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Hunchback-independent silencing of late *Ubx* enhancers by a Polycomb Group Response Element

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Drosophila **homeotic genes are kept silent outside of their appropriate expression domains by a repressive chromatin complex formed by the Polycomb Group proteins. In the case of the *Ubx* gene, it has been proposed that the early repressor HB, binding at enhancers, recruits the Polycomb complex and specifies the domain of repression. We show that some *Ubx* enhancers are activated after blastoderm. If a Polycomb Response Element (PRE) is combined with such late enhancers, repression of a reporter gene can be established everywhere in the embryo, irrespective of the presence or absence of hunchback protein. If, however, these late enhancers are combined with a *Ubx* early enhancer, as well as a PRE, repression is established only where the reporter gene was inactive at early stages. These results imply that the Polycomb complex is not dependent on hunchback and suggest that the pattern of silencing reflects rather the state of activity of the gene at the time the Polycomb complex is formed.**

Keywords: chromatin silencing/Polycomb response elements/*Ultrabithorax*/*wingless*

Introduction

The expression of the *Drosophila Ubx* gene depends on a number of enhancer modules scattered over a region which includes some 35 kb upstream and ~40 kb downstream of the promoter (Simon *et al.*, 1990; Müller and Bienz, .1991; Qian *et al.*, 1991; Pirrotta *et al.*, 1995). The activity of most of these embryonic enhancers begins at blastoderm and depends primarily on segmentation genes. Pair-rule gene products activate expression in a metameric pattern while the products of gap genes *hb* and *tll* function as repressors to limit the domain of *Ubx* activity to the region between PS5 and 13. During germ band extension these gene products begin to disappear from their segmental domains: first the repressors HB and TLL fade away, and shortly thereafter the activators themselves disappear in their pair-rule domains. However, two of the embryonic enhancers, ABX and 2218R6, are active in the nervous system and this activity persists in late embryonic development. Expression in imaginal tissues is not due to an extended activity of the embryonic enhancers but to an independent set of imaginal enhancers that are activated later. There are apparently two clusters of imaginal enhancers, one in the ABX region (Simon *et al.*, 1990; Christen and Bienz, 1994) and one in the upstream region between the PBX enhancer and the PRE (Christen and Bienz, 1994; Pirrotta *et al.*, 1995). When these enhancers are activated in the later embryo, the positional cues in the form of HB product in the anterior half and TLL product at the posterior end of the embryo are no longer present to set the limits of the expression domain. When tested in isolation, in a reporter gene construct, these enhancers in fact are expressed in imaginal discs without regard to parasegmental origin. The continued repression of the endogenous *Ubx* gene in the anterior- and posterior-most regions is due to the establishment of a repressive complex formed by the products of the Polycomb Group of genes (PcG) (Simon *et al.*, 1992). Target sites for the formation of this complex are Polycomb Response Elements (PREs), of which the principal one in the *Ubx* gene is located in the upstream region (Chan *et al.*, 1994) though weaker sites can be found elsewhere along the gene (Müller and Bienz, 1991; Chiang *et al.*, 1995). We know little about the molecular nature of this complex but the available evidence indicates that it initiates at PREs and spreads to involve a large region of chromatin, in the case of *Ubx*, spanning >100 kb (Orlando and Paro, 1993; Pirrotta, 1996). The essential feature of this complex is that, although the PcG products are present throughout the embryo, the PcG complex must form or at least function to repress the *Ubx* gene only in those regions in which the gene was initially repressed by the gap genes and it must allow expression in the regions where the gene was initially active. Furthermore, once established in a cell, the repressive complex is maintained in the cellular progeny.

What concerns us here is how the PcG products discriminate between the cells in which the complex should be established and those in which it is not. It has been proposed that the gap gene repressors bound to *Ubx* enhancers in the early embryo help to recruit PcG proteins and direct the assembly of the repressive complex (Zhang and Bienz, 1992). This is an attractive mechanism which resembles the recruitment of the silencing complex in yeast by the RAP1 protein bound to silencer target sequences (Hecht *et al.*, 1995). A major difficulty with this hypothesis is that constructs containing PREs, but no detectable HB binding sites, can also establish repression. Thus, a PcG target element from the *Abd-B* gene can repress a GAL4-activated promoter (Zink and Paro, 1995) and many PREs can partially or totally silence the expression of the mini-*white* gene, resulting in a variegated or even totally unpigmented eye in the absence of HB binding sites (Fauvarque and Dura, 1993; Chan *et al.*, 1994; Kassis, 1994; Gindhart and Kaufman, 1995). An alternative hypothesis is that the assembly of a stable PcG repressive complex can sense the state of activity of a gene, possibly

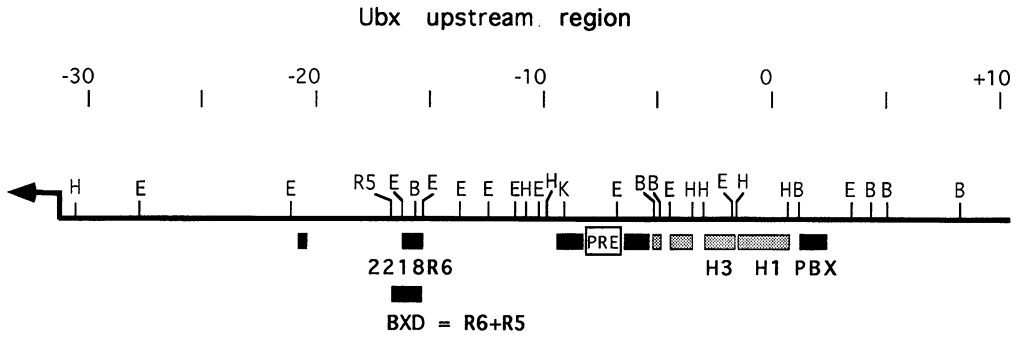

Fig. 1. Map of the *Ubx* upstream region. The position of the *Ubx* promoter is indicated by the angled arrow to the left. The parasegmental enhancer elements are shown below the line as black boxes, two of which are identified as the 2218R6 and PBX enhancers. The stippled boxes indicate the imaginal enhancers associated with the *pbx* region, two of which are identified as the 2212H3 and 2212H1 enhancers. The box labeled PRE represents the principal *Ubx* PRE. The scale is in kb, numbered according to the map of Bender *et al.* (1983). Restriction enzyme sites are shown for reference: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; R5, *Eco*RV.

because it requires a folding of the chromatin that is incompatible with the chromatin configuration created by the activation of a promoter (Chan *et al.*, 1994; Pirrotta and Rastelli, 1994). In fact, high concentrations of an activator have been shown to antagonize the PcG complex (Zink and Paro, 1995).

It is not known when the repressive complex becomes functional. Many of the PcG products are maternally supplied and at least some of them are involved in very early functions in the pre-blastoderm embryo (Jones and Gelbart, 1990; Pelegri and Lehmann, 1994). PcG repression of homeotic genes must be effective at least by the end of germ band extension, when the effects of PcG mutations first become apparent. Some evidence suggests that the repressive complex is established shortly after blastoderm. For example, the variegation that the *Ubx* PRE induces in the expression of the mini-*white* gene is often in the form of clonal patches (Chan *et al.*, 1994), suggesting that repression was established at a time when the primordia of the eye disc already consisted of several cells. It is likely therefore that the repressive complex becomes functional at this target site at some point between blastoderm and the end of germ band extension. The patterns of variegation observed suggest that repression depends on a cooperative, all-or-nothing mechanism and that it may be unstable, at least in certain chromosomal sites, resulting in a salt-and-pepper pattern of variegation when individual cells become derepressed during later development.

In this paper we examine three *Ubx* enhancer elements that, unlike the early enhancers, are activated after the blastoderm stage. One is an embryonic enhancer element first studied by Müller and Bienz (1991) who called it BXD. This element was also described by Pirrotta *et al.* (1995) who called it 2218R6 to distinguish it from three other early enhancer elements found in the *bxd* region (see map in Figure 1). We show that the expression of this enhancer begins after gastrulation and is dependent on *wingless* (*wg*), which helps explain why it is activated later than the other parasegmental enhancers. The other two enhancer elements studied are activated only in the later embryo and direct expression in the imaginal discs. The interaction of each of these three enhancers with a PRE present in the same reporter construct shows that, in these cases, repression can be established everywhere, without regard to the domains of *Ubx* or *hb* expression in the embryo. When these late-acting enhancers are combined with PBX, a typical early enhancer, they are silenced by the PRE only in the region anterior to PS6, where the PBX enhancer is initially repressed by HB. These results strongly favor the interpretation that the state of activity of the reporter gene at an early stage is a determining factor for the establishment of the PcG repressive complex, at least in the case of the *Ubx* PRE.

Results

The 2218R6 enhancer depends on wg

While all the other parasegmental enhancers so far identified are activated at syncytial blastoderm, the activity of the 2218R6 enhancer is not detectable until germ band extension is well under way (Pirrotta *et al.*, 1995). Expression of a 2218R6–*Ubx–lacZ* construct becomes visible in stage 9 embryos as a series of thin bands in PS6, 10, 11, 12 and 13 (Figure 2). Shortly afterwards, staining appears in PS 7, 8 and 9 and, at first very faintly, in the thoracic parasegments PS5, 4 and 3. The timing of appearance and the position of the bands, just in front of the posterior border of each parasegment, in front of each *engrailed* band, do not resemble those of pair-rule genes but rather those of the segment polarity gene *wingless*. Mutations in pair-rule genes *eve* or *ftz* cause little alteration in the pattern of 2218R6 other than that expected in the absence of alternate segments. Mutations in *engrailed* also have only slight and probably indirect effects on the expression pattern. In contrast, all detectable expression is abolished in embryos carrying a temperature-sensitive allele of *wg* and raised at the non-permissive temperature, while the pattern is severely disrupted if the embryos are transferred at the higher temperature after blastoderm (Figure 2G and H). The 2218R6 enhancer is unusual also in directing expression in the central nervous system (CNS) at later embryonic stages. In the *wg* embryos, this expression is also absent whether the embryos were continuously raised at the non-permissive temperature or transferred to it during germ band extension. The activity of 2218R6 is completely independent of the endogenous *Ubx* product since no alteration in the early or late expression patterns were observed in homozygous Ubx^{-} embryos.

A weak PcG response element

To determine which sequences are required for the expression in the epidermis and in the CNS, the 2218R6 enhancer

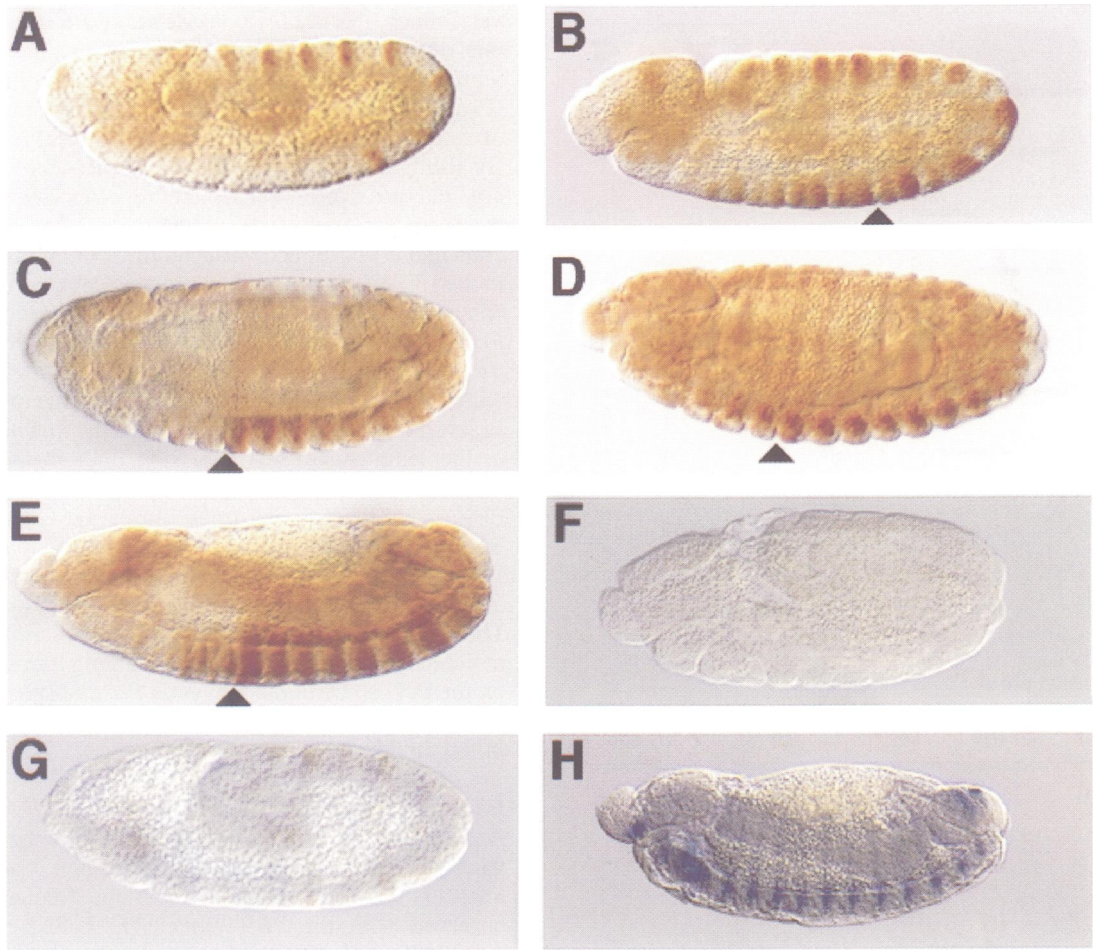

Fig. 2. Expression patterns of 2218R6. Line containing 2218R6, showing: (**A**) the early pattern repressed anterior of PS6; (**B**) at the end of germ band extension, showing expression in the epidermis of thoracic parasegments; (**C**) same line after germ band retraction, showing that expression in the CNS is repressed anterior of PS6; (**D**) the same line, crossed into a Pc^- background, is now derepressed in the thoracic region of the CNS; (**E**) line containing 2218R6 that is poorly repressed in the CNS; (**F**) line containing 2218R6 and a *hsp70-hb* transposon, heat-shocked for 30 min after 4 h of development, showing complete repression of 2218R6. (**G** and **H**) Embryos carrying 2218R6 crossed into a wg^{ts} background, raised at 25°C (G) or transferred to 25°C after 4 h of development (H). In the last two cases, the embryos were also stained with anti-invected antibody to confirm the effect of the *wg* mutation on *en* expression. The arrowhead indicates the position of PS6.

fragment of 0.8 kb was subdivided (Figure 3). First, cleavage with *Bam*HI produces two fragments of 0.3 kb and 0.5 kb, neither of which has any activity above the basal promoter pattern. This suggests that the cleavage separates elements of the enhancer that by themselves are either too weak or inactive. If the 0.8 kb fragment is trimmed at both ends with *Cla*I and *Hin*cII, the resulting 432 bp fragment retains most, if not all, the activity of the enhancer both in the epidermis and in the CNS. The two activities are therefore very closely linked and the fact that both are dependent on *wg* suggests that they may be due to the same activators. Consistent with the early repression anterior of PS6, the 2218R6 enhancer contains weak HB binding sites which were mapped by band-shifting experiments to the two central intervals: the *Hin*cII–*Bam*HI 213 bp fragment and the *Bam*HI–*Cla*I 219 fragment (Figure 4). Furthermore, ectopic expression of HB from a *hsp70-hb* transposon construct inhibits completely the expression of the 2218R6 reporter construct, even when HB expression is induced at the time of gastrulation (Figure 2F).

Expression of 2218R6 appears in the thoracic segments at the end of germ band extension. In several lines, however, thoracic expression is limited to the epidermis and remains partly or completely repressed in the CNS anterior of PS6. This effect is visible both in the 2218R6 lines and in those carrying the *Hin*cII–*Cla*I fragment. To determine whether this could represent a degree of involvement of the PcG silencing system, we tested these lines in a Pc^- background. In crosses between parents heterozygous for the Pc^3 mutation, one-quarter of the embryos showed a complete loss of maintenance both in the ectoderm and in the CNS expression pattern which now extends throughout the thorax (Figure 2D). These results suggest that the 2218R6 fragment contains a very weak PRE that can only establish partial repression in the thoracic segments. The stronger repression seen in the CNS could be due to the presence there of higher concentrations of some of the PcG proteins. We note for example that expression of the *Pc*, *Psc* and *ph* genes is much stronger in the developing CNS than in the epidermis (Franke *et al.*, 1992; Martin and Adler, 1993). None of the 2218R6 lines showed variegation of the mini-*white* gene carried by the transposon vector.

To see if PcG repression could be strengthened by adding flanking sequences, we also tested the larger

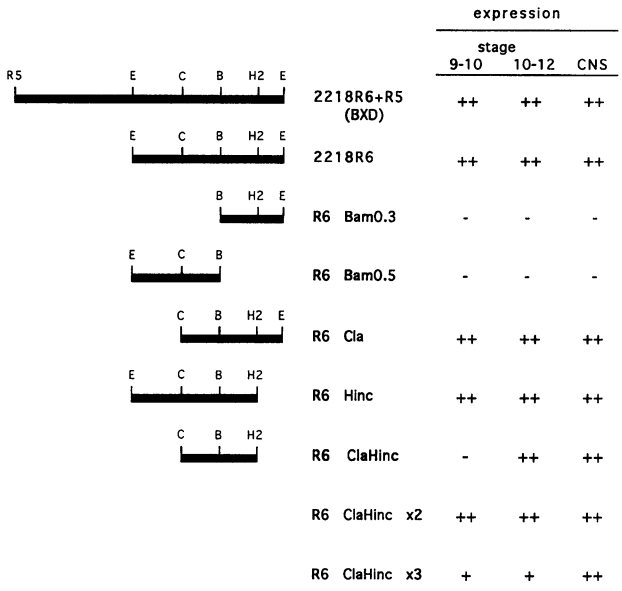

Fig. 3. 2218R6 constructs. The map of each enhancer construct is shown on the left and the level of expression in the epidermis or in the CNS is tabulated on the right. The core fragment (R6 ClaHinc) was also tested in two and three tandem copies.

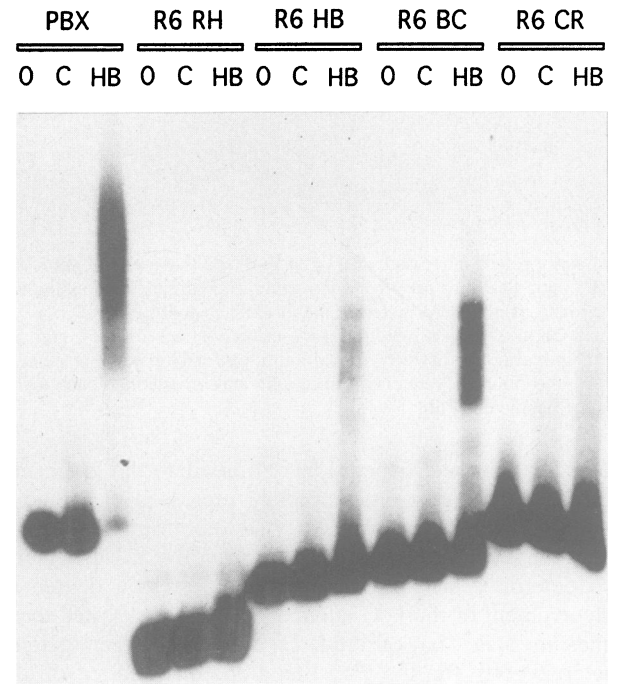

Fig. 4. HB binding of 2218R6. In these gel retardation assays, the different 2218R6 probes used are indicated above. In each case, the first lane (0) shows the probe alone, the second (C) the probe incubated with bacterial extract not containing HB, and the third lane (HB) the result of incubation with HB-containing extract. The first set on the left is a positive control using as probe a 300 bp PBX fragment containing four HB binding sites. RH, *Eco*RI–*Hin*cII fragment; HB, *Hin*cII–*Bam*HI fragment; BC, *Bam*HI–*Cla*I fragment; CR, *Cla*I–*Eco*RI fragment of the 2218R6 enhancer. Note the multiple bandshifts indicating the presence of more than one HB binding site in the two core fragments as well as in the PBX fragment.

fragment 2218R6–R5, containing an additional 700 bp *Eco*RI–*Eco*RV fragment (Figures 1 and 3). The resulting fragment is equivalent to the BXD enhancer used by Müller and Bienz (1991) and in our experiments behaves very much like 2218R6 alone in most lines. However, one out of the seven lines obtained maintained anterior repression to a considerable degree, even in the epidermis (Figure 5A and B). In this line, furthermore, the expression of the mini-*white* gene in the eye is weakly variegated. By itself, the additional 700 bp *Eco*RI–*Eco*RV fragment directed no expression of the reporter gene above the basal pattern and, even when present in three tandem copies, did not induce variegation of the mini-*white* gene in the 13 lines obtained. Transposons containing additional enhancer elements strengthen the suggestion that the *Eco*RI–*Eco*RV fragment contains sequences that can contribute to the maintenance of repression. Lines transformed with the PBX+2218R6 construct show little or no increase in anterior repression, but we found a distinct improvement in the maintenance of repression both in the epidermis and in the CNS in most of the lines transformed with the PBX+2218R6–R5 construct (Table I). One of the six lines obtained displayed a complete repression anterior of PS6 (Figure 5C and D). In this line, the mini-*white* gene is also affected, resulting in a variegated eye pigmentation. The improved degree of repression, as well as the complete maintenance displayed by the variegating line, is dependent on the PcG since in homozygous Pc^3 embryos, these lines show a complete derepression of the reporter gene both in the epidermis and in the CNS.

Effect of a strong PRE

Addition of the *Ubx* PRE to the 2218R6 fragment causes variegation of mini-*white* gene expression in ~60% of the lines (11 out of 19), about the same frequency as that observed with the PRE alone. The effect of the PRE on 2218R6 expression is, however, surprising. In the variegating lines, as well as in some of the non-variegating lines, we see a degree of repression of the R6 pattern that is highly variable from one embryo to another in extent and distribution but always unrelated to the PS6 boundary that this enhancer displays transiently (Figure 5F and G). Repression occurs in a random patchwise manner everywhere in the embryo, in extreme cases resulting in the almost complete effacement of the R6 expression pattern also in the abdominal parasegments. Even in the lines that do not display detectable variegation in eye pigmentation, the expression of the transposon in the embryo is partially repressed, with the normal pattern fading off in places. The first conclusion to be drawn from this surprising result is that the repression cannot be mediated by HB protein which is not present in the abdominal region. Addition of synthetic HB binding sites in a construct containing 2218R6+PRE+HB does not alter the pattern of ubiquitous repression, but crossing the lines into a Pc^- background restores the normal 2218R6 expression pattern. PRE-containing transposons often insert in the vicinity of chromosomal sites already containing a PRE. It might be imagined that our results are caused by insertions of the transposon in the immediate vicinity of a pre-existing PRE, in which PcG complex formation is directed by some other, HB-independent, mechanism. However, *in situ* hybridization reveals that at least some of the PRE+2218R6 transposons are inserted at chromosomal sites that do not bind Pc, Psc or Su(z)2 proteins in salivary gland chromosomes. For example, four lines that show virtually uniform repression have

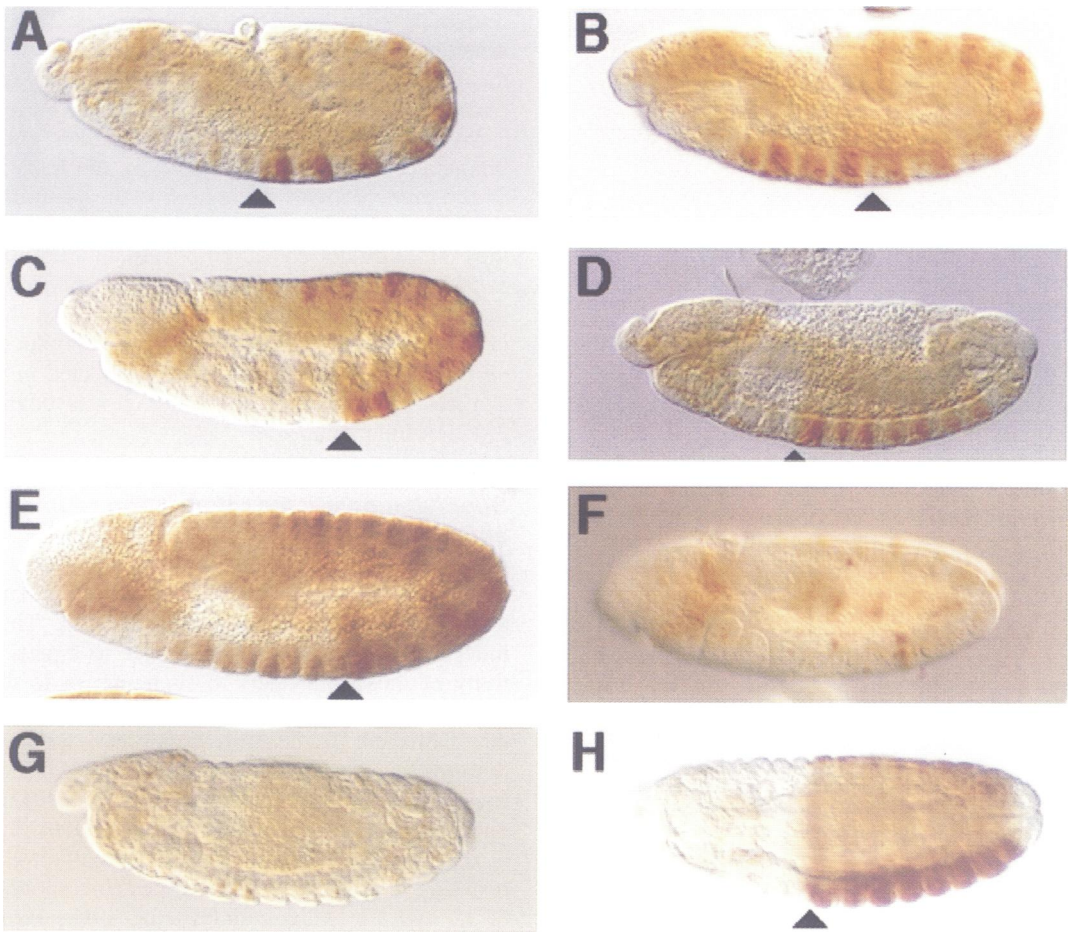

Fig. 5. PRE silencing of 2218R6. The variegating line of 2218R6–R5 shows good repression anterior of PS6 (**A**), which is lost in a Pc^{-} background (**B**). The variegating line of 2218R6–R5+PBX shows good repression anterior of PS6 in the epidermis (**C**) as well as in the CNS (**D**). Another line of 2218R6–R5+PBX shows poor repression anterior of PS6 in the epidermis (**E**). Embryos carrying 2218R6+PRE showing strong silencing with residual variegated expression (**F**) or virtually uniform repression (**G**). A transposon containing early enhancers 2212S1 and 2212S2 +PRE+2218R6 shows strong repression anterior of PS6 but continued expression in the CNS (H). The arrowhead marks the position of PS6.

insertions at 10D, 39D, 89B and 89B. Two others that are strongly repressed but with occasional patches of expression have insertions at 21F and 72D. Of these sites, only 89B is in the vicinity of a pre-existing PcG binding site on polytenic chromosomes (Rastelli *et al.*, 1993).

The ubiquitous repression observed might be interpreted as the complete shut-down of a weak enhancer by a strong PRE. However, 2218R6 does not appear particularly weaker than *Ubx* enhancers such as 2218S or 2212S1/S2, which are activated at blastoderm (Pirrotta *et al.*, 1995) and which are not indiscriminately repressed by the PRE (Chan *et al.*, 1994). Furthermore, when 2218R6 is combined with one of these early enhancers as well as the PRE, expression is silenced anteriorly but not in segments posterior to PS6 (Figure 5H). That 2218R6 is still active in the posterior segments is shown by the CNS expression, contributed by 2218R6. An alternative interpretation is that the establishment of repression depends on the state of activity of the reporter gene. In contrast to the other parasegmental enhancers, 2218R6 is not activated by pair-rule genes at blastoderm but by *wg*, during germ band extension. This delay might give the PcG complex time to establish repression and prevent activation of the enhancer.

Interaction of imaginal enhancers with the PRE

Another group of *Ubx* enhancers—the imaginal enhancers—is activated only in the late embryo. The upstream region contains at least four independent imaginal enhancer activities, each with a different pattern of expression in the discs and with a different specificity for dorsal versus ventral discs. We have looked at the activity of two of these enhancers in greater detail. The 2212H1 element is contained in a 2.3 kb *Hin*dIII fragment centered around position 0 of the *Ubx* map or 32 kb upstream from the *Ubx* promoter. It drives the expression of the reporter gene specifically in the dorsal discs: haltere, wing and, more weakly, in the eye part of the antenna disc in a virtually uniform pattern throughout these discs in most lines (Table II and Figure 6A–C). After longer staining, weak activity becomes detectable in the leg discs. A second imaginal enhancer, 2212H3, is contained in a 1.2 kb *Hin*dIII fragment adjacent to 2212H1 (Figure 1). This enhancer gives a partial coloration of wing and haltere discs, in a stripe separating the blade part from the dorsal part of the disc; in the central part of the antenna and leg discs and in the differentiated part of the eye disc. Neither of these enhancers is active in the early embryo and, when expression becomes detectable, after

Table I. Anterior repression of 2218R6 lines

Construct	Repressing lines	Anterior variegation	Eye
2218R6	1/6	++	–
	3/6	+	–
	2/6	–	–
2218R6+R5 (BXD)	1/7	+++	+
	4/7	+	–
	2/7	–	–
2218R6+PBX	3/12	++	–
	7/12	+	–
	2/12	–	–
2218R6+R5+PBX	1/6	+++	+
	4/6	++	–
	1/6	–	–

The degree of repression indicated refers to the CNS, where ++ indicates strong silencing anterior to PS6, while + indicates partial silencing in thoracic segments. In exceptional cases, complete thoracic repression was also observed in the epidermis, indicated by +++. These cases were accompanied by variegation of eye pigmentation. Smaller fragments of 2218R6 that are still active (see Figure 2) behaved as 2218R6 with respect to thoracic repression.

Table II. Imaginal disc silencing

Construct	Lines	Imaginal expression	Eye variegation
H1	12/15	uniform in dorsal discs	0
	1/15	uniform in all discs	0
	2/15	no expression	0
H3	6/7	pattern in all discs	0
	1/7	uniform in all discs	0
H1+PRE	4/7	no activity	4
	3/7	patchy H1 pattern	3
H3+PRE	3/5	no activity	3
	2/5	H3 pattern	0
H1+PRE+PBX	4/10	PS6 only	4
	3/10	PS6 + patches[a]	3
	2/10	H1 pattern	0
	1/10	no activity	0

The table indicates the constructs, the number of lines showing each pattern of expression and the lines showing eye color variegation.
[a]Patches on wing, haltere and leg discs.

germ band retraction, it is found in all anterior segments and lacks therefore anteroposterior positional information. None of the 2212H1 or the 2212H3 lines displays detectable variegation of the mini-*white* gene marker.

If these imaginal enhancers receive no direct positional information from the segmentation genes, what would be the effect of the PRE on their expression? Table II summarizes the imaginal expression patterns observed in lines carrying reporter transposons containing the 2212 H1 enhancer plus PRE or the 2212H3 fragment plus PRE. Four of the seven lines carrying 2212H1+PRE display a complete repression of the activity of this enhancer in all imaginal discs. Three of the seven show a partial repression resulting in spots of expression appearing in wing, haltere or eye discs. The size, number and position of the patches of expression within the disc varies from individual to individual in a manner typical of variegated expression. In addition, all seven lines display different degrees of variegation of mini-*white* expression. An analogous result was obtained with the 2212H3+PRE lines. Three lines of five gave complete repression of the enhancer while the remaining two lines showed little or no repression. The effect on mini-*white* gene expression was entirely parallel: the three repressed lines had variegated eyes while the two non-repressed lines were not visibly variegated.

We conclude that, like the 2218R6 enhancer, the imaginal enhancers are silenced by the PRE in all parts of the embryo, with no regard to the position along the anteroposterior axis. This implies that, by themselves, they either do not detect the positional cues present in the early embryo or they cannot maintain their memory until they are activated at later stages. Note that the silencing is once again independent of *hb* gene product. Although very weak HB binding sites were detected *in vitro* in the 2212H1 fragment, no HB binding was detected in the 2212H3 fragment.

Supplying positional information to imaginal enhancers

The effects of the PRE on the imaginal enhancers parallels that observed on the 2218R6 enhancer and suggests that they can be explained the same way. The position-independent silencing might be due to a particular sensitivity of these enhancers to silencing, or to the fact that they are activated only after the PcG repressive complex is established. To test this possibility, we constructed a reporter transposon containing the PBX enhancer in addition to the 2212H1 enhancer and the PRE. The PBX enhancer is activated at syncytial blastoderm by FTZ and is repressed by HB in the anterior half of the embryo, resulting in a pattern of four stripes corresponding to PS6, 8, 10 and 12 (Müller and Bienz, 1991; Pirrotta *et al.*, 1995). If the establishment of the silencing complex is sensitive to the state of activity of the surrounding region, we would expect silencing to occur only in compartments anterior to PS6. The 2212H1 enhancer would then be active preferentially in PS6 (the posterior half of the haltere disc) and be repressed in the wing, eye and anterior compartment of the haltere. The results, summarized in Table II, show that different lines display different degrees of repression. Of the ten lines obtained, four show total or nearly complete silencing everywhere but the posterior part of the haltere disc (Figure 6). Three lines show uniform expression in the posterior haltere and varying degrees of patchy expression in the anterior haltere and wing discs. Upon longer staining, the weaker staining of the leg discs shows a similar pattern of variegated expression. Two lines give no detectable repression and the staining of the discs resembles that observed in the absence of the PRE. One line gave no expression in any disc but we could not ascertain if this was due to a positional effect such as is occasionally seen in lines containing 2212H1 alone (see above) or to complete silencing by the PRE. The expression of the mini-*white* gene in these lines was variegated in parallel with the silencing effect on the disc expression with exception of the last line mentioned which was completely silent but showed no eye variegation. This leads us to believe that the inactivity of this line was a position effect not dependent on the PRE. We conclude that the activity of the PBX enhancer at earlier stages of development confers on 2212H1 a degree of resistance to silencing by the PRE. In contrast, a construct containing the PBX enhancer plus imaginal enhancers but

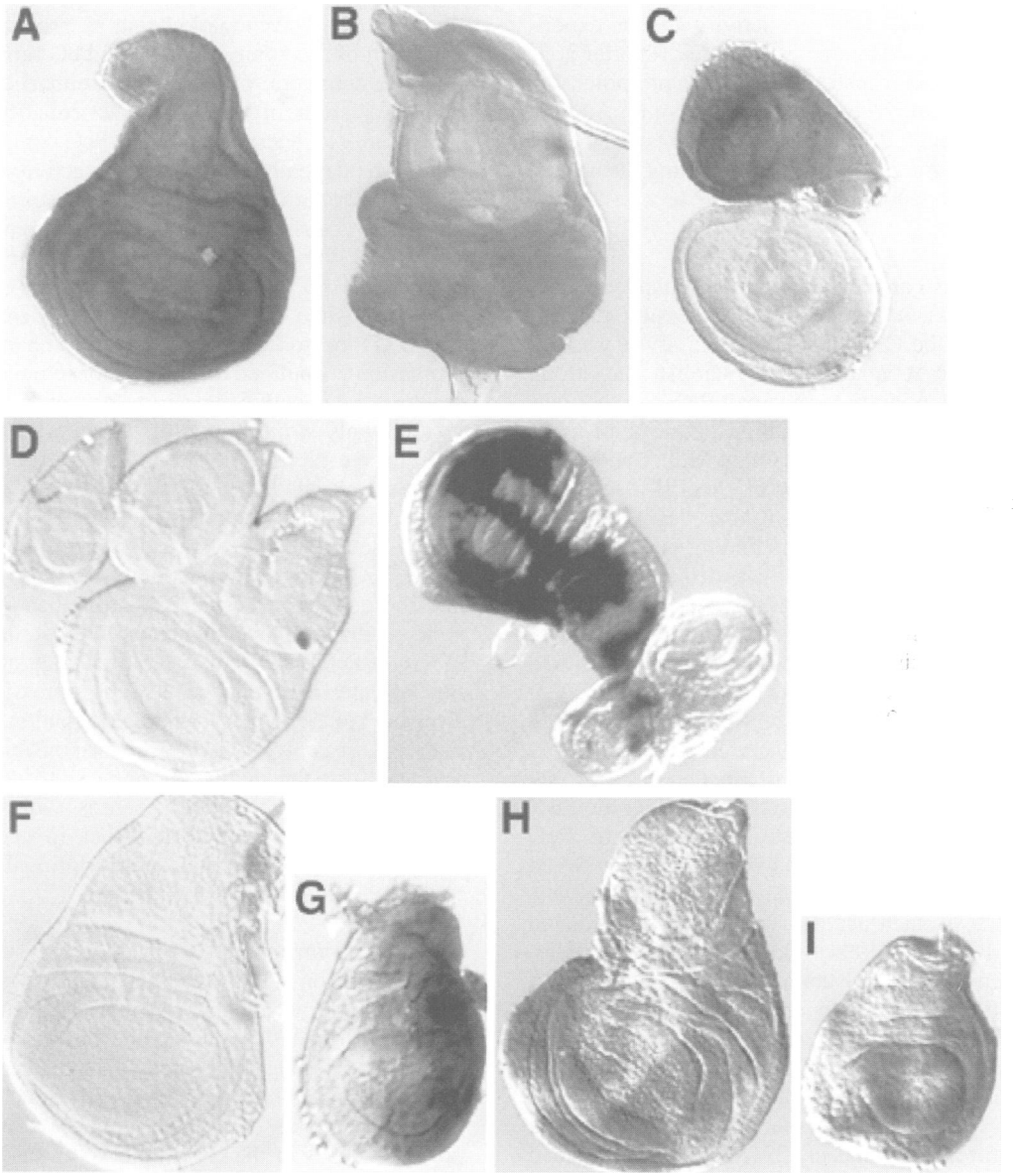

Fig. 6. PRE silencing of imaginal enhancers. The 2212H1 enhancer produces nearly uniform staining in wing and haltere discs, weaker staining in the eye disc and very weak staining in the leg discs (**A**, **B** and **C**). In some lines carrying the H1+PRE construct, expression was completely repressed everywhere (**D**). Others gave a variegated pattern of repression (**E**). In lines carrying the H1+PRE+PBX construct, expression was repressed anterior to PS6, but not in the posterior compartment of the haltere (**F** and **G**). Other lines carrying this construct gave expression in the posterior haltere and variegated repression in the wing and anterior parasegments (**H** and **I**).

no PRE gives imaginal expression in all discs, no different from that of the imaginal enhancers alone (not shown).

Discussion

Expression of the 2218R6 enhancer

The 2218R6 enhancer element (BXD) has also been studied by Müller and Bienz (1991) and by Christen and Bienz (1992) who report results somewhat different from ours. Given its late initiation and expression pattern, it was not surprising to find that 2218R6 activity is not activated by pair-rule genes but is dependent on *wg* function. The effect of *wg* mutations on endogenous *Ubx* expression is not visible during germ band extension but, at the extended germ band stage, a loss of modulation within the parasegment has been reported (Martinez-Arias *et al.*, 1988). Expression of *Ubx* in the CNS is at first apparently normal but later disappears in tracts and much of the nervous system degenerates (Perrimon and Mahowald, 1987). Because of the important effects of *wg* on the development of the parasegment and later on the differentiation of neuroblasts (Chu-Lagraff and Doe, 1993) it is difficult to say whether the effects on *Ubx* expression are direct or indirect. Furthermore, *Ubx* expression in the CNS is also due to the ABX enhancer, whose dependence on *wg* is not known and which could mask the loss of the 2218R6 enhancer activity.

The expression of 2218R6 in the CNS is apparently tightly linked with the ectodermal expression since the 432 bp *Cla*I–*Hin*cII fragment still contains both activities while cleavage at the *Bam*HI site destroys both. In contrast, Christen and Bienz (1992) find weak enhancer activities in three subfragments, the 0.7 *Eco*RI–*Eco*RV, the 0.5 *Eco*RI–*Bam*HI and the 0.3 *Bam*HI–*Eco*RI fragments

which, when separated, gave no activity in our experiments. One important difference is that their reporter gene used the *hsp70* promoter instead of the *Ubx* promoter. We have found that the *hsp70* promoter, when combined with *Ubx* enhancers or with enhancers of segmentation genes, frequently gives spurious patterns not reflecting the normal activity of the endogenous gene.

Weak PREs

Several of the lines carrying the 2218R6 enhancer show a limited but significant degree of PcG repression, particularly noticeable in the CNS. This PRE activity is weak in some respect since it is unable to establish the strong silencing observed with the principal PRE of the *Ubx* gene and never causes the variegation of the mini-*white* gene present in the transposon construct. Müller and Bienz (1991) reported that the addition of the PBX enhancer to BXD causes a significantly stronger degree of PcG repression. Our results confirm this finding but only when the 0.7 kb *Eco*RI–*Eco*RV fragment is added to the 2218R6 enhancer. We interpret this to mean that even weaker PREs are present in the PBX enhancer and in the *Eco*RI–*Eco*RV fragment, such that the combination of all three results in perceptibly better repression. The infrequent lines of 2218R6–R5 or 2218R6–R5+PBX that give strong anterior repression and *white* gene variegation are probably due to additional contributions of sequences near the transposon insertion site. Alone, however, neither the PBX nor 2218R1–R5 fragments cause any degree of PcG repression or variegation of the *white* gene even when they are present in two or three tandem copies, respectively.

The existence of such accessory PREs was postulated by Pirrotta and Rastelli (1994) to account for the stabilization and extension of PcG complexes. The presence of such a site in the 2218R6 enhancer can explain the results of Müller (1995) who found that GAL4–PC chimeric protein expressed in a short burst during early development causes only a transient repression of a reporter gene containing a GAL4 binding site but produces stable repression if the BXD enhancer (2218R6–R5) is included in the construct. We interpret this to mean that, in the presence of the weak PRE of the BXD enhancer, the complex recruited at the GAL4 binding site persists even when the recruiting GAL4–PC protein is no longer present. Additional weak response elements are very likely found elsewhere in the *Ubx* gene. One of these is situated in the *bx* region and is responsible for the weak *Pc*-dependent maintenance observed when using a large BX enhancer fragment (Qian *et al.*, 1993). The existence of this site was confirmed by Chiang *et al.* (1995), who found that it can, in some lines, give rise to a new binding site for Pc protein in polytene chromosomes. The fact that weak PRE sites are associated also with the BXD and PBX elements, indicates that there is not one PcG response site per 'domain' as suggested by Chiang *et al.* (1995) but rather a number of sites of different strength which contribute to the spread and stability of the repressed state of the entire *Ubx* region.

Determinants of PRE silencing

A critical feature of PcG repression is that it maintains the *Ubx* gene repressed in the anterior part of the embryo but allows its expression in its appropriate domain. It is this delicate discrimination that is probably responsible for much of the complexity of the PcG repression system. The late activation of the 2218R6 enhancer and the even later activation of imaginal disc enhancers offered an opportunity to determine whether PcG complex is recruited by HB or depends on the state of activity at the time of complex formation. The addition of a strong PRE to these enhancers causes the indiscriminate repression in all regions of the embryo or larva, with no regard to the domain of *hb* gene expression in the early embryo. However, when an early embryonic enhancer is added to the BXD or to the imaginal disc enhancers, the PcG repression is confined to the anterior region of the embryo, never affecting PS6 or more posterior regions. These results imply strongly that the HB repressor is not necessary to recruit the PcG complex but that some other feature, correlated with the state of activity of the reporter gene, determines whether or not repression is established. They also imply that a critical phase in the establishment of the PcG complex occurs between syncytial blastoderm and the activation of the 2218R6 enhancer during germ band extension. This phase apparently requires the function of *esc*, a PcG gene that is required only at this stage but not for later maintenance of PcG repression (Struhl and Brower, 1982). A race between activation and the establishment of repression may be responsible for the high variability in the degree of repression we observe with 2218R6+PRE. This view accords with the results of Walters *et al.* (1996) who find that, in mammalian cells, enhancer action prevents transcriptional silencing by repressive chromatin structures.

The link to imaginal enhancers

The expression of the *Ubx* and other homeotic genes in the later embryo and larva is often said to be repressed by the PcG genes but maintained in the proper domains by the trx-G genes. The analysis of the regulatory region of *Ubx* and of other homeotic genes indicates that this is not correct. The embryonic enhancers respond to the positional cues in the early embryo and are important for determining larval segmental identity but their expression in the epidermis ceases by the end of germ band expression. Expression in adult structures is determined by imaginal enhancers that lack the anteroposterior positional information necessary to distinguish the correct segmental domain of expression. Our results show that this positional information derives from the state of activity of the *Ubx* gene at the time the PcG complex is formed in the early embryo. PcG repression conveys this information to the imaginal enhancers by preventing their activation in the segments in which early expression was repressed by the HB and TLL products. The role of trx-G genes in later stages of development, in the CNS and in the imaginal discs, is thought to be to stimulate expression. Although trx-G proteins are a heterogeneous lot and probably act in different ways, the fact that some of them (*zeste*, *trx*, GAGA factor) interact specifically with the PRE or its immediate vicinity and not with the embryonic or imaginal enhancers themselves (L.Rastelli, C.Tatout and V.Pirrotta, unpublished observations) suggests that their role is to counteract or limit the effects of PcG repression, rather than to extend the activity of the embryonic enhancers. They might for example limit the phase of PcG complex

formation to the early embryonic stages and prevent the establishment of a repressed state that would block the later activation of 2218R6 and still later of the imaginal disc enhancers in appropriate segments.

Expression domains of imaginal enhancers

If the state of activity of a regulatory region governs the establishment of a repressive PcG complex, what is the relative importance of the different early enhancers in controlling the later activity of the imaginal disc enhancers? The *Ubx* gene contains two sets of imaginal enhancers: one in the *pbx* region and one in the *abx* region. The *pbx* imaginal enhancers, including 2212H1 and H3 used in this work, are in the vicinity of several embryonic enhancers such as PBX itself, 2212S1 and S2, all of which are active in even-numbered parasegments and are physically close to the major *Ubx* PRE. The *abx* imaginal enhancers are closely associated with the ABX enhancer, active in odd-numbered parasegments, and more than 15 kb distant from the next embryonic enhancer, BX, which is active in even-numbered parasegments. To account for the fact that *abx* mutations affect primarily PS5 imaginal discs, it has been proposed that the *abx* region constitutes a distinct chromatin domain which is active only in PS5, while the upstream enhancers are in a chromatin domain that is active only in PS6 (Chiang *et al.*, 1995). Our results suggest an alternative way to interpret the PS5 specificity of the *abx* imaginal enhancers. We suppose that the PcG complex normally depends on PcG-responsive elements at the major PRE, supported by the involvement of weaker elements scattered through the regulatory region, such as those associated with 2218R6, PBX, the BX enhancer and, possibly, in the *abx* region itself. If the spread of PcG repression to the more distant regions is particularly dependent on the state of activity of the neighboring enhancers, imaginal enhancers in the *pbx* region, such as 2212H1 and H3, would contribute principally to imaginal expression in PS6 while the imaginal enhancers in the *abx* region would be active mainly in odd-numbered parasegments, the domain of activity of the ABX enhancer. We suggest that this, rather than the existence of a distinct chromatin domain, is responsible for the apparent PS5 specificity of the ABX embryonic and imaginal enhancers.

In a paper that appeared while this work was under review, McCall and Bender (1996) found that a GAL4-driven promoter inserted in the *bx* region of the endogenous *Ubx* gene is not repressed at early embryonic stages but becomes repressed by the PcG products anterior to PS6 in the later embryo. We have little to add to this interesting result except that it must surely be possible for PcG complexes to repress active genes like *engrailed,* which has been shown to be regulated by PcG genes at later developmental stages (Moazed and O'Farrell, 1992). The issue in this paper is whether *Ubx* gene expression is repressed by the PRE in cells in which it was inactive but not in cells in which it was initially active. The difference between our results and those of McCall and Bender may perhaps lie in the *Ubx* promoter, whose proximal sequence has been shown to be important for its response to silencing (Chang *et al.*, 1995), or in the *Ubx* enhancers, or both.

Materials and methods

Fly strains and mutants

The host flies for germ line transformation were $Df(1)w^{67c22}$ in which the *white* gene is partially deleted, causing complete lack of eye pigmentation (Pirrotta *et al.*, 1983). The mutant stocks used for analysis of genetic interactions are: Pc^{3}; ftz^{9H34}; eve^{R13}; en^{B}; Ubx^{P18}; wg^{IL114}, a temperature-sensitive allele that behaves as a null at 25°C. Flies containing a *hsp70-hb* transposon were obtained from W.McGinnis.

Transposon constructs

The constructs were assembled in the CaSpeR Ubx-lacZ transposon vector previously used (Qian *et al.*, 1991), which contains the mini-*white* gene as a visible marker for transformation (Pirrotta, 1988) and for PRE activity. The different enhancer fragments used are described in this work or in Pirrotta *et al.* (1995). The PRE-containing fragment used is the 1.5 kb *Sty*I–*Eco*RI fragment described by Chan *et al.* (1994).

Staining of embryos and discs

For routine staining, embryos were collected for 8–12 h at room temperature. In the case of *wg* temperature-sensitive mutants, collections were made either at 25°C or for 4 h at 18°C, followed by 3 h incubation at 25°C. Embryos were fixed, stained and mounted according to Lawrence and Johnston (1989), using rabbit anti-β-galactosidase antibody (Cappel) preadsorbed against 0–14 h wild-type embryos. Secondary biotinylated goat anti-rabbit and Vectastain ABC-HRP (Vector Labs) were used to reveal the antibody complexes. In some experiments the stained embryos were restained with a monoclonal anti-invected antibody (a gift from T.Kornberg) to identify the mutant embryos. In this case the second antibody was goat anti-mouse and the color was developed with the Vectastain ABC-AP kit (Vector Labs). Imaginal discs were dissected from third instar larvae, fixed with 1% glutaraldehyde in 50 mM sodium cacodylate, pH 8.0, washed and stained with 0.2% X-gal in 100 mM $NaHPO_4$, pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$. The stained preparations were mounted in Canada balsam (embryos) or in 50% glycerol (imaginal discs). Images were taken using a Sony 3CCD camera and edited with Photoshop.

In vitro binding

The four subfragments produced by cutting the 2218R6 fragment with *Hin*cII, *Cla*I and *Bam*HI were isolated and end-labeled with the Klenow DNA polymerase. Extracts of bacterial cultures expressing HB protein as well as control extracts not expressing HB were prepared as described by Qian *et al.* (1991). The extracts were incubated in ice with each of the four probes in 15 μl containing 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll, 0.25 mg/ml bovine serum albumen and 0.5 μg salmon sperm DNA. After 30 min on ice, the reaction was loaded on a 4.5% non-denaturing acrylamide gel to display the retardation effects. A control reaction was carried out using as probe a 300 bp *Bam*HI–*Sau*3A DNA fragment from the PBX enhancer containing four HB binding sites.

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