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The DNA-binding Polycomb group protein Pleiohomeotic mediates silencing of a *Drosophila* homeotic gene

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SUMMARY

Polycomb group (PcG) proteins repress homeotic genes in cells where these genes must remain inactive during development. This repression requires *cis*-acting silencers, also called PcG response elements. Currently, these silencers are ill-defined sequences and it is not known how PcG proteins associate with DNA. Here, we show that the *Drosophila* PcG protein Pleiohomeotic binds to specific sites in a silencer of the homeotic gene *Ultrabithorax*. In an *Ultrabithorax* reporter gene, point mutations in these Pleiohomeotic binding sites abolish PcG repression in vivo. Hence, DNA-bound Pleiohomeotic protein may function in the recruitment of other non-DNA-binding PcG proteins to homeotic gene silencers.

Key words: *Drosophila*, PcG genes, PRE, *pho*, YY1

INTRODUCTION

The body plan of higher eukaryotes depends on spatially restricted expression of homeotic genes (Lewis, 1978; Duboule and Dollé, 1989; Graham et al., 1989, McGinnis and Krumlauf, 1992; Salser and Kenyon, 1994). *Cis*-regulatory sequences controlling homeotic gene expression have been characterised in *Drosophila* and in vertebrates (Simon et al., 1990; Müller and Bienz, 1991; Qian et al., 1991; Zink et al., 1991; Püschel et al., 1991; Gérard et al., 1993; Sharpe et al., 1998). In *Drosophila* homeotic genes, two types of regulatory sequences have been identified by reporter gene assays in transformed animals: enhancers and silencers (for review see Bienz and Müller, 1995). Enhancers fall into two broad classes: early enhancers, which transiently direct transcription at the blastoderm stage, and late enhancers, which become active only after gastrulation and direct expression in embryonic or imaginal tissues. Early enhancers activate transcription exclusively within the limits of the corresponding homeotic gene expression domain, whereas most late enhancers – if linked individually to a reporter gene – are active not only within but also outside of homeotic gene expression domains. Such misexpression is suppressed if certain DNA fragments, called silencers, are linked to late enhancers in reporter gene constructs (Müller and Bienz, 1991; Busturia and Bienz, 1993; Simon et al., 1993; Chan et al., 1994; Christen and Bienz, 1994). Since this silencing depends on PcG gene function (Müller and Bienz, 1991; Busturia and Bienz, 1993; Simon et al., 1993; Chan et al., 1994; Christen and Bienz, 1994), these silencers were called PcG response elements (Simon et al., 1993). Most known PcG proteins do not bind to DNA directly but bind to the chromatin of homeotic genes (Zink and Paro, 1989; Zink et al., 1991; DeCamillis et al., 1992). PcG protein binding regions in chromatin have been mapped by formaldehyde cross-linking PcG proteins to DNA (Orlando and Paro, 1993). This approach identified the same DNA fragments that were previously found to act as silencers in functional assays (Strutt et al., 1997; Orlando et al., 1998). Currently, PcG response elements are only poorly defined sequences, several hundred base pairs in length and, despite extensive cross-linking studies it is not known how PcG proteins bind to DNA (Orlando and Paro, 1993; Strutt and Paro, 1997; Strutt et al., 1997; Orlando et al., 1998).

The product of the PcG gene *pleiohomeotic* (*pho*), a zinc finger protein related to the mammalian transcription factor YY1, was recently identified as a factor that binds to a silencing fragment from the *engrailed* gene (Brown et al., 1998). The role of this DNA fragment in the regulation of *engrailed* expression is not known but it functions as a pairing-sensitive silencer if linked to a mini-*white* reporter gene (Kassis, 1994). This pairing-sensitive silencing of mini-*white* requires an intact PHO protein binding site and is partially impaired in *pho* mutants (Brown et al., 1998); however, the effects of mutations in PcG proteins on pairing-sensitive silencing are variable and highly dependent on the chromosomal insertion site (Kassis, 1994 and J. K., unpublished data). Thus, it remains unclear which PcG proteins actually mediate the pairing-sensitive silencing. Here we analysed the requirement for PHO protein binding sites in a bona fide PcG response element of a homeotic gene. One of

the best-studied PcG response elements is a 1.6 kb fragment from the *Ultrabithorax* (*Ubx*) gene (Chan et al., 1994). This fragment was named PRE (Chan et al., 1994) and we shall refer to it as *PRE* to distinguish it from the term PRE, the generally used abbreviation for PcG response elements (Simon et al., 1993). We show here that PHO binds to *PRE* and that the PHO binding sites are essential for PcG repression in vivo. These experiments establish a direct link between a PcG protein and its target site in a homeotic gene.

MATERIALS AND METHODS

Drosophila transformation, mutant strains and staining procedures

Transformants were generated as previously described (Bienz et al., 1988). Mutations used in this study were Pc^{XT109}, a protein null allele (Franke et al., 1995), and pho^{1} and pho^{b} alleles, which contain stop codons upstream of the DNA-binding domain and are presumed nulls (Brown et al., 1998). *pho* homozygotes were identified either by their misexpression phenotype or by using a 4th chromosome marked with *ci-lacZ*. A *TM6B Tb* chromosome was used to identify *Pc* heterozygotes. We found that pho^{1} and pho^{b} homozygotes are indistinguishable with respect to misexpression of reporter genes and *Ubx*. X-gal stainings were done as described (Christen and Bienz, 1994) and antibody stainings using fluorescence were done following standard protocols.

Plasmid constructions

The basic *IDE-Ubx-lacZ* reporter gene (based on a Carnegie 20 transformation vector) containing the 2.8 kb PBX-41 fragment called *IDE* linked to the proximal *Ubx* promoter and *lacZ*-coding region has been described (Christen and Bienz, 1994). This construct was modified to contain the 0.6 kb embryonic *PBX* enhancer pbxSB (Zhang et al., 1991) upstream of *IDE*. Unique *Kpn*I and *Xba*I sites were engineered between *PBX* and *IDE* to insert the various *PRE* subfragments. Transformants carrying the *PBX-IDE-Ubx-lacZ* and the *PBX-*$PRE_{1.6}$*-IDE-Ubx-lacZ* construct were kindly provided by M. Bienz and G. Struhl (personal communication); in these two cases, the basic *lacZ* reporter gene had been further modified to contain a nuclear localisation signal N-terminal to the β-gal coding region and, in addition, the transformation vector backbone carried the *yellow* gene rather than *rosy* as selective marker.

$PRE_{1.6}$ corresponds to 2212StR1.6 (Chan et al., 1994) and subfragments of it were obtained by subcloning or by PCR. The sequence of $PRE_{1.6}$ can be obtained from GenBank (acc. no. L32205). For simplicity, the sequence of the ten first and ten last nucleotides of each fragment preceded and followed by the nucleotide position in L32205 are given here:

$PRE_{1.6}$ (1.56 kb): 33106gaattcaaaa... agcgccaagg34667;
PRE_A (0.58 kb): 33106gaattcaaaa... tgataaggtc33683;
PRE_B (0.96 kb): 33519atatgcaaccc... aagagcgtgc34479;
PRE_C (0.48 kb): 34184gctccgtcgc... agcgccaagg34667;
PRE_D (0.57 kb): 33683ccataatctt... ctcataatcg34249.

Sequencing of the PRE_D fragment used in this study suggests that there are several deviations from the database sequence, two of these putative polymorphisms affect PHO-binding sites. The first deviation generates the sequence GCCATCTC that corresponds to site 3 in our PRE_D fragment while the database sequence is GCCTTCTC; this latter sequence probably would not constitute a PHO protein binding site. The second deviation is at site 5: in our PRE_D fragment it has the sequence ACCATTAC, the database sequence is GCCATTAC, which binds PHO even stonger than site 4 (see below).

$PRE_{DPho\ mut}$ was obtained by substituting two to three nucleotides (shown in bold) in the conserved core of all six PHO sites using site-directed mutagenesis; see Fig. 3 for wild-type sequence.

site 1: CACGGAAGC**ACG**AACGGCAG
site 2: CGCAGC**TG**TTA**GCA**TGCGCG (note that in this case we also mutated a second potential PHO binding site on the opposite strand)
site 3: ACGGTTAG**AT**ATCTCGCTCG
site 4: CTCCGTCGC**ACG**AACTGTCG
site 5: TAAAACGA**T**C**GG**TACGAACG
site 6: TTATGAGGC**ACG**CTCAGTCG

The distal end of PRE_D starts with CCATA and is preceded by polylinker sequences, this sequence was altered to CCGCG in $PRE_{DPho\ mut}$.

The complete *pho* coding region from the *pho* 12a cDNA (Brown et al., 1998) was subcloned downstream of the *β-globin* 5′ UTR and ATG in *pT7link* (provided by R. Treisman) to obtain $PHO_{2\text{-}520}pT7$.

Detailed maps of plasmids are available on request.

Electromobility shift assays

Radiolabeled double-stranded probes of the sequences listed in Fig. 3B were generated by annealing the corresponding single-stranded oligonucleotides containing a $(dG)_3$ overhang at the 5′ end. The ends were then filled with [^{32}P]-dCTP using Klenow polymerase followed by phenol extraction and separation of the probe from unincorporated nucleotides over a G-25 column. [^{14}C]-Leu-labeled full-length PHO protein was in vivo translated from the $PHO_{2\text{-}520}pT7$ template using the TNT reticulocyte lysate system (Promega); integrity of the labeled protein was checked by SDS-page followed by autoradiography. For binding tests, 2-5 fmol DNA probe and 2 μl of the in vivo translation reaction were incubated for 20 minutes on ice in a 20 μl reaction (100 mM KCl, 35 mM Hepes 7.9, 1 mM DTT, 50 μM $ZnCl_2$, 12% glycerol, 2 mM spermidine, 1 mg/ml BSA, 0.1 mg/ml dI:dC). DNA-protein complexes were resolved at 4°C on a native 4% polyacrylamide gel at 10 V/cm using 0.5× TB as running buffer; the gel was pre-run for 1 hour prior to loading. The gel was fixed, dried and exposed for autoradiography.

In addition to the oligos shown in Fig. 2, we also tested additional oligos using the same gel-shift assay. Of three further double-stranded oligos with CCAT motifs from the *PBX* region, the oligos $hb_{2/3}$ (5′ATAATTTTTTGCCATGGCTAATAAAA3′) and hb_6 (5′ACGGGA-ATGCGCCATAAAAAATGTGT3′) were not bound by PHO protein whereas hb_4 (5′AGAGCCGTCGGCCATT-AAAAAAGGTG3′) was bound by PHO. We also note that PHO sites 1, 2, 3, 4 and 6 in PRE_D all have a G 5′ to the CCAT motif whereas PHO site 5 lacks such a G. In our PRE_D fragment, PHO site 5 has the sequence ACCATTAC whereas the database sequence is GCCATTAC. We found that changing the 5′ A to a G in the PHO site 5 oligo increases its affinity for PHO protein binding; this altered site binds even stronger than site 4 (C. F. and J. M., data not shown). Conversely, although oligos 'D' from PRE_D (see Fig. 2 for sequence) as well as oligos $hb_{2/3}$ and hb_6 from *PBX* do contain a GCCAT motif, they still failed to bind in our assay. Thus, it appears that, besides the critical CCAT core motif, a 5′ G is required but this G can be compensated for by appropriate nucleotides 3′ to the CCAT motif, i.e. the sequence TAC as in the case of PHO site 5 from PRE_D.

Immunostaining of polytene chromosomes

Rabbit polyclonal PHO antibodies were generated against a gel-isolated HIS-tag/PHO full-length fusion protein. The production, affinity purification and characterization of the antibody will be presented elsewhere (J. L. B. and J. A. K., unpublished data). The specificity of the antibody was demonstrated by inhibition or supershifting of PHO/DNA complexes in the gel-shift assay, by the detection of a single protein species on westerns of 0-12 hour nuclear embryonic extracts and by the absence of PHO staining on polytene chromosomes of pho^{1} mutant larvae. Affinity-purified and crude PHO antisera give the same banding pattern on polytene chromosomes.

Antibody staining of polytene chromosomes

Polytene chromosomes were fixed in 2% formaldehyde, 40% glacial acidic acid for 2.5 minutes. Under these fixation conditions, approximately 35 PHO bands are observed. Slides were washed in phosphate-buffered saline for 30 minutes, incubated in blocking buffer (PBS, 0.5% BSA, 0.1% Tween 20) for 30 minutes and then incubated with a 1/200 dilution of the crude PHO serum overnight at 4°C. Signals were developed using the secondary antibody and HRP detection system from the Vectastain ABC Elite kit (Vector Laboratories). The signal was enhanced by the inclusion of 0.008% $NiCl_2$ and 0.008% $CoCl_2$ in the HRP reaction.

RESULTS

To dissect the 1.6 kb *Ubx PRE*, we used a *Ubx-lacZ* reporter gene to monitor silencing capacity of *PRE* subfragments. We previously identified an embryonic enhancer, called *PBX* and an imaginal disc enhancer, called *IDE*, which are both located about 30 kb upstream of the *Ubx* transcription start site (Müller and Bienz, 1991; Castelli-Gair et al., 1992). *PBX* directs expression in early embryos in a pattern similar to *Ubx* with a sharp anterior boundary in parasegment 6 (ps 6) (Müller and Bienz, 1991). In contrast, if *IDE* is linked to a reporter gene it activates transcription not only in haltere discs where endogenous *Ubx* is expressed but also in wing discs where *Ubx* is not expressed (Castelli-Gair et al., 1992; Chan et al., 1994; Christen and Bienz, 1994; White and Wilcox, 1984; Beachy et al., 1985). A *PBX-IDE* reporter gene is thus active within *Ubx* expression boundaries in early embryos but is later expressed also outside of the *Ubx* domain, i.e. in the wing disc. We therefore tested whether *PRE* or subfragments thereof would silence this misexpression if inserted into the *PBX-IDE* reporter gene.

First, we inserted the 1.6 kb *PRE* (Chan et al., 1994) between the *PBX* and *IDE* enhancers and introduced this reporter gene ($PRE_{1.6}$) into flies (Fig. 1). Whereas *PBX-IDE* transformants

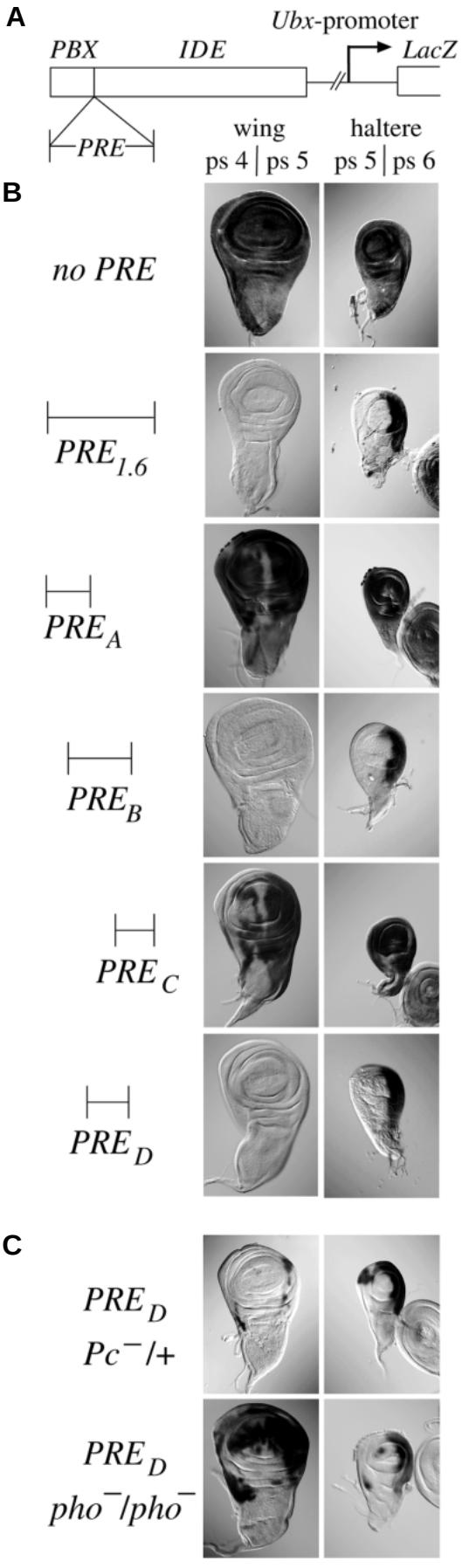

Fig. 1. *PRE*-mediated silencing in imaginal discs. (A) *PRE* subfragments (black bars) were inserted upstream of *IDE* into the *PBX-IDE-Ubx-lacZ* reporter gene (top) to avoid unspecific blocking of *IDE* by *PRE*. The subfragments are drawn to scale (see Materials and Methods). (B) β-gal expression in wing and haltere discs of transformant lines carrying the reporter gene indicated on the left was visualized by X-gal staining. In all cases, the anterior compartment of the disc is to the left. Transformants carrying the *PBX-IDE* reporter gene without a *PRE* insert (*no PRE*) and PRE_A transformants show β-gal expression throughout ps 4, 5 and 6 although in wing discs typically small patches without β-gal expression are present; the wing disc of the *no PRE* line shown here lacks such unstained patches. In $PRE_{1.6}$, PRE_B and PRE_D transformants β-gal expression is restricted to the posterior compartment of the haltere disc (ps 6) and no staining is present in wing and anterior haltere discs (ps 4 and 5) due to silencing by the *PRE* fragment. Partial silencing is observed in several PRE_C lines; shown here are discs from the weakly silenced line PRE_C line 33.1. (C) Loss of *PRE*-mediated silencing in PcG mutants. In *Pc* heterozygotes, the PRE_D reporter gene is misexpressed in small patches in the wing and anterior haltere disc; such misexpression was never observed in wild-type discs (see above). Note that these animals still carry one wild-type copy of the *Pc* gene. *pho* homozygotes show extensive misexpression of the reporter gene in wing and haltere discs. See Table 1 for more details.

Table 1. Transformant lines and their expression pattern in individual compartments of wing and haltere discs

Construct	Transformant line	Wing		Haltere		Silencing	Loss of silencing in	
		ps 4	ps 5	ps 5	ps 6		Pc⁻/+	pho⁻/pho⁻
no *PRE*	206.3F	+°	+	+°	+	no	n.d.	n.d.
	206.8 F	+	+	+	+	no	n.d.	n.d.
	206.11 F	+	+	+	+	no	n.d.	n.d.
$PRE_{1.6}$	205.1	–	–	–	+	yes	n.d.	n.d.
	205.3	–	–	–	+	yes	n.d.	n.d.
	205.4	–	–	–	+	yes	n.d.	n.d.
	205.5	–	–	–	+	yes	n.d.	n.d.
	205.6	Position effect	Position effect	Position effect	Position effect		n.d.	n.d.
	205.7	–	–	–	+	yes	n.d.	n.d.
	205.8	–	–	–	+	yes	n.d.	n.d.
	205.9	–	–	–	+	yes	yes	n.d.
	205.10	–	–	–	+	yes	n.d.	n.d.
	205.11	+	+	+	+	no	n.d.	n.d.
	205.12	–	–	–	+	yes	yes	n.d.
	205.13	–	–	–	+	yes	yes	n.d.
PRE_A	40.1	–	–	–	–	–	no	n.d.
	40.4	+°	+	+	+	no	n.d.	n.d.
	48.1	–	–	–	–	–	n.d.	n.d.
	48.2	+	+	+	+	no	n.d.	n.d.
	60.3	+	+	+	+	–	no	n.d.
	64.1	+	+	+	+	no	n.d.	n.d.
	71.1	+	+	+	+	no	n.d.	n.d.
	73.1	+	+	+	+	no	n.d.	n.d.
	82.1	+	+	+	+	no	n.d.	n.d.
	82.2	–	–	–	–	–	n.d.	n.d.
PRE_B	1	–	–	–	+	yes	n.d.	n.d.
	2	Position effect	Position effect	Position effect	Position effect		n.d.	n.d.
	3	–	–	–	+	yes	n.d.	n.d.
	4	–	–	–	–	–	no	n.d.
PRE_C	6.3	–	–*	–	+	yes	n.d.	n.d.
	6.6	+	+	+	+	no	n.d.	n.d.
	12.4	+	+	+	+	no	n.d.	n.d.
	16.5	–	+°	–	+	yes (ps5)	n.d.	n.d.
	33.1	+°	+	+	+	yes (ps5)	n.d.	n.d.
PRE_D	2	–	–*	–	+	yes	yes	n.d.
	3	–	–	–	+	yes	yes	yes
	4	–	–	–	+	yes	yes	yes
	5B	–	–	–	+	yes	yes	yes
$PRE_{Dmut\ pho}$	1.3	+	+	+	+	no	n.d.	n.d.
	7.2	–	–	–	–	–	no	n.d.
	7.4	+	+	+	+	no	n.d.	n.d.
	8.1	+	+	+	+	no	n.d.	n.d.
	33.1	+°	+	+	+	no	n.d.	n.d.

+, strong and nearly uniform β-gal expression with only small unstained patches; –, no *β-gal* expression detectable; +°, β-gal expression not uniform but in big patches filling large areas of the compartment; –*, incomplete silencing with β-gal expression in very small patches. All transformant lines obtained with a particular construct are listed, including lines that lacked β-gal expression altogether and lines that showed additional β-gal expression patterns caused by flanking chromosomal enhancers at the insertion site (position effect). We note that Poux et al. (1996) used a construct similar to our $PRE_{1.6}$ reporter gene but found that only a fraction of their lines show reliable silencing anterior to ps 6 (4/10 lines with complete silencing and 3/10 with partial silencing) whereas we found excellent silencing in most $PRE_{1.6}$ lines (x/x). These differences might arise from using slightly different *Ubx* fragments or, more likely, from using different marker genes on the transformation vector. The mini-*white* marker gene used by Poux et al. (1996) can be silenced by $PRE_{1.6}$ and therefore might have precluded isolation of lines with excellent silencing.

without the *PRE* fragment show nearly uniform β-galactosidase (β-gal) expression in wing and haltere discs, β-gal expression in $PRE_{1.6}$ transformants is confined to the posterior compartment of haltere discs (Fig. 1; Table 1). The boundary between β-gal-positive and β-gal-negative cells runs through the middle of the haltere disc and apparently coincides with the ps 6 compartment boundary. Thus, *IDE* activity is completely suppressed anterior to ps 6 but is unaffected in ps 6 itself. This suggests that $PRE_{1.6}$ silences the reporter gene anterior to ps 6 and thereby preserves the anterior expression boundary delimited by *PBX* in the embryo. We note that the expression pattern directed by *PBX* in the embryo is not silenced by $PRE_{1.6}$ (data not shown, see also Discussion). After we intiated these experiments, an independent study by Pirrotta and co-workers showed that the 1.6 kb *PRE* is able to silence misexpression of a reporter construct that is very similar to our *PBX-IDE* reporter gene (Poux et al., 1996).

We next tested subfragments of the 1.6 kb *PRE* for silencing function. Silencing anterior to ps 6 was also observed in imaginal discs of two PRE_B lines and in all four PRE_D lines

(Fig. 1; Table 1). In contrast, only one of five PRE_C lines showed substantial silencing anterior to ps 6; two lines showed partial silencing and two lines showed no silencing at all (Fig. 1; Table 1). None of the PRE_A lines showed silencing; these transformants showed β-gal staining in imaginal discs similar to transformants carrying the *PBX-IDE* reporter gene without *PRE* (Fig. 1; Table 1). Taken together, these data suggest that the *PRE* silencer is contained in the central 567 bp PRE_D fragment.

We asked whether the silencing mediated by the *PRE* fragments depends on PcG gene function. We tested all PRE_D and several $PRE_{1.6}$ lines in *Pc* heterozygotes and found in each case small patches of β-gal staining in the wing disc and in the anterior part of the haltere disc (Fig. 1; Table 1). Thus, a reduction in *Pc* gene dosage leads to a partial loss of silencing; the extent of the observed misexpression is comparable to the misexpression of the endogenous *Ubx* gene in *Pc* heterozygotes (Fig. 4). We then examined the patterns of PRE_D lines in larvae homozygous for a *pho* mutation. We found in each case that *pho* mutant wing and haltere discs show an extensive loss of silencing (Fig. 1). These results demonstrate that silencing by PRE_D requires PcG gene function.

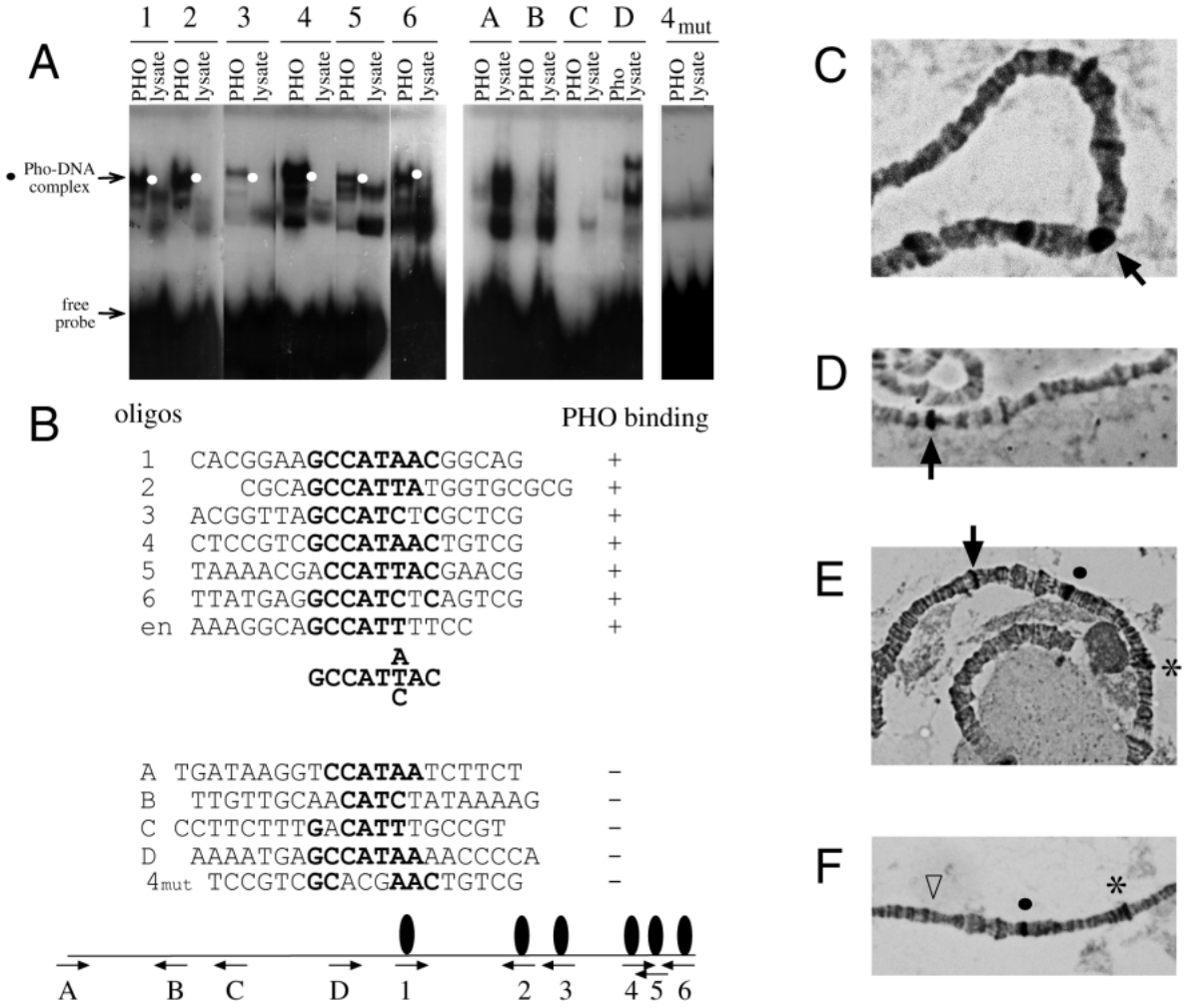

Fig. 2. PHO protein binds to PRE_D in vivo. (A) EMSA with radiolabeled PRE_D subfragments 1-6 and A-D. Labeled DNA probes were incubated with in vivo translated PHO ('PHO' lanes) or with reticulocyte lysate from a mock translation reaction ('lysate' lanes). In the presence of Pho, probes 1-6 formed specific complexes (white dots) that were not observed with lysate alone. Note that a 3-base pair substitution in the strongest binding site 4 abolishes binding of PHO protein (4_{mut}). No stable PHO-specific complexes were observed with probes A-D; it is unclear why in these cases complex formation by other binding activities in the lysate is reduced in the presence of PHO. (B) Sequences of PRE_D subfragments used for EMSA. A consensus sequence derived from fragments 1-6 and from the binding site in *engrailed* originally used to isolate PHO (Brown et al., 1998) suggests GCCATTAC is the optimal PHO binding site (see Materials and Methods for further discussion of this). Below, arrows, drawn to scale, mark the position, length and orientation (5′>3′) of these subfragments within PRE_D (long thin line); black ovals indicate PHO protein. (C-F) Binding of PHO protein to BXC and PRE_D in vivo. PHO protein bound to polytene chromosomes was visualized by histochemical staining. (C) A strong PHO signal (arrow) is present at band 89E, the location of the BXC. (D) In situ hybridization of a *lacZ* probe to polytene chromosomes from a PRE_D transformant line shows a chromosomal insertion site of 46D (arrow). (E) This reporter gene generates a new PHO protein binding site (arrow) that is not present (open arrowhead) in control animals (F); two endogenous chromosomal PHO binding sites are marked for reference (dot and asterisk, respectively). The dot corresponds to the *engrailed* locus (48A).

We next examined whether PHO protein binds directly to PRE_D. PHO contains a DNA-binding domain with very high similarity to the DNA-binding domain of YY1, which is known to bind to the sequence G/t C/t/a CATN T/a T/g/c (Hyde-DeRuyscher et al., 1995). The PRE_D fragment contains several motifs that match versions of this YY1 protein binding site. Oligos spanning each of these motifs were tested for PHO binding in gel-shift assays (Fig. 2). We found that PHO protein formed a specific complex with six of the ten tested oligos (Fig. 2A). These and additional binding tests with other oligos suggest GCCATTAC as an optimal binding site for PHO (see Fig. 2B and Material and Methods for further details). To test whether PHO protein binds to the PRE_D construct in vivo, we generated antibodies against the PHO protein. On polytene chromosomes from salivary glands, PHO antibodies bind to approximately 35 different loci. The strongest signal was found at the location of the Bithorax-Complex (BXC), suggesting that PHO protein is bound to the BXC genes (Fig. 2C). Furthermore, on polytene chromosomes of a PRE_D transformant line, we found a strong additional signal at the transposon insertion site (Fig. 2D-F). These data suggest that PHO protein binds directly to PRE_D in vitro and in vivo.

We then tested whether PHO protein binding sites are needed for silencing in imaginal discs. We mutated all six PHO binding sites in the PRE_D fragment by altering two or three nucleotides in each CCAT core motif (see Materials and Methods). The introduced base changes abolish binding of PHO protein in vitro (Fig. 2). The mutated PRE_D fragment was inserted into the *PBX-IDE* reporter gene to obtain $PRE_{D\ pho\ mut}$. $PRE_{D\ pho\ mut}$ transformants show uniform β-gal staining in wing and haltere discs comparable to transformants carrying the reporter gene without *PRE* (Fig. 3). Thus, mutations in the PHO binding sites abolish *PRE* function. Note that PHO protein binding sites 4-6 are also present in the PRE_C fragment that overlaps PRE_D (Fig. 1 and Materials and Methods). It is possible that these sites are responsible for the partial silencing observed in some of the PRE_C transformant lines (Fig. 1; Table 1). Taken together, these experiments provide strong evidence that PHO protein binds directly to *PRE* and is required for silencing.

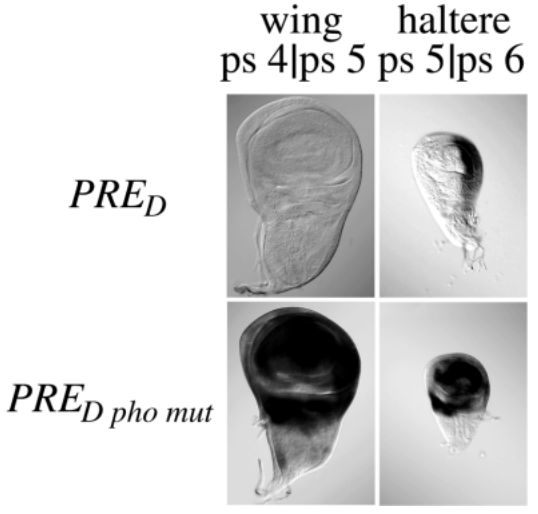

Fig. 3. PHO binding sites in PRE_D are essential for *PRE* function in vivo. β-gal expression in wild-type imaginal discs of PRE_D and $PRE_{D\ pho\ mut}$ transformants visualized by X-gal staining. Point mutations in PHO binding sites 1-6 in PRE_D abolish silencing function; the $PRE_{D\ pho\ mut}$ reporter gene is expressed in wing and haltere discs comparable to the *PBX-IDE* reporter gene without a *PRE* insert (*no PRE*, Fig. 1).

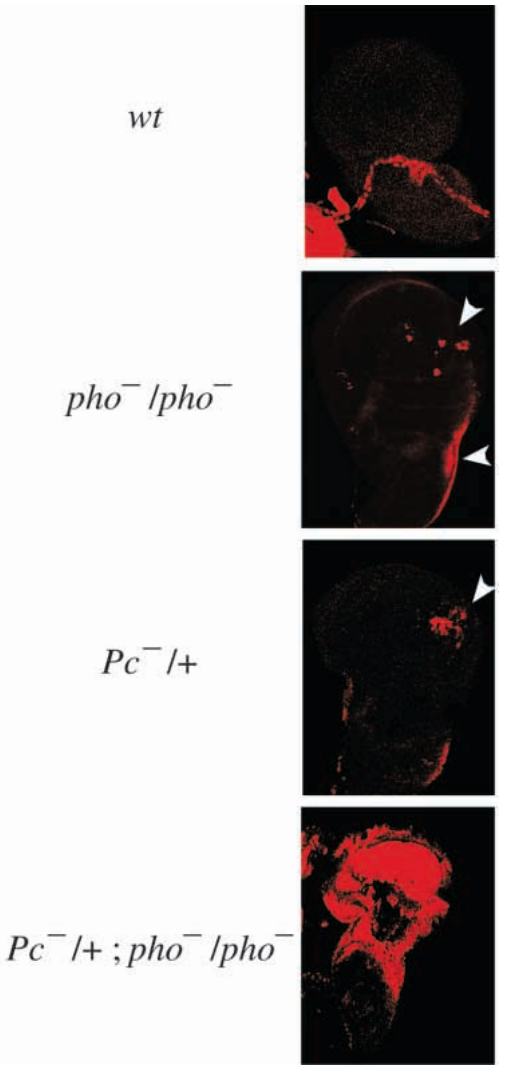

Fig. 4. *pho* represses the endogenous *Ubx* gene in wing discs. Confocal images of wing discs stained with *Ubx* antibody. In the wild type, no *Ubx* expression is detected in the wing disc proper; the labeled cells discernible here and out of focus in the pictures below are part of the peripodial membrane and/or trachea (cf. White and Wilcox, 1984; Beachy et al., 1985). pho^1 homozygotes alone and Pc^{XT109} heterozygotes alone show misexpression of *Ubx* in the wing disc, but only in a few cells (white arrowheads). In pho^1 homozygotes that are also heterozygous for Pc^{XT109} silencing of *Ubx* is almost completely lost and Ubx protein is strongly expressed in most wing disc cells.

Finally, we analysed expression of the endogenous *Ubx* gene in imaginal discs of *pho* mutants. Animals that are homozygous for *pho* null mutations develop into pharate adults with only relatively mild homeotic transformations (Gehring, 1970; Breen and Duncan, 1986; Girton and Jeon, 1994). Consistent with this, we found that pho^1 and pho^b homozygotes show only slight misexpression of *Ubx* in wing and antennal

discs (Fig. 4 and data not shown). The observed misexpression is comparable to the misexpression of *Ubx* in *Pc* heterozygotes (Fig. 4). We note that, in *pho* mutants, the PRE_D reporter gene shows substantially more misexpression than the endogenous *Ubx* gene (Fig. 1). Thus, silencing of the reporter gene is more sensitive to the lack of *pho* product than the native *Ubx* gene. Animals that are mutant for two different PcG mutations often show more severe misexpression of homeotic genes and consequently enhanced homeotic transformations compared to the single mutants by themselves (Jürgens, 1985). *pho* homozygotes that are also heterozygous for *Pc* show very dramatic misexpression of *Ubx* in wing and other discs (Fig. 4). Thus, in this genetically sensitized background due to only one rather than two copies of *Pc*, *pho* is required to repress *Ubx* in all imaginal disc cells.

DISCUSSION

Previous studies using a formaldehyde cross-linking assay showed that Polycomb protein is specifically associated with chromatin encompassing $PRE_{1.6}$ (Strutt et al., 1997; Orlando et al., 1998). However, no direct link has been made between Pc or any other PcG protein and this DNA. Our experiments here establish a direct physical link between the PcG protein PHO and $PRE_{1.6}$ and demonstrate that PHO binding is essential for repression. In a database search, sequence motifs resembling PHO binding sites have been noted in *cis*-regulatory regions from many *Drosophila* homeotic genes (Mihaly et al., 1998). Our functional data suggest that PHO protein binding sites might constitute an essential, integral part of PcG response elements. The results presented here support the view that PHO may act to recruit and anchor PcG proteins to the DNA (see below).

Early function of *pho*

Most *pho* mutant embryos, which lack maternal wild-type *pho* product, fail to develop altogether and the rare putatively paternally rescued embryos which do develop die with segmentation defects and homeotic transformations (Breen and Duncan, 1986). In contrast, if maternal *pho* product is present, *pho* homozygotes survive to pharate adults. This suggests that *pho* function is particularly important in the very early embryo. Here, we found that mutation of the PHO binding sites in the $PRE_{D\ pho\ mut}$ reporter gene abolish silencing in all disc cells. Thus, it appears that if PHO protein is prevented from binding to *PRE*, i.e. in the $PRE_{D\ pho\ mut}$ reporter gene, silencing is probably never established. Conversely, silencing of the PRE_D reporter gene is only partially lost in larvae homozygous for a *pho* null mutation (compare Figs 1 and 3). Thus, in *pho* homozygous embryos (which contain maternal PHO protein) silencing of the PRE_D reporter is probably established but is subsequently lost in imaginal discs. In summary, these observations strongly suggest that maternally deposited PHO protein is crucial for the establishment of silencing but that zygotic PHO protein is required for complete silencing.

Repression by PHO

How does PHO repress transcription? One possible model is that PHO protein recruits other non-DNA-binding PcG proteins to DNA to form 'silencing complexes' (Bienz and Müller, 1995). We imagine that such silencing complexes interact with proteins at the proximal promoter to prevent recruitment or formation of active RNA-Polymerase II complexes (Bienz and Müller, 1995). However, PHO protein binds to DNA in a sequence-specific manner and *pho* RNA and protein are expressed in all cells throughout embryonic development (Brown et al., 1998 and J. L. B. and J. A. K., unpublished observations). Why then does PHO protein not repress *Ubx* in all cells? One possibility is that PHO protein only has access to *PRE* DNA in cells where *Ubx* is repressed. A more likely scenario is that PHO protein also binds to *PRE* in cells where *Ubx* is expressed but that PHO is unable to repress on its own, e.g. because it cannot recruit functional PcG complexes by itself. Previous studies showed that repression by the gap protein Hunchback (HB) determines where *Ubx* is turned off in the early embryo (White and Lehmann, 1986; Irish et al., 1989; Qian et al., 1991; Müller and Bienz, 1992; Zhang and Bienz, 1992). dMi-2, the fly homologue of a mammalian histone deacetylase subunit, was recently identified as a HB-interacting protein that is needed for repression of *Ubx* by HB (Kehle et al., 1998). It is possible that chromatin modifying activities of a HB:dMi-2 complex are a prerequisite for DNA-bound PHO to recruit other PcG proteins in the embryo. A different scenario would be that assembly of repressive PcG complexes occurs by default unless the linked homeotic gene promoter is transcriptionally activated in the early embryo (Pirrotta et al., 1995; Poux et al., 1996). This model is based on previous studies on the 1.6 kb *PRE* which revealed an unusually potent silencing property of this fragment (Pirrotta et al., 1995; Poux et al., 1996). In particular, the 1.6 kb *PRE* fragment could silence linked imaginal disc enhancers even within the *Ubx* expression domain in some reporter constructs (Pirrotta et al., 1995; Poux et al., 1995). Since this unusual silencing occurred only in reporter genes lacking an early embryonic enhancer (e.g. *PBX*), this lead to the suggestion that early transcriptional activation is needed to prevent ubiquitous silencing by the 1.6 kb *PRE* fragment. According to this view, assembly of silencing complexes (e.g. recruited by PHO) and consequent repression would occur by default unless the reporter gene is transcriptionally activated in the early embryo (Poux et al., 1996; discussed in Bienz and Müller, 1995). It should be noted that at present none of the other identified silencers thought to contain PcG response elements has been found to indiscriminately prevent transcriptional activation by late-acting enhancers (Busturia and Bienz, 1993; Christen and Bienz, 1994). Furthermore, studies on a reporter gene that is inserted in the endogenous *Ubx* gene suggest that early transcriptional activation of this reporter gene alone is insufficient to prevent its subsequent repression by PcG proteins (McCall and Bender, 1996). Thus, it remains to be seen to what extent in the endogenous *Ubx* gene PcG-mediated repression occurs by default and to what extent establishment of this repression requires direct cooperation from gap proteins.

Maintenance function of PHO

Previous studies on PcG protein function suggest that both PcG proteins and PcG response elements are required throughout development to maintain silencing. In particular, experiments on a PcG response element from the *Drosophila* homeotic gene *Abd-B* showed that the silencer DNA itself is continuously

required for PcG repression (Busturia et al., 1997). Furthermore, analysis of *Pc* mutant clones showed that Pc protein is required throughout development to silence homeotic genes (Busturia and Morata, 1988). The simplest model to explain these observations is that sequence-specific DNA-binding proteins are required for anchoring PcG proteins to the DNA throughout development. Since maternal PHO is not sufficient for complete silencing of the PRE_D reporter gene or of *Ubx* (see Figs 1, 4), we suggest that PHO may play a role in continuously anchoring PcG proteins to the DNA. Why then is the derepression of Ubx in *pho* homozygotes not more severe? It is unlikely that maternally deposited PHO is responsible for the residual silencing in *pho* mutant discs for two reasons. First, in embryos homozygous for a *pho* protein-null mutation, maternal PHO protein becomes undetectable by 9 hours of development (J. L. B. and J. A. K., unpublished data). Second, the extensive dilution during postembryonic cell divisions would virtually eliminate any persisting PHO protein molecules in imaginal disc cells of *pho* homozygotes. Thus, we imagine that PHO protein is required to continuously anchor PcG protein complexes on DNA but suggest that other, currently unidentified DNA-binding proteins can partially substitute for PHO protein in *pho* mutant larvae. We note that other PcG genes are present in duplicate in the *Drosophila* genome and can partially substitute for each other (Dura et al., 1987; Brunk et al., 1991; van Lohuizen et al., 1991; DeCamillis et al., 1992; Soto et al., 1995). Although we have not found another YY1- or PHO-related sequence by database searches (J. M., unpublished observations), there remains the possiblity that there is a second *pho*-like gene in *Drosophila*.

In summary, we have presented strong evidence that PHO is required for the activity of a Polycomb group response element from the *Ubx* gene. We have also shown that this element responds to *Pc*. Further, our observation that $Pc^{-/+}$; $pho^{-/-}$ animals show nearly ubiquitous misexpression of *Ubx* in third instar larvae shows that PHO protein strongly synergises with Pc. However, we have no evidence that PHO and Pc interact and it remains to be seen whether recruitment of Pc to *PRE* occurs by PHO protein itself or through another DNA-binding protein.

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