNaseI primer extension footprinting revealed that PHO and PCC binding to the *bxd* PRE is highly synergistic (Figure 3A). After addition of PCC to a sub-saturating amount of PHO, which by itself did not yield a footprint (lane 2), DNA-binding was readily detected (lanes 3-5). As expected, PCC did not bind DNA by itself (lane 6). The PHO/PCC complex made extensive contacts with the DNA, resulting in dramatic changes in the DNaseI digestion pattern over ~420 bps. This large footprinted area revealed protein-DNA contacts extending well beyond the PHO/PBE modules. As we showed previously (Mohd-Sarip et al., 2005), DNA-binding by PHO and PCC was highly cooperative. Firstly, these proteins failed to bind DNA by themselves, but together they docked efficiently onto the PHO/PBE sites. Secondly, PRE binding by PHO/PCC showed an all-or-nothing dynamic, and we never observed occupancy of only a subset of PHO/PBE modules.

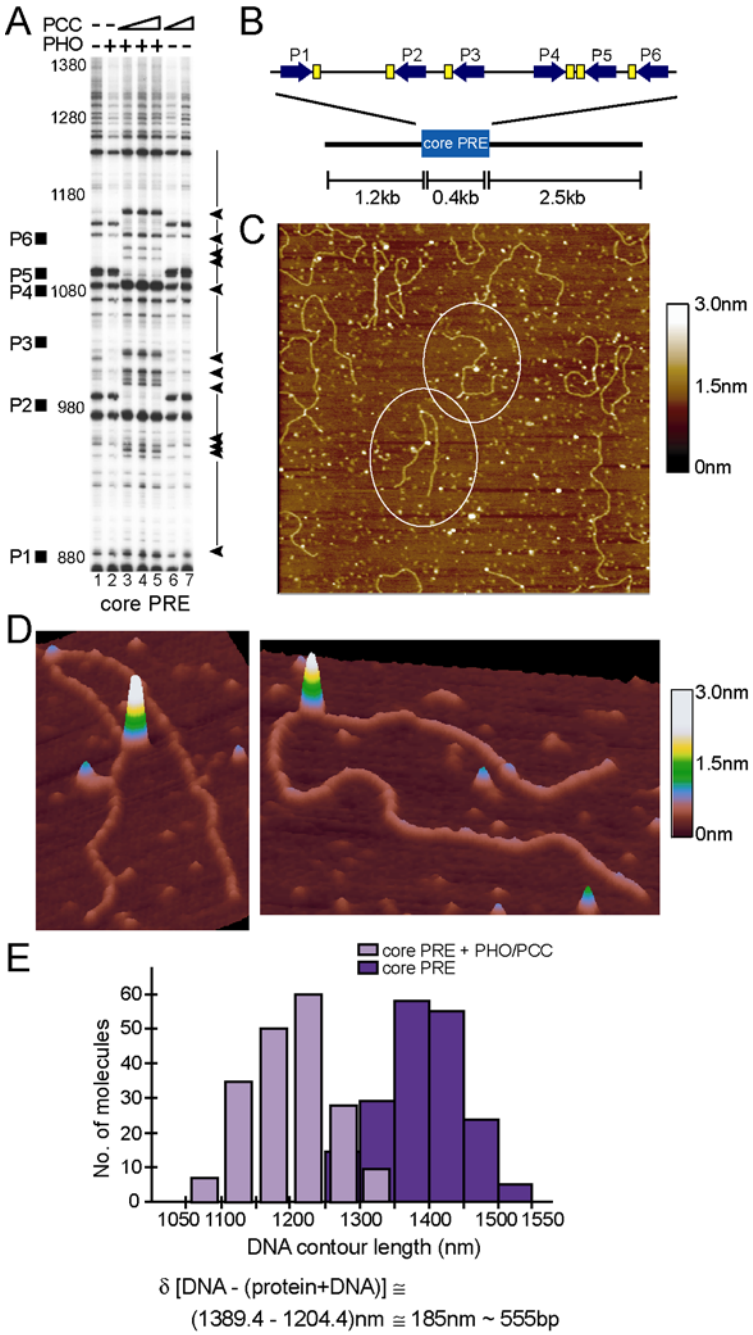

Figure 3. SFM images of PHO/PCC bound to the *bxd* PRE

(A) Binding of PHO/PCC to the core *bxd* PRE was analyzed by primer extension DNaseI footprinting as described in the legend to Figure 2. (B) A schematic representation of the position of the 6 PHO/PBE modules within the 4100 bp linear DNA fragment used in the SFM studies. (C) SFM images of the formation of PHO/PCC complexes on linear DNA harboring the core *bxd* PRE in a 2 X 2 µm scan. The circles indicate DNA-bound PHO/PCC. (D) Tilted view of a zoom of the PHO/PCC/PRE complexes, processing was as described in the legend to Figure 2. (E) Histogram displaying the contour length distribution of naked- and PHO/PCC-bound DNA molecules. For DNA-protein complexes, the contour length was traced as the shortest possible DNA path through the bound protein. Dark purple bars represent free DNA molecules, while lighter purple represent DNA bound by PHO/PCC. We measured 179 unbound DNA molecules and 192 PHO/PCC/PRE complexes. The average contour length of the unbound 4100 bp DNA fragments was ~1389.4 ($\pm$ 4.4) nm, whereas DNA molecules bound at the PRE by PHO/PCC had an average contour length of ~ 1204.4 ($\pm$ 4.1) nm. The difference was highly significant as demonstrated by the Mann-Whitney U test ($p < 0.001$). The apparent shortening of the DNA corresponds ~ 555 bp.

The appearance of alternating protected and DNaseI hypersensitive regions upon PHO/PCC binding, suggested that the PRE DNA is wrapped around PHO/PCC. To investigate the consequences for DNA conformation of PHO/PCC binding, we analyzed

PHO/PCC-*bxd* PRE complexes by SFM imaging. We used a linear 4100 bp DNA fragment containing the 400 bp core of the *bxd* PRE, harboring 6 PHO/PBE modules (Figure 3B). PHO/PCC binding to the *bxd* PRE was readily detected and approximately 10 % of the DNA molecules were bound by PHO/PCC (Figure 3C). We observed a confined protein mass at the precise location of the *bxd* PRE core sequences, without the formation of protein-coated filaments (Figure 3D). Again, no specific complex formation was detected in the presence of either PHO- or PCC alone or in the absence of PHO/PBE sites (not shown).

The SFM images of the PHO/PCC/DNA complexes were again suggestive of DNA wrapping. Measurement of the DNA contour lengths of naked and protein-bound DNA molecules in the same deposition revealed a striking reduction following PHO/PCC binding (Figure 3E). The average contour length of the free 4100 bp fragment was ~1389.4 ($\pm$ 4.4) nm, whereas the PHO/PCC bound DNA had an average contour length of ~ 1204.4 ($\pm$ 4.1) nm. This difference was statistically significant as demonstrated by the Mann-Whitney U test ($p < 0.001$). The reduction in contour length corresponds to an apparent shortening of roughly 546 bp. Our DNaseI footprinting results suggested that ~420 bp of DNA was contacted directly by PHO/PCC (Figure 3A). Thus, two different techniques revealed that PHO/PCC contact over 400 bp of the *bxd* PRE DNA. In summary, the large DNaseI footprinted area displaying alternating protection and hypersensitivity, appearance of the PHO/PCC/PRE complex in SFM images, and shortening of the DNA contour length, all indicated that the *bxd* PRE is bound and wrapped by a multimeric PHO/PCC complex.

PRE-mediated PHO/PCC oligomerization

The size of a protein can be estimated roughly by measuring its volume in SFM images. Although the absolute dimensions will vary depending on the deposition and tip used, there is a linear relationship between SFM-determined volume and molecular mass (Ratcliff and Erie, 2001; Wyman et al., 1997). Thus, this approach provides a powerful tool to compare the stoichiometry of free and DNA-bound protein complexes. We determined the apparent volumes of free PCC, PHO/PCC bound to the 2 PHO/PBE sites in the PHO4/5-PRE and PHO/PCC bound to the *bxd* PRE, harboring 6 PHO/PBE modules (Figure 4A). Assuming PCC comprises of one of each of its subunits, it has a calculated molecular mass of ~ 434 kDa. Because of its relatively small size (~ 57 kDa), we did not measure the volume of PHO alone. The measurements of free PCC molecules were taken from the depositions containing PHO/PCC bound to the PHO4/5-PRE and *bxd* PRE. The free PCC volumes determined on these depositions were very similar, establishing the absence of deposition-dependent effects.

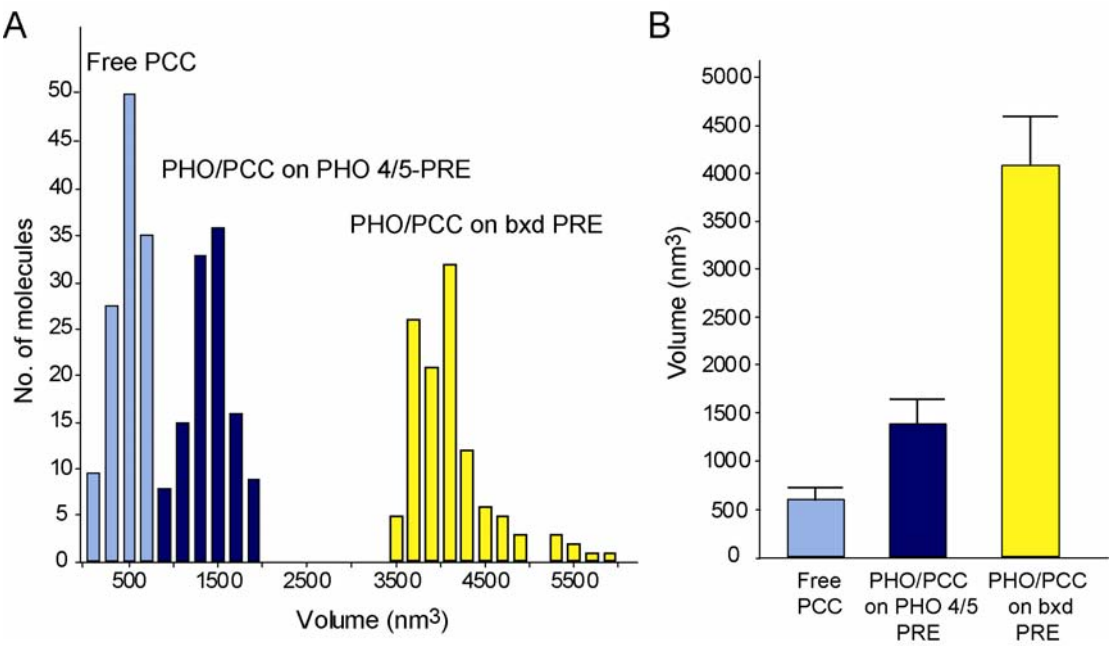

Figure 4. Relative size of PHO/PCC complexes bound to distinct DNA elements.
Representative SFM images of free PCC and PHO/PCC associated with either PHO 4/5-PRE and or the core *bxd* PRE are shown in Figures 2D and 3D. (A) Histograms show the apparent volume distribution in nm^3 of free PCC (light blue), PHO/PCC bound to PHO4/5-PRE DNA (harboring 2 PHO/PBE modules; dark blue) or PHO/PCC bound to the *bxd* PRE (harboring 6 PHO/PBE modules; yellow). Histograms show the volume distribution of unbound PCC protein complex (light blue bars), PHO 4/5-PRE-bound PHO-PCC (dark blue bars) and core bxd PRE-bound PHO/PCC (yellow bars). (B) Mean volume of PHO/PCC derived from Figure 4A depicted as bar graph. The volumes were determined of 120 free PCC complexes, and 117 each of PHO/PCC bound to either PHO4/5-PRE or the *bxd* PRE. Mean volumes of 566.4 ($\pm$ 14.1) nm^3 for free PCC, 1410.8 ($\pm$ 23.2) nm^3 for PHO/PCC bound to PHO4/5-PRE and 4110.0 ($\pm$ 43.7) nm^3 for PHO/PCC on the *bxd* PRE were calculated. The Kruskal-Wallis test showed that the differences in volume differences are highly significantly ($p < 0.001$).

Determination of the mean volumes of free and PRE-associated proteins revealed a striking correlation with the number of PHO/PBE sites present (Figure 4B). The volume of the PHO/PCC protein mass binding the 2 elements within PHO4/5-PRE is ~ 2.5 times larger than that of free PCC. This increase fits surprisingly well with the predicted 2.3-fold increase if 2 PHO molecules and 2 PCCs would assemble onto the 2 PHO/PBE docking sites present. The estimated volume of the protein mass on the *bxd* PRE, harboring 6 PHO/PBE sites, is ~ 3-fold larger than that on the PHO4/5-PRE. This is consistent with the binding of 6 PHO molecules and 6 PCC complexes. The Kruskal-Wallis test showed that these volume differences determined by SFM are significant ($p < 0.001$). We conclude that the number of PHO/PBE modules determines the stoichiometry of the DNA-bound PHO/PCC complex.

These volume measurements revealed again the highly co-operative nature of PHO/PCC binding to the PRE. One particularly striking feature of Figure 4A is the gap between the protein volumes measured on DNA templates harboring either 2- (PHO4/5-PRE) or 6 (*bxd* PRE) PHO/PBE docking sites. The absence of intermediate protein complexes strongly suggests that once PHO/PCC binds to the *bxd* PRE, all six sites are occupied co-operatively. We note that these experiments were not performed using excess amounts of proteins, as illustrated by the presence of free DNA (Figure 3B). Thus, consistent with our DNaseI footprinting results, volume measurements by SFM revealed highly co-operative PRE binding by PHO and PCC. Although the PHO/PBE modules are spread out over ~ 257 bp of

bxd PRE DNA, the images revealed a highly compact nucleoprotein complex, rather than an elongated structure. This indicates extensive DNA-induced protein-protein interactions between individual PHO and PCC complexes.

PHO/PCC binding to the PRE induces a negative superhelical turn in DNA

Our previous results suggested that the binding of PHO/PCC to the PRE changes the conformation of the DNA double helix. To gain more insight into the molecular mechanism, we decided to utilize a topological assay. Linking number is a parameter of double stranded DNA topology that corresponds to the number of times the 2 strands of the DNA double helix wind around each other. The linking number is the sum of two geometrical parameters, twist and writhe. Twist describes the number of times one DNA strand of the double helix crosses the other. Writhe describes the number of crossings of the axis of the double helix over itself. To determine the effect of PHO/PCC binding on DNA conformation, we incubated singly nicked plasmid DNA harboring either the PHO4/5-PRE (Figure 5A) or the core *bxd* PRE (Figure 5B) with purified PHO and PCC. To fix protein-induced DNA conformational changes, the nicks were closed by the addition of DNA ligase. Changes in DNA linking number were visualized by analyses of the topoisomer distribution after agarose gel electrophoresis in the presence of chloroquine (Figure 5A,B). The addition of 0.5 µg/ml chloroquine in the gel system used caused positive supercoiling of DNA.

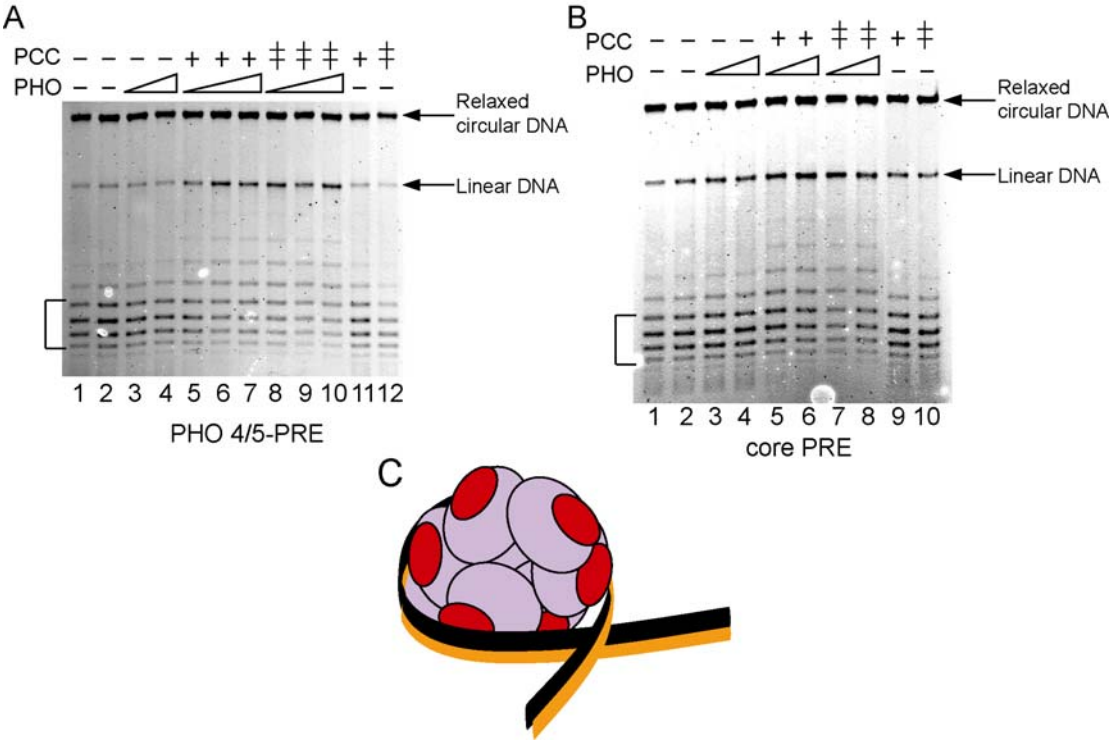

Figure 5. PHO/PCC binding to the PRE induces negative supercoiling
Singly nicked plasmid DNA harboring either (A) the PHO 4/5-PRE or (B) the core *bxd* PRE was incubated with 30-90 nM of PHO (indicated as triangles; lanes 3-4), or 40 nM (+) or 120 nM (‡) PCC (lanes 11-12 and 9-11 respectively), PHO and PCC (lanes 5-10 and 5-8 respectively). The nick was ligated, and the resulting topoisomer distribution was analyzed on a 0.8% agarose gel containing 0.5 µg/ml chloroquine. As a control reaction, the nicked DNA was ligated in the absence of PHO and PCC (lanes 1-2). (C) Cartoon illustrating PHO/PCC binding and wrapping of PRE DNA around its surface in a left-handed manner, constraining a negative supercoil.

In the combined presence of PHO and PCC, we observed a clear shift in the topoisomer distribution towards more negative supercoiling. The faster migrating bands became weaker, whereas the slower migrating bands gained in intensity compared to the nicked plasmid DNA ligated in the absence of protein (Figure 5A, compare lanes 5-10 with lanes 1 and 2; Figure 5B, compare lanes 5-8 with lanes 1 and 2). As expected, given the dependency on co-operative interactions for DNA-binding, PHO- or PCC alone did not affect DNA conformation. Likewise, the shift towards negative supercoiling required the presence of the PHO/PBE sites (not shown). We conclude that PHO/PCC binding to the PRE changes the DNA double helix conformation by constraining negative supercoiling.

The reduction in linking number in the topological assay caused by PHO/PCC binding to the PRE is the result of a change in either twist or writhe. Proteins can change twist by stretching the DNA helix in a protein filament (Kosikov et al., 1999; Ogawa et al., 1993). By wrapping the DNA around their surface, proteins can affect writhe (Klug et al., 1980; White et al., 1988). Our SFM results allowed us to distinguish between these two possibilities, and strongly favored a change in writhe, not twist. We observed no evidence for protein-coated filaments of elongated DNA in our SFM images. In contrast, PHO/PCC/PRE complexes were compact with the DNA entry and exit on the same side of the protein (Figures 2D and 3D). Moreover, the alternating protected and hypersensitive regions in the PHO/PCC/PRE DNaseI footprints are consistent with PRE DNA being wrapped around the protein complex (Figures 2A and 3A). Most telling, the DNA contour length was significantly shortened upon PHO/PCC binding, which is highly indicative of DNA wrapping (Figures 2E and 3E).

Collectively, our results suggest that PHO/PCC actively binds and wraps the PRE DNA left-handed around its surface, constraining a negative supercoil (Figure 5C). An interesting corollary is that these topological constraints and extensive DNA-contacts made by PHO/PCC are difficult to reconcile with the simultaneous presence of nucleosomes.

The *bxd* PRE maps to a nucleosome-free region of chromatin in vivo

Several lines of evidence suggest that nucleosomes are displaced by PHO/PCC binding. (1) Assembly of a PRE into chromatin does not hamper PHO/PCC binding, showing that the binding energy of silenceosome formation overcomes chromatinization (Mohd-Sarip et al., 2005; and data not shown). (2) The DNaseI footprints upon PHO/PCC binding to either naked DNA or a nucleosomal array are virtually identical (Mohd-Sarip et al., 2005; data not shown). (3) PHO/PCC interacts extensively with the PRE DNA, covering hundreds of base pairs, leaving little room for histone-DNA binding (Mohd-Sarip et al., 2005; this study). (4) PHO/PCC wraps the PRE DNA left-handedly around its surface constraining a negative supercoil (this study). These properties of the PHO/PCC/PRE nucleoprotein complex, make it highly improbable that normal nucleosomes are present simultaneously. In agreement with this notion, it was shown previously that the *iab-7* PRE, which is bound by PHO/PCC (Mishra et al., 2001; Mohd-Sarip et al., 2005), maps to a nucleosome-free region of chromatin (Mishra et al., 2001).

To investigate whether the *bxd* PRE studied here maps to a nucleosome-free region in vivo, we performed nuclease-hypersensitivity assays. Nuclei were isolated from KC cells, in which the *Ubx* gene is silenced, or 12-24 hr *Drosophila* embryos were digested with either micrococcal nuclease (MNase) or DNaseI. The pattern of nuclease cleavage in chromatin across the *bxd* PRE region was then analyzed by indirect end-labeling. As shown in Figure 6, we detected a large nuclease hypersensitive region of about 0.4 kb in length that coincides closely with the "core" *bxd* PRE in KC cells and in embryos. The *bxd* core is hypersensitive to both MNase and DNaseI in late as well as early embryos (data not shown). The large nuclease hypersensitive PRE region is separated from a less prominent hypersensitive region by about 220 bp, suggesting the presence of a positioned nucleosome. As indicated in the diagram, other weaker bands flanking the core *bxd* PRE on either side in chromatin digests are also spaced at nucleosome length intervals. We conclude that both in KC cells and in fly embryos, the core of the bxd PRE forms a ~ 0.4 kb nuclease-hypersensitive region. The nucleosome-free PRE core domain appears to be directly flanked by positioned nucleosomes.

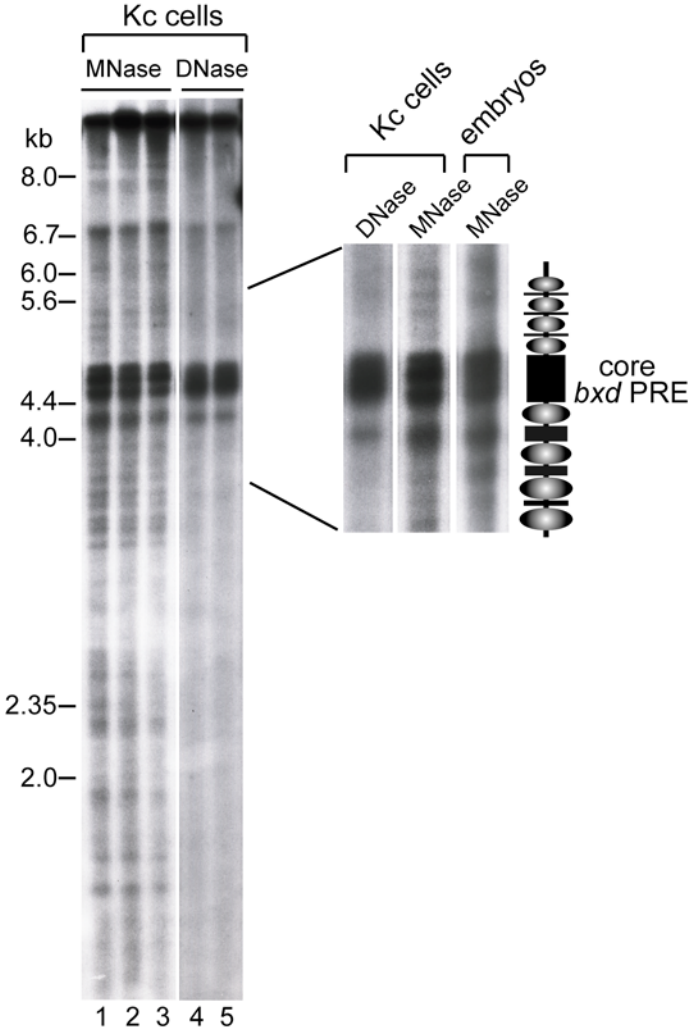

Figure 6. The core *bxd* PRE is nuclease hypersensitive in vivo
The left panel shows an autoradiograph of an indirect end-labeling experiment visualizing MNase and DNaseI digests from KC nuclei. Digests were restricted with Xho I and probed with a 1,724 bp Xho I- Hind III fragment located ~19 kb upstream of the start of the *Ubx* transcription unit. This fragment end-labels a large Xho I fragment extending from this promoter proximal Xho I site towards the telomere that includes the *bxd* PRE. According to the genomic sequence of the region, the Xho I fragment should be 18.45 kb in length. An Xho I fragment of this length is

observed in digests of embryo DNA. However, there is also cleavage at a polymorphic Xho I site located ~9.4 kb from the proximal Xho I site. In KC DNA, all of the DNA is cleaved at a different polymorphic distal Xho I site located ~9.6 kb from the proximal end-labeling site. Two different types of size markers were used to localize the *bxd* PRE in this Xho I fragment. The first was embryonic genomic DNA digested to completion with Xho I and then digested partially with Eco R1 or Hind III. The second was a 2 kb ladder. The position of each marker band in the original autoradiograph is indicated on the left, while the location of the *bxd* PRE (bp 4,049-5,605) is indicated by the bar on the right. There are two major MNase and DNaseI hypersensitive regions mapping to the 1.6 kb *bxd* PRE fragment. The less prominent hypersensitive region maps 4.1-4.18 kb from the Xho I end labeling site. The proximal end of the larger of the hypersensitive regions coincides closely with the marker band at 4.4 kb (which is 4,404 bp from the Xho I site). The large hypersensitive region is 0.4 kb in length and its distal end is 4.8 kb from the Xho I end-labeling site. As shown in the blow-up on the right, the large hypersensitive region coincides closely with the "core" *bxd* PRE. The "core" bxd PRE begins 4,416 bp from the Xho I end labeling site and ends at 4, 835 bp. In addition to KC cell MNase and DNaseI digests, the blow up shows the *bxd* PRE region from a MNase digest of 12-24 hr embryos. Like KC cells, the *bxd* core is hypersensitive to MNase and DNaseI (not shown) in late embryos collections and also in early embryo collections (not shown). The large hypersensitive region is separated from the less prominent hypersensitive region by 220 bp. This would be sufficient to accommodate one nucleosome. As indicated in the diagram, other weaker bands seen near the core *bxd* PRE in chromatin digests are also spaced at nucleosome length intervals.

Discussion

How specialized DNA elements such as PREs can bring a linked gene under epigenetic control remains poorly understood. An important breakthrough was the identification of PHO as a sequence-specific PcG protein (Brown et al., 1998). Subsequent research firmly established that PHO forms a critical component of the "PRE code" (Brown et al., 1998, 2003; Busturia et al., 2001; Fritsch et al., 1999; Mishra et al., 2001; Kwon et al., 2003). We recently identified another building block of PREs: The PBE, which is located directly downstream of PHO (Mohd-Sarip et al., 2005). PHO and PCC only interact weakly in solution, but docking onto PHO/PBE modules drives the assemblage of a stable PHO/PCC/PRE silenceosome. The mechanistical properties of silenceosome and enhanceosome formation are strikingly similar (Mohd-Sarip et al, 2005; Blastyak et al., 2006). Both involve synergistic interactions between a stereo-specific arrangement of binding sites and a reciprocal network of protein-protein interactions. Here, we investigated the architecture of a PcG silenceosome.

Silenceosome architecture and chromatin

Our results revealed that PHO/PCC contacts the *bxd* PRE over ~400 bp and wraps the PRE DNA around its surface in a constrained negative supercoil. PHO/PCC binding to the PRE overcomes chromatinization and the DNaseI digestion pattern of the PHO/PCC/PRE complex strongly suggests nucleosome eviction from the PRE (Mohd-Sarip et al., 2005; data not shown). We confirmed this notion by demonstrating that the ~400 bp *bxd* PRE core is nucleosome free in vivo, as revealed by MNase- and DNaseI mapping in cells and embryos. Although PcG silencing is generally believed to be associated with decreased rather than increased chromatin accessibility, the *bxd* PRE (this study) and PREs from the *Abd-B cis*-regulatoy domains are nuclease-hypersensitive in chromatin digests (Barges et al., 2000; Karch et al., 1994; P.S. unpublished results). Recent high resolution ChIP analysis independently revealed that PREs are depleted for histones (B. Papp and J. Mueller, personal communication; R. Paro, personal communication). Taken together, these result show that PREs are in a histone-depleted, open chromatin conformation.

These in vivo findings fit well with the molecular features of PcG silenceosome architecture we have established in this study. Our estimates suggested that a PHO/PCC oligomer can wrap more DNA around its surface than a nucleosome. The extensive contacts between PHO/PCC and PRE DNA, together with the left-handed wrapping are likely to affect histone-DNA interactions and cause nucleosome displacement. Previous studies have shown that the Pc component of PCC can directly bind histones, modulated by H3K27 methylation (Breiling et al., 1999; Wang L. et al. 2004). The simultaneous binding of DNA and nucleosomal histones, would constrain the free rotation of PHO/PCC, and now DNA wrapping would create torsion to mediate histone eviction.

The extend of nuclease hypersensitivity varies, depending on PRE size. For example, the hypersensitive regions associated with the *Abd-B* PREs are smaller than the *bxd* PRE, ranging in size from 300 bp for the *Mcp* PRE to about 150 bp for the *iab-8* PRE. Hypersensitivity in chromatin is also closely correlated with the silencing activity of the *Abd-B* PREs in vivo. In the case of the *iab-7* PRE, the sequences required for the pairing sensitive silencing of *mini-white* in transgene assays closely coincide with the nuclease hypersensitive region bound by PHO/PCC (Mishra et al., 2001). In the case of *Mcp*, the 300 bp core PRE sequence is more sensitive to DNaseI in chromatin digests in a transgenic insert that shows pairing sensitive silencing of *mini-white* than it is in a transgenic insert which does not show *mini-white* silencing (Muller *et al*., l999). Thus, there appears to be a good correlation between PRE-activity and nuclease hypersensitivity.

Our findings dovetail nicely with the results of recent genome-scale determination of nucleosome positioning in yeast. These studies suggested that RNA polymerase II promoters comprise a nucleosome-free region flanked by positioned nucleosomes, bearing a stereotyped pattern of histone modifications (Yuan et al., 2005; Liu et al., 2005; Pokholok et al., 2005). We propose that, like promoters and enhancers, PREs are in an open, nucleosome-free conformation in vivo.

Mechanism of PRE-mediated silencing

We note that our results are not inconsistent with an important role for histone modifications in the establishment of silent chromatin. PREs are required to initiate PcG-directed gene silencing. We propose that this is a multi-step process, starting with silenceosome formation. The next step would involve the spreading of the silenced state to linked genes. PCC-histone interactions, rather than DNA-binding, are likely to be the main driving force of sequence-independent spreading over a target gene. Such a spreading of PcG complexes over a target gene is likely to be modulated by covalent histone modifications, such as H3K27 methylation or histone (de)ubiquitylation. This, in turn, can lead to the creation of closed chromatin domains (Ringrose and Paro, 2004; Levine et al. 2004; Francis et al., 2004; Mohd-Sarip and Verrijzer, 2004; Knaap et al., 2005; Wang H. et al., 2004). Thus, we imagine that histone modifications would generally follow, rather than precede silenceosome assembly.

Like most cis-acting DNA elements, PREs are complex and their activity involves the combinatorial function of distinct recognition elements and their cognate factors. Although essential, the docking of PHO/PCC onto PHO/PBE elements is only part of the silencing mechanism. Our proposal to view PcG silencing complexes bound to PREs as silenceosomes provides a simple framework to accommodate additional data available. For instance, the HMGB2 related DSP1 might induce DNA bending upon binding to its GAAA motif, assisting the formation of a higher-order nucleoprotein structure (Dejardin et al., 2005). Other PRE-binding factors, such as GAGA, might assist chromatin remodeling (Mahmoudi et al., 2003), contribute to reorganization of the PRE topology (Katsani et al., 1999), or mediate long-range interactions, for example with the promoter DNA (Mahmoudi et al., 2002). PRE binding factors other than PHO, may also directly contact PcG complexes and aid in their recruitment (reviewed by Ringrose and Paro, 2004; Levine et al., 2004; see also Blastyak et al., 2006; Brown et al., 2005).

Collectively, the available evidence enforces the notion that a co-operative network of contextual protein-DNA and protein-protein interactions nucleates silenceosome formation. We suggest that PRE-binding by PcG complexes causes the eviction of histones, creating a nucleosome-free PRE that is flanked by positioned nucleosomes. Our work presented here provided a first view of the architecture of a PHO/PCC/PRE silenceosome and provides a framework for models explaining PRE function.

Experimental Procedures

DNA constructs and proteins

All cloning was performed using standard methods. Details are available upon request. The PHO 4/5-PRE and core bxd PRE constructs used in the footprinting assays have been described previously (Mahmoudi et al., 2003; Mohd-Sarip et al., 2005). The linear DNA substrates for SFM studies were generated by PCR (PHO 4/5-PRE) or by restriction enzyme digestion, followed by dephosphorylation (bxd PRE). Fragments were purified by subsequent phenol:chloroform- and chloroform extraction, ethanol precipitation, and dissolved in water (glass-distilled, Sigma). For topological assays, plasmids were singly nicked in a 30-µl reaction containing 0.5 ug of DNA, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 360 µg/ml ethidium bromide, and 1 µg/ml DNaseI at 30°C for 30 min. The reaction was stopped by the addition of 0.1 volume of stop mix (5% SDS, 50 mM EDTA, 30 µg/ml proteinase K) and incubation at 65°C for 30 min. DNA was purified as described above. Recombinant proteins were expressed in Sf9 cells using the baculovirus system and immunopurified as described (Mohd-Sarip et al., 2002; Francis et al., 2001).

DNA-binding and SFM imaging

DNaseI primer extension footprinting assays were performed as described (Mahmoudi et al., 2003; Mohd-Sarip et al., 2005). PHO/PCC/DNA complexes for SFM were prepared by incubating 250 ng of linear PHO 4/5-PRE or core bxd PRE with 30 ng of PHO and 100 ng PCC in a 20-µl volume containing 12.5 mM HEPES-KOH, pH 7.6, 6.25 mM MgCl2, 1 mM DTT, 0.05% Nonidet P-40 and 80 mM KCl. The binding reaction was incubated on ice for 30 min. Binding reactions were diluted 15- to 20-fold in deposition buffer (10 mM HEPES-KOH, pH 7.5, and 10 mM $MgCl_2$) and directly deposited onto freshly cleaved mica. After a 1-min incubation, the mica surface was washed with H_2O (glass-distilled, Sigma) and dried with a stream of filtered air. The protein-DNA complexes were imaged using a Nanoscope IIIa (Digital Instruments) operating in tapping mode with a type E scanner. Images were collected as 2 x 2 µm scans. The raw data were processed by flattening only to remove background slope using Nanoscope software. Silicon tips (Nanoprobes) were obtained from Digital Instruments. The DNA contour length and the size of protein complexes on DNA were measured from NanoScope images imported into IMAGE SXM 1.62 (National Institutes of Health IMAGE version modified by Steve Barrett, Surface Science Research Centre, Univ. of Liverpool, Liverpool, U.K.). DNA contours were manually traced and subsequently smoothed. For DNA-protein complexes, the contour length was traced as the

shortest possible DNA path through the bound protein. The volume of DNA-bound protein complexes was determined as described (Wyman et al., 1997). The protein was manually traced, and its area and average height were measured. A background volume determined from the same size area, including a segment of DNA, was subtracted. Volume measurements are given in nm^3. Statistical analysis was performed using XL Stat.

Topological assays

The singly nicked plasmids PHO 4/5-PRE (50ng) or core bxd PRE (50 ng) was incubated with the indicated amounts of PHO or PCC in a 60-µl reaction volume containing 20 mM Tris-HCl, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. After 45 min at 25°C, one unit of E. coli T4 DNA ligase and 6 µl 10x ligation buffer containing NAD was added followed by an additional 1 hr incubation at 25°C. DNA was purified as described above and topoisomers were resolved by electrophoresis on 0.8% agarose gels containing 0.5 µg/ml chloroquine. Gels were run in 1 x Tris borate EDTA for 27 h at 70 V. At the end of the run, chloroquine was washed out with 1 x TBE, and followed DNA was visualized by staining with ethidium bromide.

Nuclease hypersensitive assays

Nuclei were prepared from *Drosophila* tissue culture cells or from embryos as described in Udvardy and Schedl (l984). Nuclease hypersensitivity assays were performed essentially as described previously (Udvardy and Schedl, l984). Briefly, the nuclei were incubated with either MNase or DNaseI for variable lengths of time, and the digested DNA was then purified by proteinase K treatment and phenol extraction. The DNA at each time point was analyzed by gel electrophoresis and the time points pooled so that on average there was less than one double strand break induced by nuclease treatment every 10-15 kb. As a control, total genomic DNA (not treated with nuclease) was also isolated. For the indirect end-labeling experiments, the DNA samples were restricted with the appropriate restriction enzyme, resolved by electrophoreses on 40 cm agarose gels, followed by Southern blotting and hybridization with the appropriate probes (see legend to Figure 6).

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References

Bender,W., Akam,M., Karch,F., Beachy,P., Peifer,M., Spierer,P., Lewis,E. and Hogness,D. (1983) Molecular genetics of the bithorax complex in Drosophila melanogaster. Science *221*, 23-29.

Barges, S., Mihaly, J., Galloni, M., Hagstrom, K., Muller, M., Shanower, G., Schedl, P., Gyurkovics, H., and Karch, F. (2000). The Fab-8 boundary defines the distal limit of the bithorax complex iab-7 domain and insulates iab-7 from initiation elements and a PRE in the adjacent iab-8 domain. Development *127*, 779-90.

Blastyak, A., Mishra, R.K., Karch, F., and Gyurkovics, H. (2006). Efficient and specific targeting of Polycomb group proteins requires cooperative interaction between grainyhead and pleiohomeotic. Mol. Cell. Biol. *26*, 1434-1444.

Breiling, A., Bonte, E., Ferrari, S., Becker, P.B., and Paro, R. (1999). The Drosophila Polycomb protein Iinteracts with nucleosomal core particles in vitro via its repression domain. Mol. Cell. Biol. *19*, 8451-8460.

Brown J.L., Fritsch, C., Mueller, J., and Kassis, J.A. (2003). The Drosophila pho-like gene encodes a YY1-related DNA-binding protein that is redundant with pleiohomeotic in homeotic gene silencing. Development 130, 285-294.

Brown, J.L., Grau, D.J., DeVido, S.K., and Kassis, J.A. (2005). An Sp1/KLF binding site is important for the activity of a Polycomb group response element from the Drosophila engrailed gene. Nucleic Acids Res. *33*, 5181-5189.

Brown J.L., Mucci, D., Whiteley, M., Dirksen, M.L., and Kassis, J.A. (1998). The Drosophila Polycomb group gen pleiohomeotic encodes a DNA-binding protein with homology to the transcription factor YY1. Mol. Cell *1*, 1057-1064.

Busturia, A., Lloyd, A., Bejarano, F., Zavortink, M., Xin, H. and Sakonju, S. (2001). The MCP silencer of the Drosophila Abd-B gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression. Development *128*, 2163-2173.

Carey, M. (1998). The enhanceosome and transcriptional synergy. Cell *92*, 5-8.

Dejardin, J., Rappaillles, A., Cuvier, O., Grimaud, C., Decoville, M., Locker, D., and Cavalli, G. (2005). Recruitment of Drosophila Polycomb group proteins to chromatin by DSP1. Nature *434*, 533-538.

Francis, N.J., and Kingston, R.E. (2001). Mechanisms of transcriptional memory. Nature Rev. Mol. Cell. Biol. *2*, 409-421.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core Polycomb repressive complex. Mol. Cell *8*, 545-556.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science. 306,1574-7.

Fritsch, C., Brown, J.L., Kassis, J.A., and Muller, J. (1999). The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a Drosophila homeotic gene. Development *126*, 3905-3913.

Karch, F., Galloni, M., Sipos, L., Gausz, J., Gyurkovics, H., and Schedl, P. (1994). Mcp and Fab-7: molecular analysis of putative boundaries of cis-regulatory domains in the bithorax complex of Drosophila melanogaster. Nucleic Acids Res. *22*, 3138-3146.

Katsani, K.R., Hajibagheri, M.A., and Verrijzer, C.P. **(**1999). Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/ POZ domain and reorganizes promoter topology. EMBO J. *18*, 698-708.

Klug, A., Rhodes, D., Smith, J., Finch, J. T., and Thomas, J. O. (1980). A low resolution structure for the histone core of the nucleosome. Nature *287*, 509-516.

Klymenko, T., Papp, B., Fischle, W., Kocher, T., Schelder, M., Fritsch, C., Wild, B., Wilm, M., and Muller, J. (2006). A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. Genes Dev. *20*, *in press.*

Kosikov, K. M., Gorin, A. A., Zhurkin, V. B., and Olson, W. K. (1999). DNA stretching and compression: large-scale simulations of double helical structures. J. Mol. Biol. *289*, 1301-1326.

Kwon, S-H., Kim, S.H., Chung, H-M., Girton, J.R., and Jeon, S-H. (2003). The Drosophila pleiohomeotic mutation enhances the Polycomblike and Polycomb mutant phenotypes during embryogenesis and in the adult. Int. J. Dev. Biol. *47*, 389-395

Levine, S.S., King, I.F.G., and Kingston, R.E. (2004). Division of labour in Polycomb group repression. Trends Biochem. Sci. *29*, 478-485.

Liu, C.L., Kaplan, T., Kim, M., Buratowski, S., Schreiber, S.L., Friedman, N., and

Rando, O.J. (2005). Single-nucleosome mapping of histone modifications in S. cerevisiae. PLoS Biol. *3*, e328.

Mahmoudi, T., Zuijderduijn, L.M.P., Mohd-Sarip, A., and Verrijzer, C.P. (2003). GAGA facilitates binding of Pleiohomeotic to a chromatinized Polycomb response elements. Nucleic Acids Res. *31*, 4147-4156.

Mahmoudi, T. and Verrijzer, C.P. (2001). Chromatin silencing and activation by Polycomb and trithorax group proteins. Oncogene *20*, 3055-3066.

Mahmoudi, T., Katsani, K.R., and Verrijzer, C.P. (2002). GAGA can mediate enhancer function in trans by linking two separate DNA molecules. EMBO J. *21*, 1775-1781.

Mishra, R.K., Mihaly, J., Barges, S., Spierer, A., Karch, F., Hagstrom, K., Schweinsberg, S.E., and Schedl, P. (2001). The iab-7 polycomb response element maps to a nucleosome-free region of chromatin and requires both GAGA and Pleiohomeotic for silencing activity. Mol. Cell. Biol. *21*, 1311-1318.

Mohd-Sarip, A., and Verrijzer, C.P. (2004). Molecular Biology. A higher order of silence. Science *306*, 1484-5.

Mohd-Sarip, A., Venturini, F., Chalkley, G.E., and Verrijzer, C.P. (2002). Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. Mol. Cell. Biol. *22*, 7473-7483.

Mohd-Sarip, A., Cleard, F., Mishra, R.K., Karch, F., and Verrijzer, C.P. (2005) Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex. Genes Dev. *19*, 1755-1760.

Muller, M., Hagstrom, K., Gyurkovics, H., Pirrotta, V., and Schedl, P. (1999). The Mcp element from the Drosophila melanogaster bithorax complex mediates long-distance regulatory interactions. Genetics *153*, 1333-1356.

Ogawa, T., Yu, X., Shinohara, A., and Egelman, E. H. (1993). Similarity of the yeast RAD51 filament to the bacterial RecA filament. Science *259*, 1896-1899.

Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell, G.W., Walker, K., Rolfe, P.A., Herbolsheimer, E., Zeitlinger, J., Lewitter, F., Gifford, D.K., and R.A. Young. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. Cell *122*, 517-527.

Ratcliff, G.C., and Erie, D.A. (2001). A novel single-molecule study to determine protein-protein association constants. J. Am. Chem. Soc. *123*, 5632-5635.

Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb group and trithorax group proteins. Annu. Rev. Genet. *38*, 413-443.

Udvardy, A., and Schedl, P. (l984). Chromatin organization of the 87A7 heat shock locus of Drosophila melanogaster. J. Mol. Bio. *172*, 385-402.

van der Knaap, J.A., Kumar, B.R.P., Moshkin, Y.M., Langenberg, K., J., Krijgsveld, Heck, A.J.R., Karch, F., and Verrijzer, C.P. (2005). GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. Mol. Cell. *17*, 695-707.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004). Hierarchical recruitment of Polyomb group silencing complexes. Mol. Cell *14*, 637-646.

Wang, H., Wang, L.J., Erdjument-Bromage, H., Vidal, M., Tempst, P, Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in polycomb silencing. Nature *431*, 873-878.

White, J. H., Cozzarelli, N. R., and Bauer, W. R. (1988). Helical repeat and linking number of surface-wrapped DNA. Science *241*, 323-327.

Wyman, C., Rombel, I., North, A.K., Bustamante, C., and Kustu, S. (1997). Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. Science *275*, 1658-1661.

Yuan, G-C., Liu, Y-J., Dion, M.F., Slack, M.D., Wu, L.F., Altschuler, S.J., Rando, O.J., (2005) Genome-scale identification of nucleosome positions in S. cerevisiae. Science *309*, 626-630.

Summary

Nederlandse samenvatting

Summary

Epigenetic regulation refers to effects on eukaryotic gene expression that are inherited through cell divisions. 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 *cis*-acting epigenetic DNA elements that maintain segment-specific silencing of linked enhancers in a PcG gene-dependent manner and function as chromosomal tethers for Polycomb group proteins (PcG) proteins. The mechanism by which they are targeted to PREs remains largely unclear. One of the major classes of PcG repressor complexes (PRCs) is PRC1. PRC1-type complexes harbor the core subunits Posterior sex combs (PSC), Polyhomeotic (PH), Polycomb (PC) and dRING1, and several other proteins. Because PREs function in transgenes, there is possibly a DNA sequence code that can impose PcG control. However, the nature of this PRE code is still elusive. Recent studies have shed more light in this area. Sequence-specific DNA-binding proteins have been implicated in recruiting PcG complexes to PREs. One of the key players is the *Drosophila* homologue of the mammalian transcription factor Yin Yang 1 (YY1), which is the DNA-binding protein Pleiohomeotic (PHO). PHO binding elements are essential in PcG silencing *in vivo* and PHO mutants result in PcG phenotypes in flies. In this thesis, we report on the role of PHO in targeted gene silencing by PcG proteins.

Previous studies indicate that PHO and PC cooperate *in vivo* during fly development. An explanation for this cooperation would be a direct interaction between PHO and PcG proteins. In general, proteins that regulate transcription are not stably associated with their corepressors, we set out to identify a putative repression domain in PHO and whether it interacted with a PC-containing repressive complex. In Chapter 2, we demonstrated that a distinct domain of PHO containing a conserved motif contacts the PcG proteins PC and PH. With mobility shift assays and DNA pulldown experiments, we demonstrated that PHO is able to link PC, which lacks sequence-specific DNA-binding activity, to the DNA. Importantly, we found that the PC-binding domain of PHO (PBD) can mediate transcriptional repression in transfected *Drosophila* Schneider cells. Concomitant overexpression of PC resulted in stronger PHO-directed repression that was dependent on its PC-binding domain. Together, these results suggest that PHO can contribute to PRE-mediated silencing by direct recruitment of a PC complex to repress transcription.

PHO sites in many PREs form part of a larger conserved sequence motif. Deciphering the sequence requirements for PRE function is critical to understanding how DNA elements can direct cellular memory during development. In Chapter 3 we identified a conserved sequence motif present in most PREs, named PCC-binding element (PBE), flanking the core PHO binding sites, 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. To reflect the generality of these rules, we refer to our PRE-bound PcG silencing complexes as silenceosomes.

We reveal the architecture of the PHO/PCC/PRE silenceosome in Chapter 4, by employing scanning force microscopy (SFM) and a variety of biochemical approaches. Our results suggest a model whereby the PRE DNA is wrapped around PHO/PCC in a negatively supercoiled fashion. This reorganization of DNA topology could rule out nucleosomes formation. To support that, MNaseI and DNaseI hypersensitive assays in cells and embryos showed that PREs are indeed nucleosomes free. We propose that the PHO/PCC/PRE silenceosome forms a nucleoprotein complex and that it shares architectural features with nucleosomes.

Collectively, we have come closer in identifying the PRE code required for silenceosome formation and elucidating the role of how PcG proteins are targeted to PREs. It remains to be seen in the future as to how the orchestration of the PRE code and/or histone code (though there is probably not a simple explanation to it) are involved in maintaining epigenetic silencing of the homeotic genes, in flies and perhaps in mammals too. We have resolved the nature of PHO-PCC induced change in DNA conformation, which will therefore provide important information on the mechanism of action for both targeting and silencing in PcG proteins. In this case, the assembly of the silenceosome employs the principle of DNA-binding as an efficient way to recruit, target and dock PcG proteins to specific DNA sites.

Nederlandse samenvatting

Epigenetische regulatie verwijst naar effecten op eukaryotische gen expressie, die doorgegeven worden bij celdelingen. Onderzoek gedurende het laatste decennium heeft vastgesteld dat covalente chromatine modificaties een essentiële rol spelen bij de handhaving van genexpressie patronen. Hoe gespecialiseerde DNA sequentie elementen het daarbij behorende gen onder epigenetische controlle brengt, is echter nog onduidelijk. *Drosophila* Polycomb respons elementen (PRE's) zijn *cis*-werkende epigenetische DNA elementen die segment-specifieke repressie van de bijbehorende enhancers handhaven op een PcG gen afhankelijke wijze, en functioneren als chromosomale bindingsplaatsen voor Polycomb groep (PcG) eiwitten. Het mechanisme waardoor deze eiwitten geselecteerd worden naar de PRE's is grotendeels onduidelijk. Een van de belangrijkste klassen van PcG repressor complexen (PRC's) is PRC1. Complexen behorende tot de PRC1-klasse bestaan uit de centrale subeenheden Posterior sex combs (PSC), Polyhomeotic (PH), Polycomb (PC) en dRING1, alsmede verscheidene andere eiwitten. Omdat PRE's functioneren in transgenen, is er misschien een code in de DNA sequentie, die tot PcG regulering kan leiden. De aard van deze PRE code is nog onbekend. Recent onderzoek heeft hier wel enige duidelijkheid in gebracht. Sequentie specifieke DNA-bindende eiwitten zijn geïmpliceerd in de rekrutering van PcG complexen naar de PRE's. Een van de sleutelelementen is de *Drosophila* homoloog van de transcriptie factor Yin Yang 1 (YY1) in zoogdieren. Dit is het DNA-bindend eiwit Pleiohomeotic (PHO). PHO-bindende elementen zijn essentieel voor PcG repressie *in vivo* en PHO mutaties leiden tot PcG fenotypes in vliegen. In dit proefschrift doen we verslag van de rol van PHO in selectieve genrepressie door PcG eiwitten.

Eerdere studies tonen dat PHO en PC samenwerken *in vivo* gedurende de ontwikkeling van de vlieg. Een verklaring voor deze samenwerking zou een directe interactie tussen PHO- en PcG-eiwitten kunnen zijn. In het algemeen zijn transcriptie-regulerende eiwitten niet stabiel geassocieerd met hun co-repressoren. Ons doel was een mogelijk repressie domein in PHO te identificeren en hebben we bekeken of het interacteert met een PC-bevattend repressief complex. In hoofdstuk 2 hebben we aangetoond dat een duidelijk domein van PHO, wat een geconserveerd motief bevat, bindt aan de PcG eiwitten PC en PH. Met mobility shift assays en DNA pulldown experimenten hebben we aangetoond dat PHO in staat is PC, dat geen sequentie specifiek DNA bindende activiteit heeft, kan verbinden met DNA. Relevant is dat we gevonden hebben dat het PC-bindende domein van PHO (PBD) betrokken is bij transcriptionele repressie in getransfecteerde *Drosophila* Schneider cellen. Daarbovenop komende overexpressie van PC resulteerde in sterkere PHO afhankelijke repressie, wat afhankelijk was van zijn PC-bindende domein. Gezamenlijk suggereren deze resultaten dat PHO kan bijdragen aan PRE-afhankelijke repressie door directe rekrutering van een PC complex, wat tot transcriptionele repressie leidt.

In veel PRE's zijn PHO-bindingsplaatsen een onderdeel van een groter geconserveerd sequentiemotief. Het ophelderen van de sequentievereisten, die van belang zijn voor het functioneren van een PRE, is van groot belang om te begrijpen hoe DNA

elementen het cellulaire geheugen kunnen regelen. In hoofdstuk 3 hebben we een geconserveerde sequentiemotief, het PCC-bindend element geheten (PBE), geïdentificeerd. Dit motief is aanwezig in de meeste PRE's. Deze sequentie flankeert de centrale PHO bindingsplaatsen en is vereist voor PcG repressie *in vivo*. We hebben aangetoond dat PHO bindingsplaatsen en PBE's samen een geïntegreerd platform vormen voor sterke coöperatieve DNA binding door PHO en PCC. Gebaseerd op deze resultaten stellen we dat het moleculaire ontwerp van een epigenetische silencer analoog is aan die van enhanceosomen. Om de algemeenheid van deze regels weer te geven, verwijzen we naar onze PRE-gebonden PcG repressie complexen met de term "silenceosomen".

In hoofdstuk 4 onthullen we de architectuur van een PHO/PCC/PRE silenceosoom, door gebruik te maken van scanning force microscopy (SFM) en een scala van biochemische methoden. Onze resultaten suggereren een model waarbij het PRE DNA rond PHO/PCC gewikkeld is, in een negatieve supercoiled structuur. (overeenkomstig negatieve supercoiling). Deze reorganisatie van DNA topologie zou de vorming van nucleosomen kunnen uitsluiten. Ter ondersteuning hiervan tonen MNaseI en DNaseI hypersensitiviteits assays in cellen en embryo's aan dat PRE's inderdaad geen nucleosomen bevatten. We stellen voor dat de PHO/PCC/PRE silenceosoom een nucleïne-eiwit complex vormt en dat het de architecturele kenmerken deelt met nucleosomen.

Over het geheel genomen zijn we dichterbij gekomen bij de identificatie van de PRE code, die vereist is voor silenceosoomvorming, en bij het ophelderen van de vraag hoe PcG eiwitten gerekruteerd worden naar de PRE's. Verder onderzoek is nodig om te verklaren hoe hoe de compositie van de PRE code en/of de histon code (hoewel er waarschijnlijk geen simpele verklaring voor is) is betrokken bij de handhaving van epigenetische repressie van de homeotische genen in de vlieg en misschien ook in zoogdieren. We hebben de aard van de door PHO-PCC geïnduceerde verandering in DNA conformatie opgelost, wat belangrijke informatie zal verschaffen over het mechanisme van rekrutering en repressie door PcG eiwitten. De assemblage van de silenceosoom maakt gebruik van het principe van DNA-binding als een efficiënte manier om PcG eiwitten te rekruteren en te clusteren op specifieke DNA plaatsen.

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- **Mohd-Sarip A.**, Cleard F., Mishra R.K., Karch F., and Verrijzer C.P. (2005) Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb Core Complex. Genes Dev. 19(15): 1755-1760.

- **Mohd-Sarip A.**, and Verrijzer C.P. (2004) Molecular Biology. A higher order of silence. Science, 306(5701): 1484-1485.

- Oruetxebarria I.,Venturini F., Kekarainen T., Zuijderduijn L.M., Houweling A., **Mohd-Sarip A.**, Vries R.G., Hoeben R.C. and Verrijzer C.P. (2004) p16INK4a is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumour cells. J. Biol. Chem. 279(5): 3807-3816.

- Mahmoudi T., Zuijderduijn L.M., **Mohd-Sarip A.**, and Verrijzer C.P. (2003) GAGA facilitates binding of Pleiohomeotic to a chromatinized Polycomb response element. Nucleic Acids Res. 31(14): 4147-4156.

- **Mohd-Sarip A.**, Venturini F., Chalkley G.E., and Verrijzer C.P. (2002) Pleiohomeotic can link Polycomb to DNA and mediate transcriptional repression. Mol. Cell. Biol. 22(21): 7473-7483.

- Linder B., Jones L., Chaplin T., **Mohd-Sarip A.**, Heinlein, U.A., Young B.D., and Saha V. (1998) Expression pattern and cellular distribution of the murine homologue of AF10. Biochim Biophys Acta. 1443(3): 285-296.

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--Ralph Waldo Emerson

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