General transcriptional silencing by a Polycomb response element in *Drosophila*

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Summary

Polycomb response elements (PREs) are cis-regulatory sequences required for Polycomb repression of Hox genes in *Drosophila*. PREs function as potent silencers in the context of Hox reporter genes and they have been shown to partially repress a linked *miniwhite* reporter gene. The silencing capacity of PREs has not been systematically tested and, therefore, it has remained unclear whether only specific enhancers and promoters can respond to Polycomb silencing. Here, using a reporter gene assay in imaginal discs, we show that a PRE from the *Drosophila* Hox gene *Ultrabithorax* potently silences different heterologous enhancers and promoters that are normally not subject to Polycomb repression. Silencing of these reporter genes is abolished in PcG mutants and excision of the PRE from the reporter gene during development results in loss of silencing within one cell generation. Together, these results suggest that PREs function as general silencer elements through which PcG proteins mediate transcriptional repression.

Key words: Silencing, Polycomb, Polycomb response element

Introduction

The regulation of Hox gene expression in *Drosophila* represents a paradigm for understanding how heritable transcriptional states are established and maintained during development. In the early *Drosophila* embryo, transiently acting transcriptional regulators that are encoded by segmentation genes determine in which cells Hox genes are to be expressed and in which cells these genes should stay inactive. After the decay of segmentation gene products, transcriptional ON and OFF states of Hox genes are heritably maintained by Polycomb group (PcG) and trithorax group (trxG) proteins which, however, are present in all cells. PcG repressors keep Hox genes inactive in cells in which these genes must remain inactive whereas trxG regulators are needed to maintain the active state of Hox genes in appropriate cells.

Recent progress towards understanding the PcG/trxG system has come from the biochemical characterization of PcG and trxG protein complexes. Two distinct PcG protein complexes have been characterized to date; PRC1 functions by inhibiting chromatin remodeling by SWI/SNF complexes in in vitro assays (Shao et al., 1999; Francis et al., 2001), whereas the Esc-E(z) complex functions as a histone methyltransferase (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). Similarly, the trxG proteins Trithorax and Ash1 exist in two distinct multiprotein complexes (Papoulas et al., 1998; Petruk, 2001) and both function as histone methyltransferases (Milne et al., 2002; Nakamura et al., 2002; Beisel et al., 2002; Byrd and Shearn, 2003). Thus, it appears that both PcG and trxG proteins regulate gene expression by modifying the structure of chromatin.

Nevertheless, silencing by Polycomb group proteins requires specific cis-acting sequences, called Polycomb response elements (PREs). PREs were initially identified as regulatory sequences that prevent inappropriate activation of Hox reporter genes in a PcG protein-dependent fashion in transgenic *Drosophila* embryos and larvae (Müller and Bienz, 1991; Simon et al., 1993; Chan et al., 1994; Christen and Bienz, 1994). PREs contain binding sites for Pleiohomeotic (Pho) and Pho-like (Phol), the only known DNA-binding PcG proteins, and binding of these proteins to PREs is crucially required for silencing in *Drosophila* (Brown et al., 1998; Brown et al., 2003; Fritsch et al., 1999; Shimell et al., 2000; Busturia et al., 2001; Mishra et al., 2001). Pho and Phol do not co-purify with PRC1 or the Esc-E(z) complex, and neither PRC1 nor the Esc-E(z) complex bind to DNA in a sequence-specific fashion. However, formaldehyde cross-linking studies showed that components of both PRC1 and the Esc-E(z) complex specifically associate with the chromatin of PREs in tissue culture cells and in developing embryos and larvae (Strutt and Paro, 1997; Orlando et al., 1998; Cao et al., 2002). This association is crucial for the long-term repression of Hox genes as most PcG proteins are needed throughout development to keep Hox genes silenced (Beuchle et al., 2001). Moreover, excision of a PRE from a silenced Hox reporter gene results in loss of repression, even if the PRE is removed late in development (Busturia et al., 1997). Taken together, these findings support the idea that PREs are silencer elements in

Hox genes through which PcG proteins mediate long-term repression by modifying chromatin structure.

Although PREs function as very potent silencers within Hox reporter genes, their ability to silence transcription in the context of other enhancers and promoters has not been systematically tested. Several PREs have been reported to partially repress transcription of a linked *miniwhite* reporter gene (Chan et al., 1994; Zink and Paro, 1995; Hagstrom et al., 1997) (reviewed by Kassis, 2002). In those studies, the effect of a PRE on *miniwhite* expression was analyzed by monitoring eye pigmentation in adult flies, and repression of *miniwhite* by the linked PRE was revealed by an increase in eye pigmentation in animals that are heterozygous for PcG mutations. It is important to note that the *miniwhite* reporter gene was never completely repressed in those studies, even though this process is often referred to as '*miniwhite* silencing'. A major limitation in the interpretation of this incomplete silencing of *miniwhite* is the fact that the *miniwhite* gene in the reporter construct also served as transformation marker to isolate transgenic lines harboring the reporter gene and, hence, only lines showing incomplete silencing of *miniwhite* were isolated and analyzed. Thus, it has remained unclear whether PREs function as general transcriptional silencers, or whether they only function effectively in the context of Hox genes and require specific target sequences in enhancers and/or promoters.

Here, using a reporter gene assay in imaginal discs, we test a PRE from the Hox gene *Ultrabithorax* (*Ubx*) for its capacity to silence reporter genes that contain enhancer and promoter sequences from genes that are normally not under PcG control. We find that the *Ubx* PRE very potently prevents transcription of each of the tested reporter genes, and we show that this silencing depends on PcG gene function. Excision of the PRE from the reporter gene by flp-mediated recombination results in the complete loss of repression within 12 hours of flp induction. These results imply that, after removal of the PRE, changes in the chromatin state generated by the action of PcG proteins cannot be propagated by the flanking chromatin.

Materials and methods

Drosophila strains and plasmid constructs

The $Su(z)12^2$ and $Su(z)12^3$ mutant alleles have been described (Birve et al., 2001); $Su(z)12^2/Su(z)12^3$ transheterozygous larvae shown in Fig. 3 were identified by their mutant phenotype. The fragments used for the constructing the *lacZ* reporter genes have been described in earlier studies; the 1.6 kb PRE fragment corresponds to $PRE_{1.6}$ (Fritsch et al., 1999), the FRT sequences are derived from J33R (Struhl and Basler, 1993), the vg^{QE} enhancer corresponds to the 806 bp fragment described as '*vg* quadrant enhancer' by Kim et al. (Kim et al., 1996), the vg^{BE} enhancer corresponds to the 750 bp *Eco*RI-*Eco*RI fragment described as '*vg* D/V boundary response element' by Williams et al. (Williams et al., 1994), the dpp^{WE} enhancer corresponds to the 817 bp *Ssp*I-*Mln*I fragment described as 'construct 10' by Müller and Basler (Müller and Basler, 2000). The *lacZ* reporter genes containing a 4.1 kb fragment from the *Ubx* promoter or the *TATA* box minimal promoter from *hsp70* have been described (Müller and Bienz, 1991). All enhancers were cloned upstream of these promoters in the same 5′→3′ orientation that the enhancers have with respect to their promoter within the endogenous loci; we note that, in this orientation, the vg^{BE} enhancer directs expression in a distinct pattern than in the reverse orientation (Williams et al., 1994). All reporter genes were cloned into a transformation vector containing the *rosy* (*ry*) gene as transformation marker and the constructs were injected into *cn; ry* hosts. We note that, in contrast to the transformation marker $white^+$, ry^+ function is cell non-autonomous and a few percent of ry^+ product in the animal are sufficient to rescue the ry^- eye color phenotype. Nevertheless, we cannot exclude that, at some insertion sites, the ry^+ transgene was completely silenced by the PRE and that this has precluded the isolation of transgene insertions at some chromosomal locations. Detailed plasmid maps are available on request.

Flp-mediated excision and analysis of βgal expression

Excision of the PRE either in the germ line or during larval development was done by introducing a hs-flp transgene into the strain carrying the reporter gene and heat-shocking larvae for 1 hour at 37°C in a water bath.

X-gal stainings were performed as described (Christen and Bienz, 1994).

Results and Discussion

PREs act as general silencer elements

We tested a 1.6 kb fragment encompassing the PRE from the *Ubx* upstream control region (Chan et al., 1994; Fritsch et al., 1999) for its capacity to prevent transcriptional activation by enhancers from genes that are normally not under PcG control. For this purpose, three different enhancers were tested in a *lacZ* reporter gene assay in imaginal discs: dpp^{WE}, the imaginal disc enhancer from the *decapentaplegic* (*dpp*) gene (Müller and Basler, 2000); vg^{QE} the quadrant enhancer from the *vestigial* (*vg*) gene (Kim et al., 1996); and vg^{BE}, the *vg* D/V boundary enhancer (Williams et al., 1994). If linked to a reporter gene, each of these enhancers directs a distinct pattern of expression in the wing imaginal disc and activation by each enhancer is regulated by transcription factors that are controlled by a different signaling pathway. Specifically, the *dpp* enhancer contains binding sites for the Ci protein and is activated in response to *hedgehog* signaling (Müller and Basler, 2000), the *vg* quadrant enhancer contains binding sites for the Mad transcriptional regulator and is activated in response to *dpp* signaling (Kim et al., 1997), and the *vg* boundary enhancer contains binding sites for the Su(H) transcription factor and is regulated by *Notch* signaling (Kim et al., 1996). Here, we inserted the dpp^{WE}, vg^{QE} and vg^{BE} enhancers individually into a *lacZ* reporter gene construct that contains the *PRE* fragment and either a TATA box minimal promoter from the *hsp70* gene (here referred to as *TATA*), or a 4.1 kb fragment of the proximal *Ubx* promoter (here referred to as Ubx_P), fused to *lacZ*. The structure of these six constructs is shown in Figs 1, 2. In each construct, the *PRE* fragment is flanked by FRT sites that permit excision of the *PRE* fragment by flp recombinase. We first generated several independent transgenic lines for each of the six *PRE* transgenes. From individual transgene insertions, we then generated derivative transgenic lines by flp-mediated excision of the *PRE* in the germline (Figs 1, 2). We could thus compare expression of individual transgene insertions in the presence and absence of the *PRE* by staining wing imaginal discs for β-galactosidase (β-gal) activity. In the absence of the *PRE*, each of the three enhancers tested directs β-gal expression in the characteristic pattern previously reported (Figs 1, 2) (Williams et al., 1994; Kim et al., 1996; Müller and Basler, 2000). We find that each enhancer activates expression in the same pattern from either the TATA box minimal

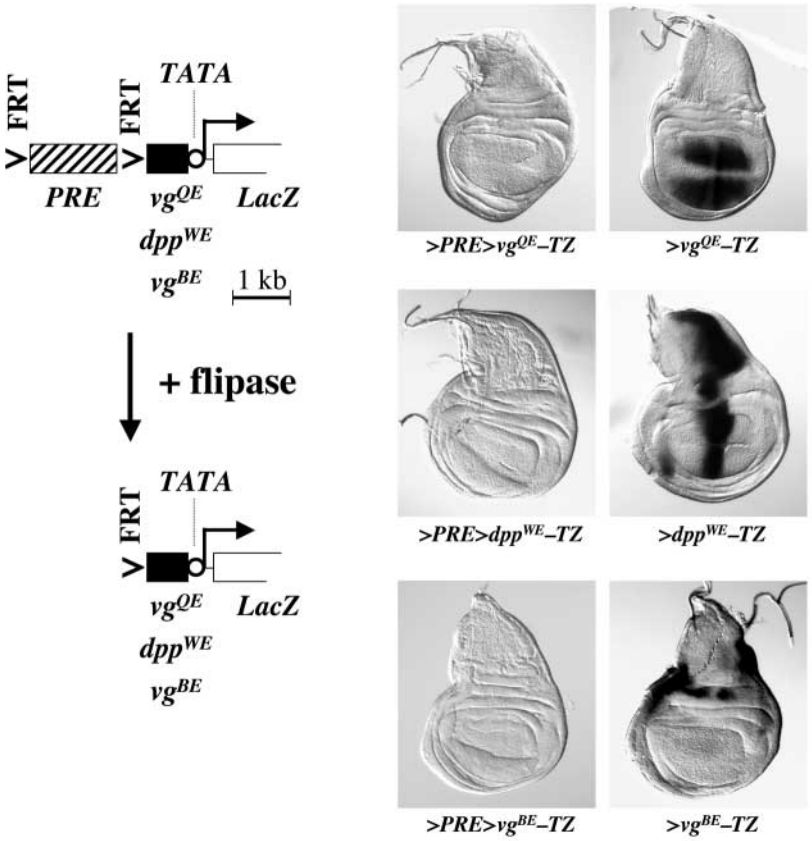

Fig. 1. The *Ubx PRE* indiscriminately silences reporter genes that contain an enhancer and a TATA box minimal promoter. (Left) Schematic drawing of the reporter constructs that contain the 1.6 kb *Ubx PRE* fragment (hatched rectangle) flanked by FRT sequences upstream of either the vg^{QE}, vg^{BE} or dpp^{WE} enhancer (black rectangle) linked to hsp70 TATA box minimal promoter (white circle) followed by hsp70 5′ UTR (thin line) and *lacZ* gene (white box). A transgene derivative that lacks the *PRE* (below) can be created by flp-mediated excision of the *PRE* fragment. (Right) X-gal staining of wing imaginal discs from transformant lines carrying the indicated reporter gene construct with (left column) and without (right column) the *PRE* fragment. In each case, the disc on the left is from the original transformant line and the disc on the right is from its derivative line, obtained after excision of the *PRE* in the germline. In the absence of the *PRE* fragment, the vg^{QE}, dpp^{WE} and vg^{BE} enhancer each direct *lacZ* expression in the pattern previously described (Williams et al., 1994; Kim et al., 1996; Müller and Basler, 2000). The pattern directed by the vg^{BE} enhancer depends on its relative orientation to the TATA box promoter; in this orientation it activates expression primarily in the hinge and notum region (Williams et al., 1994). Note that in the presence of the *PRE* fragment, all three reporter genes are completely silenced.

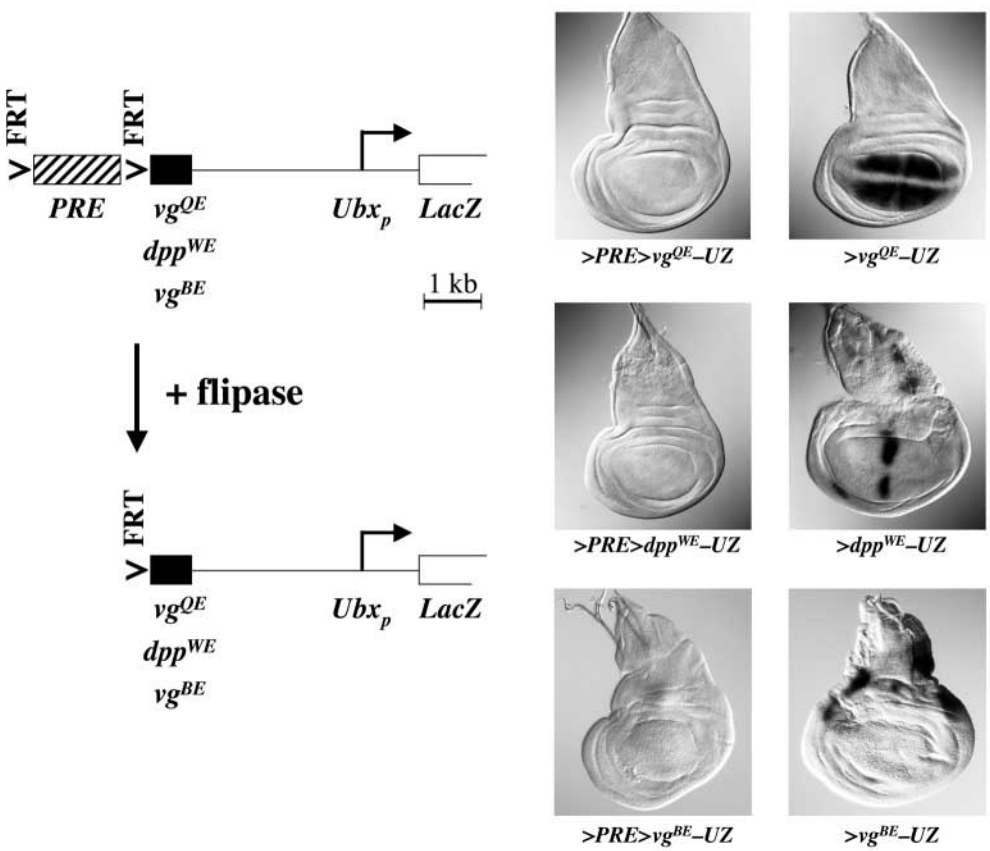

Fig. 2. The *Ubx PRE* indiscriminately silences reporter genes that contain an enhancer and the *Ubx* proximal promoter. (Left) The reporter genes are very similar to those shown in Fig. 1 but the TATA box minimal promoter is replaced by a 4.1 kb fragment that contains the proximal *Ubx* promoter and 5′ UTR (thin line, transcription start site marked by arrow). (Right) X-gal staining of wing imaginal discs from transformant lines carrying the indicated reporter gene construct with (left column) and without (right column) the *PRE* fragment; in each case, the disc on the left is from the original transformant line and the disc on the right is from its derivative line, obtained after excision of the *PRE* in the germline. Note that in the presence of the *PRE* fragment all three reporter genes are completely silenced. In the derivative lines lacking *PRE*, the intensity of X-gal staining suggests that the vg^{QE} enhancer directs higher expression levels from the *Ubx* promoter than from the TATA box promoter; conversely, the dpp^{WE} enhancer activates expression more potently from the TATA box promoter than from the *Ubx* promoter (compare with Fig. 1).

promoter or the *Ubx* promoter with some minor, promoter-specific differences with respect to the expression levels (Figs 1, 2). By contrast, in most of the parental transformant lines, i.e. those carrying the corresponding reporter gene with the PRE, β-gal expression is completely suppressed. These observations suggest that the *PRE* fragment very potently silences each of the six reporter genes (Figs 1, 2). We note, however, that, at some transgene insertion sites, efficiency of silencing by the *PRE* fragment appears to be impeded by flanking chromosomal sequences; in these cases, we find that β-gal expression is activated even in the presence of the *PRE*. The extent to which individual transgene insertions are silenced is summarized in Table 1.

Silencing by the PRE requires PcG gene function

To test whether silencing of our reporter genes by the *PRE* depends on PcG gene function, we introduced the *PRE*-containing transgenes *>PRE>dpp^{WE}-TATA-lacZ* and *>PRE>vg^{QE}-Ubx-lacZ* into larvae that carry mutations in the PcG gene *Suppressor of zeste 12* [*Su(z)12*] (Birve et al., 2001). *Su(z)12* encodes a core component of the Esc-E(z) histone methyltransferase (Czermin et al., 2002; Müller et al., 2002). We find that silencing of both transgenes is lost in *Su(z)12^{2}/Su(z)12^{3}* mutant larvae, and the transgenes express β-gal expression at levels comparable with the transgene derivatives that lack the *PRE* fragment (Fig. 3). Taken together with the results described above, these observations suggest that the 1.6 kb *PRE* fragment from *Ubx* is a very potent general transcriptional silencer element that represses transcription in a PcG protein-dependent manner. Thus, it appears that this PRE acts indiscriminately to block transcriptional activation by a variety of different activator proteins.

Long-term silencing requires the continuous presence of the PRE

To test the long-term requirement for the PRE for silencing of these reporter genes, we excised the PRE during larval development and we then monitored β-gal expression at different time points after excision. Forty-eight hours after induction of flp expression, all six reporter genes show robust derepression of β-gal, suggesting that, in each case, removal of the PRE resulted in the loss of PcG silencing (Fig. 4 and data not shown). Among the different enhancer-promoter combinations used in this study, the *dpp^{W}* enhancer fused to

Table 1. Silencing of β-galactosidase expression in wing imaginal discs of transformant lines

		β-galactosidase expression		
Construct	Transformant number	*+PRE*	*–PRE*	Silencing
>PRE> vg^{QE}-TZ	10a	None*	*vg^{Q}* pattern	Yes
	18a	PE	PE + *vg^{Q}* pattern	Yes
>PRE> vg^{QE}-UZ	6.1	None	*vg^{Q}* pattern	Yes
	14.2	PE	PE + *vg^{Q}* pattern	Yes
	17.1	None	*vg^{Q}* pattern	Yes
	18.2	PE	PE + *vg^{Q}* pattern	Yes
	41.1	PE + *vg^{B}* pattern	PE + *vg^{B}* pattern	No
	42.1	None	*vg^{Q}* pattern	Yes
	44.1	None	PE	–
>PRE> dpp^{WE}-TZ	1a	PE + *dpp^{W}* pattern	PE + *dpp^{W}* pattern	No
	1b	PE	PE	–
	7a	None	*dpp^{W}* pattern	Yes
	17a	None	*dpp^{W}* pattern	Yes
	22a	None	*dpp^{W}* pattern	Yes
	30b	None	*dpp^{W}* pattern	Yes
	36a	None	*dpp^{W}* pattern	Yes
>PRE> dpp^{WE}-UZ	17.1	None	*dpp^{W}* pattern	Yes
	17.2	PE	n.d.	–
>PRE> vg^{BE}-TZ	8a	*vg^{B}* pattern	*vg^{B}* pattern	No
	8b	None	*vg^{B}* pattern	Yes
	20a	None	*vg^{B}* pattern	Yes
	21a	Weak *vg^{B}* pattern	*vg^{B}* pattern	(Yes)
	26a	PE	PE + *vg^{B}* pattern	Yes
	45a6a	None	*vg^{B}* pattern	Yes
	46a8a	*vg^{B}* pattern	*vg^{B}* pattern	No
	47a	None	*vg^{B}* pattern	Yes
	50a	PE	PE + *vg^{B}* pattern	Yes
>PRE> vg^{BE}-UZ	8.1	PE	PE	–
	12.1	None	*vg^{B}* pattern	Yes
	13.1	None	*vg^{B}* pattern	Yes
	19.1	None	*vg^{B}* pattern	Yes
	20.1	None	None	–

Transformant number indicates the number of the specific transformant line listed below; *+PRE* indicates the original transformant line, whereas *–PRE* indicates the same transformant line after excision of the PRE either in the germline or during larval development. None indicates no β-gal expression is detected in any of the discs because of complete silencing by the PRE,

*Incomplete silencing in the presence of the PRE. In these cases, a fraction of discs shows small patches of β-gal-expressing cells within the pattern that is observed after excision of the PRE.

PE (position effect) indicates a specific β-gal pattern that is distinct from the pattern directed by the enhancer in the construct and is most probably activated by sequences flanking the transgene insertion site. Yes and No indicate whether transcriptional activation by the enhancer is silenced in the presence of the PRE; – indicates cases in which the enhancer-activated pattern was not detected even after excision of the PRE.

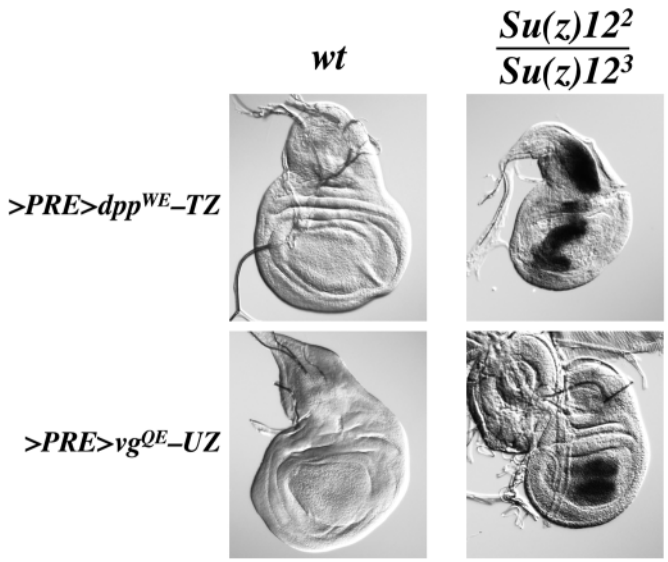

Fig. 3. Silencing by *PRE* depends on PcG gene function. X-gal stainings of wing imaginal discs from *wt* and *Su(z)12* mutant larvae carrying the indicated reporter genes. Note that silencing of both reporter genes is lost in $Su(z)12^{2}/Su(z)12^{3}$ transheterozygous larvae. The *Su(z)12* mutant wing discs are smaller than *wt* wing discs, presumably as they are partially transformed into haltere discs due to misexpression of Hox genes (see Birve et al., 2001).

the TATA box minimal promoter appears to direct the highest levels of *lacZ* expression; *>PRE>dpp^{W}–TZ* transformant lines consistently show the strongest β-gal staining after excision of the PRE (see Figs 1, 2). We therefore analyzed *>PRE>dpp^{W}-TZ* transformants at 4, 8, 12 and 24 hours after induction of flp expression to study the kinetics of this derepression. We did not detect β-gal signal at 4 hours or even at 8 hours after flp induction, but 12 hours after flp induction, all discs show robust β-gal expression (Fig. 4). Thus, even in the case of the most potent enhancer-promoter combination used here (i.e. *dpp^{W}* enhancer and TATA box minimal promoter), we observe a delay of 12 hours between flp induction and β-gal expression. As the average cell cycle length of imaginal disc cells in third instar larvae is 12 hours (Neufeld et al., 1998), this implies that most disc cells have undergone a full division cycle within this period. Derepression of the reporter gene in this experiment requires several steps: (1) excision of the PRE by the flp recombinase; (2) dissociation of the PRE and PcG proteins attached to it – possibly by disrupting PcG protein complexes formed between the PRE and factors bound at the promoter (Breiling et al., 2001; Saurin et al., 2001); and (3) transcriptional activation by factors binding to the enhancer in the construct. It is possible that one or several steps in this process require a specific process during the cell cycle (e.g. passage through S phase).

Finally, we note that removal of the PRE from our transgenes results in the loss of silencing in all imaginal discs within 12 hours of induction of flp expression. This finding provides an interesting contrast to similar PRE excision experiments reported in an earlier study (Busturia et al., 1997). Busturia et al. (Busturia et al., 1997) used a reporter gene that contained the *MCP* PRE from the Hox gene *Abd-B*, the potent imaginal disc enhancer *PBX* from the *Ubx* gene and the *Ubx* promoter, fused to lacZ. The authors reported that excision of the *MCP* PRE results in derepression of the reporter gene but they found that only about 20% of the discs show derepression, if discs are analyzed 24 hours after flp induction (Busturia et al., 1997). Derepression in 100% of the discs was only observed if discs

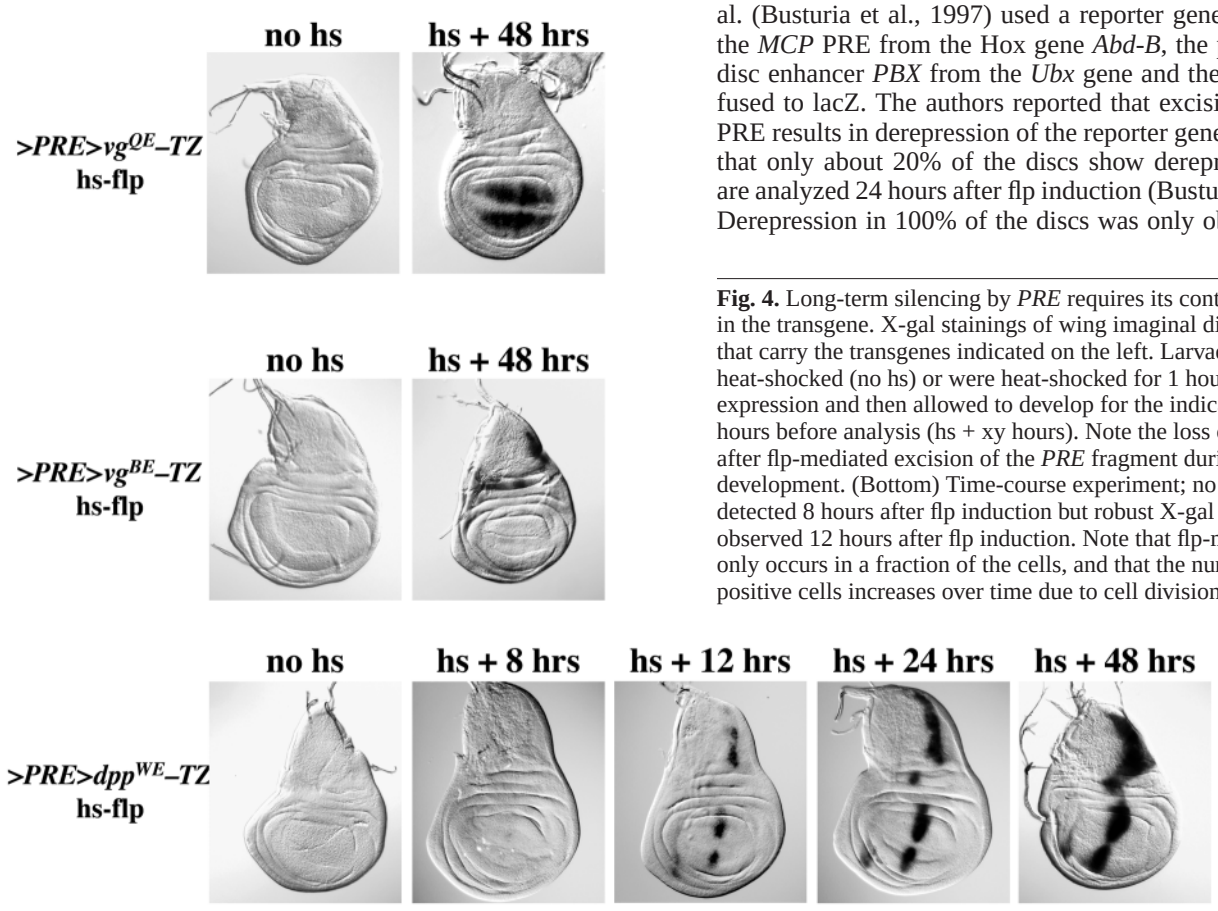

Fig. 4. Long-term silencing by *PRE* requires its continuous presence in the transgene. X-gal stainings of wing imaginal discs from larvae that carry the transgenes indicated on the left. Larvae were either not heat-shocked (no hs) or were heat-shocked for 1 hour to induce flp expression and then allowed to develop for the indicated number of hours before analysis (hs + xy hours). Note the loss of expression after flp-mediated excision of the *PRE* fragment during larval development. (Bottom) Time-course experiment; no X-gal signal is detected 8 hours after flp induction but robust X-gal staining is observed 12 hours after flp induction. Note that flp-mediated excision only occurs in a fraction of the cells, and that the number of X-gal positive cells increases over time due to cell division.

were analyzed 72 or more hours after flp induction (Busturia et al., 1997). Thus, in the construct from Busturia et al. (Busturia et al., 1997), the release from silencing after PRE excision occurs with a considerably longer delay than in our constructs, suggesting that PcG silencing can be partially maintained for a few cell generations after removal of the *MCP* PRE. One possible explanation for this longer maintenance of silencing after PRE excision could be the presence of a weak PRE in the *PBX* imaginal disc enhancer (Christen and Bienz, 1994). It is possible that such weak or cryptic PREs help PcG proteins to maintain silencing imposed by a strong PRE and that they thus contribute to the stability of the silenced state within Hox genes (Christen and Bienz, 1994; Müller, 1995; Pirrotta, 1998). We imagine that the constructs used in our study here lack such cryptic PREs and that excision of the PRE thus directly eliminates PcG silencing.

Concluding remarks

Our experiments here show that three reporter genes, each containing a different enhancer linked to a canonical TATA box promoter, are completely silenced by a PRE placed upstream of the enhancer. Our data suggest that PcG proteins that act through this PRE prevent indiscriminately activation by a variety of different transcription factors. The PcG machinery thus does not seem to require any specific enhancer and/or promoter sequences for repression.

Two points deserve to be discussed in more detail. The first concerns the stability of silencing imposed by a PRE. Previous studies suggested that transcriptional activation in the early embryo could prevent the establishment of PcG silencing by PREs (Müller and Bienz, 1991; Poux et al., 1996). More specifically, early transcriptional activation of Hox genes by blastoderm enhancers may play an important role in preventing the establishment of permanent PcG silencing in segment primordia in which Hox genes need to be expressed at later developmental stages (Poux et al., 1996). Importantly, none of the three enhancers used in this study is active in the early embryo. Moreover, these enhancers probably do not contain binding sites for specific transcriptional repressors, such as the gap repressors, which are required for establishment of PcG silencing at some PREs in the early embryo (Zhang and Bienz, 1992). We therefore imagine that, in our constructs, PcG silencing complexes assemble by default on the 1.6 kb *Ubx* PRE in the early embryo and that PcG silencing is thus firmly established by the stage when the imaginal discs enhancers would become active. Silencing by the PRE during larval stages therefore appears to be dominant overactivation and cannot be overcome by any of the enhancers used here. There is other evidence in support of the idea that PcG silencing during larval development is more stable than in embryos. In particular, a PRE reporter gene that contains a Gal4-inducible promoter is only transiently activated if a pulse of the transcriptional activator Gal4 is supplied during larval development; by contrast, a pulse of Gal4 during embryogenesis switches the PRE into an ‘active mode’ that supports transcriptional activation throughout development (Cavalli and Paro, 1998; Cavalli and Paro, 1999). Furthermore, recent studies in imaginal discs suggest that there is a distinction between transcriptional repression and the inheritance of the silenced state; the silenced state can be propagated for some period even if repression is lost (Beuchle et al., 2001). Specifically, loss of Hox gene silencing after removal of PcG proteins in proliferating cells can be reversed if the depleted PcG protein is resupplied within a few cell generations (Beuchle et al., 2001). Taken together, it thus appears that PcG silencing during postembryonic development is a remarkably stable process. Finally, the results reported in this study also imply that, once PcG silencing is established, Hox genes can ‘make use’ of virtually any type of transcriptional activator to maintain their expression; PcG silencing will ensure that activation by these factors only occurs in cells in which the Hox gene should be active. The analysis of *Ubx* control sequences supports this view; if individually linked to a reporter gene, most late-acting enhancers direct expression both within as well as outside of the normal *Ubx* expression domain (Müller and Bienz, 1991; Castelli-Gair et al., 1992).

The second point to discuss here concerns the repression mechanism used by PcG proteins. Biochemical purification of PRC1 revealed that several TFIID components co-purify with the PcG proteins that constitute the core of PRC1 (Saurin et al., 2001; Francis et al., 2001). Moreover, formaldehyde crosslinking experiments in tissue culture cells showed that TFIID components are associated with promoters, even if these are repressed by PcG proteins (Breiling et al., 2001). This suggests that PcG protein complexes anchored at the PRE interact with general transcription factors bound at the promoter. One possibility would be that PcG repressors directly target components of the general transcription machinery to prevent transcriptional activation by enhancer-binding factors. As mentioned above, three distinct activators act through the three enhancers used here (Kim et al., 1996; Kim et al., 1997; Müller and Basler, 2000) and, according to our results, none of them is able to overcome the block imposed by the PcG machinery. But how do the known activities of PcG protein complexes [i.e. histone methylation by the Esc-E(z) complex and inhibition of chromatin remodeling by PRC1] fit into this scenario? Both these activities may be required for the repression process by altering the structure of chromatin around the transcription start site and thus preventing the formation of productive RNA Pol II complexes. Other scenarios are possible. For example, histone methylation may primarily serve to mark the chromatin for binding of PRC1 through Pc (Fischle et al., 2003; Min et al., 2003), and PRC1 components such as Psc then perform the actual repression process (Beuchle et al., 2001; Francis et al., 2001). Whatever the exact repression mechanism may be, our PRE-excision experiment shows that this repression is lost within one cell generation after removal of the PRE. This implies that changes in the chromatin generated by the action of PcG proteins cannot be propagated by the flanking chromatin.

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