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Transcriptional silencing by the Polycomb protein in *Drosophila* embryos

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Polycomb group (Pc-G) proteins act to keep homeotic genes stably and heritably silenced during *Drosophila* development. Here, it is shown that Polycomb (Pc), one of the Pc-G proteins, acts as a transcriptional silencer in *Drosophila* embryos if tethered to reporter genes by the DNA binding domain of GAL4 (i.e. as a GAL−Pc fusion protein). The results suggest that silencing by GAL−Pc requires the C-terminal portion of Pc, but not the chromodomain. If a pulse of GAL−Pc is provided, synthetic reporter genes are repressed, though only transiently. In contrast, reporter genes containing homeotic gene sequences remain stably and heritably silenced in a *Pc-G* gene-dependent fashion, even when GAL−Pc is no longer present. This implies that GAL−Pc recruits Pc-G proteins to DNA and suggests that maintenance of silencing requires the anchoring of Pc-G proteins to specific *cis*-regulatory sequences present in homeotic genes. The extent of DNA over which the *Pc-G* machinery acts is quite selective, as silencing established on one enhancer does not necessarily 'spread' to a juxtaposed synthetic enhancer.

Key words: *Drosophila*/homeotic gene/Polycomb response elements/transcriptional silencing

Introduction

In eukaryotic cells, specific proteins bind to control regions of genes to activate or repress their transcription. Usually these proteins have to be continuously present in the cell to act. The regulation of homeotic genes in *Drosophila* seems to be an exception to this. At least some of the proteins which determine whether homeotic genes will be permanently active or inactive are present only early in development.

Most higher eukaryotes have homeotic genes; these encode transcription factors (reviewed in Scott *et al.*, 1989; Affolter *et al.*, 1990) which are expressed in particular parts of the body to determine position-specific patterns (e.g. Lewis, 1963, 1978; Kaufman *et al.*, 1980; Levine *et al.*, 1983; Kenyon, 1986; Duboule and Dollé, 1989a,b; Wilkinson *et al.*, 1989; Chisaka and Cappechi, 1991; Lufkin *et al.*, 1991; Clark *et al.*, 1993; Wang *et al.*, 1993; for reviews see Akam, 1987; Lawrence, 1992; McGinnis and Krumlauf, 1992). Ectopic expression of homeotic genes in inappropriate body regions usually misdirects development and is thus often lethal (e.g. Schneuwly *et al.*, 1987; Gibson and Gehring, 1988; Balling *et al.*, 1989; Gonzalez-Reyes and Morata, 1990; Mann and Hogness, 1990; Lufkin *et al.*, 1992; Morgan *et al.*, 1992). The spatial regulation of homeotic gene expression is therefore crucially important. In *Drosophila*, this process can be subdivided into two steps: the first determines the domains of homeotic gene expression, whereas the second maintains these domains. In both steps, repression plays a central role (Bienz, 1992).

The establishment of domains of homeotic gene expression in the *Drosophila* embryo along the anteroposterior axis occurs in response to positional information. This information is provided by the early-acting segmentation genes (Nüsslein-Volhard and Wieschaus, 1980), e.g. the gap genes which encode DNA binding proteins (Pankratz *et al.*, 1989; Stanojevic *et al.*, 1989; Treisman and Desplan, 1989). Gap gene products, distributed in broad bands, determine the limits of homeotic gene expression (White and Lehmann, 1986; Harding and Levine, 1988; Irish *et al.*, 1989; Reinitz and Levine, 1990). The gap protein hunchback (hb) is expressed anteriorly and binds directly as a repressor to regulatory sequences of the homeotic gene *Ultrabithorax* (*Ubx*; Qian *et al.*, 1991; Zhang *et al.*, 1991), where it prevents more generally distributed factors from activating *Ubx* (Qian *et al.*, 1991, 1993; Müller and Bienz, 1992). *Ubx* is therefore repressed wherever hb protein is present. There is evidence that other gap proteins act in a similar way as repressors to delimit other homeotic genes (Busturia and Bienz, 1993; Shimell *et al.*, 1994). By mid-embryogenesis, the gap proteins decay and become undetectable (e.g. Gaul *et al.*, 1987; Tautz, 1988).

Homeotic genes act throughout development (Morata and Garcia-Bellido, 1976; Struhl, 1982; Kaufman and Abbott, 1984; Hoppler and Bienz, 1994) within the domains defined in the early embryo. At later stages of development, genes of the *Polycomb-group* (*Pc-G*) are used to repress homeotic genes outside their domains of expression (Lewis, 1978; Struhl, 1981; Jürgens, 1985). In *Pc-G* mutant embryos, homeotic genes are initially expressed within appropriate domains but become ectopically expressed by mid-embryogenesis, suggesting that *Pc-G* genes are required to maintain but not establish domains of homeotic gene expression (Struhl and Akam, 1985; Jones and Gelbart, 1990). However, Pc-G proteins are almost uniformly distributed in the embryo (Franke *et al.*, 1992; Zink and Paro, 1992; Martin and Adler, 1993), and thus do not appear to carry positional information.

Pc-G proteins bind to shared sets of sites on salivary gland chromosomes (Zink and Paro, 1989; DeCamillis *et al.*, 1992; Franke *et al.*, 1992; Martin and Adler, 1993; Rastelli *et al.*, 1993). On salivary gland chromosomes and in tissue culture cells, Polycomb (Pc) and polyhomeotic (ph) proteins, two Pc-G members, are associated with control regions of homeotic genes (Zink *et al.*, 1991;

DeCamillis *et al.*, 1992; Orlando and Paro, 1993). However, attempts to show sequence-specific DNA binding of Pc and ph proteins have failed so far (Zink *et al.*, 1991; Franke *et al.*, 1992), suggesting that they are tethered to DNA by protein–protein interactions. Pc and ph proteins are part of large multimeric protein complexes which can be precipitated from embryonic nuclei (Franke *et al.*, 1992).

What is the mechanism of *Pc-G* gene action? A *Ubx* enhancer, called *BXD*, directs an expression pattern from head to tail throughout most of embryogenesis (Müller and Bienz, 1991). This pattern becomes restricted to the *Ubx* domain if hb binding sites, or *Ubx* control regions containing hb binding sites, are linked to *BXD* (Müller and Bienz, 1991; Zhang *et al.*, 1991; Zhang and Bienz, 1992). This suppression, or 'silencing' (as it acts at long range, and in some cases also on heterologous promoters), depends on *Pc* (Müller and Bienz, 1991; Zhang and Bienz, 1992; Busturia and Bienz, 1993). Remarkably, hb binding sites continue silencing BXD activity in older embryos when hb protein is no longer detectable. This suggests a model in which hb protein, in a hit-and-run fashion, promotes the formation of heritable 'silencing complexes' by recruiting Pc-G proteins to DNA (Zhang and Bienz, 1992). Silencing complexes are thought to contain Pc-G proteins (discussed in Bienz, 1992).

If the role of hb protein is to recruit Pc-G proteins to DNA, tethering Pc-G proteins to DNA by other means should lead to silencing and thus bypass the requirement for *hb*. Here, this prediction is tested by tethering Pc protein to DNA with the DNA binding domain of the yeast transcription factor GAL4 (Keegan *et al.*, 1986; Fischer *et al.*, 1988). It is shown that this GAL4–Pc fusion protein (GAL–Pc) represses transcription and establishes stable and heritable silencing of *BXD* activity. The evidence suggests that in order to act, Pc-G proteins have to be anchored to DNA through specific *cis*-regulatory sequences.

Results

Tethering GAL–Pc protein to DNA

To test for silencing activity of the Pc protein (Figure 1A), I constructed a set of three β-galactosidase (β*gal*) reporter genes containing the *BXD* enhancer (Müller and Bienz, 1991) and synthetic *GAL4* binding sites (Giniger *et al.*, 1985; Webster *et al.*, 1987. In these *GAL4–BXD* reporter genes, *GAL4* binding sites are either upstream or downstream of the *BXD* enhancer and they contain either the proximal *Ubx* promoter or a minimal *hsp70* promoter linked to *LacZ* (Figure 1B; see figure legend for details). These constructs were introduced into flies. Several independent transformant lines were isolated in each case and embryos were analysed for β*gal* expression. All three types of *GAL4–BXD* transformants showed head-to-tail patterns indistinguishable from those observed in *BXD* transformants (Figure 2; cf. Müller and Bienz, 1991).

For the *GAL–Pc* effector construct, the coding sequence of *Pc* fused to the *GAL4* DNA binding domain ($GAL4_{1-147}$) was linked to a 0.7 kb fragment of the *hb* promoter (Figure 1C); several transformant lines were obtained with this construct (*hb-GAL–Pc*). As this *hb* promoter fragment directs expression exclusively at the blastoderm stage in a domain including the prospective head and thorax segments (Schröder *et al.*, 1988), the *hb-GAL–Pc* effector construct produces a pulse of GAL–Pc protein in the anterior part of the early embryo.

GAL4–BXD and *hb-GAL–Pc* transformants were crossed and the β*gal* patterns of their progeny were analysed (Table I). In embryos carrying the *hb-GAL–Pc* effector and any of the *GAL4–BXD* reporter genes, β*gal* expression in the head and thorax is essentially suppressed at both early and late embryonic stages (Figure 2). This suppression is strictly dependent on *hb-GAL–Pc*. A *hb-GAL* effector construct (expressing only the GAL4 DNA binding domain under the *hb* promoter; Figure 1C) did not confer any suppression whatsoever (Figure 2). Thus, GAL–Pc, but not GAL alone, suppresses reporter gene activity, presumably via the Pc moiety of the fusion protein. Two other GAL fusion proteins were tested, but these did not confer any suppression either (results not shown; see Materials and methods). Note that the suppression of *BXD* activity by GAL–Pc is maintained throughout embryogenesis, although GAL–Pc protein is produced only in the very young embryo and is no longer synthesized at later stages (see below). This suggests that the GAL–Pc protein establishes stable and heritable silencing.

I next asked whether GAL–Pc acts by binding to the *GAL4* binding sites in the reporter gene. First, I deleted the *GAL4* DNA binding domain in the *hb-GAL–Pc* effector construct (*hb-Pc*, Figure 1C). In embryos carrying *hb-Pc* there was no silencing of any of the *GAL4–BXD* reporter genes (Figure 2). Second, I tested the *hb-GAL–Pc* effector construct in the original *BXD* transformants (in the absence of *GAL4* binding sites): these showed a virtually normal *BXD* pattern, although β*gal* levels appeared slightly reduced in the brain lobes (data not shown). These two experiments strongly suggest that silencing is not simply due to overexpression of GAL–Pc protein, but that the GAL–Pc protein has to bind to the *GAL4* binding sites to act as a silencer.

Note that GAL–Pc protein suppresses all *BXD* activity, whether *GAL4* binding sites are placed upstream or downstream of the 1.6 kb *BXD* enhancer. This suggests that GAL–Pc protein acts at a distance to silence *BXD* reporter genes.

Functional domains in Pc

The Pc protein contains a conserved protein motif, the chromodomain, also present in a *Drosophila* chromatin-associated protein (James and Elgin, 1986; Paro and Hogness, 1991) and in a mouse protein (Pearce *et al.*, 1992). The chromodomain is essential for the binding of Pc to salivary gland chromosomes (Messmer *et al.*, 1992). I therefore asked whether the chromodomain is required for the function of GAL–Pc. To test this, I introduced a point mutation which abolishes the binding of Pc protein to chromosomes (Messmer *et al.*, 1992) in the chromodomain of *GAL–Pc* (*hb-GAL–*Pc^{CHD}, Figure 1C). This mutant GAL–Pc^{CHD} protein silenced all types of *GAL4–BXD* reporter genes as efficiently as wild-type GAL–Pc protein at early and late embryonic stages (Figure 2). This shows that the Pc protein without a functional chromodomain is capable of silencing, provided it is tethered to DNA.

The C-terminus of Pc contains a conserved stretch of

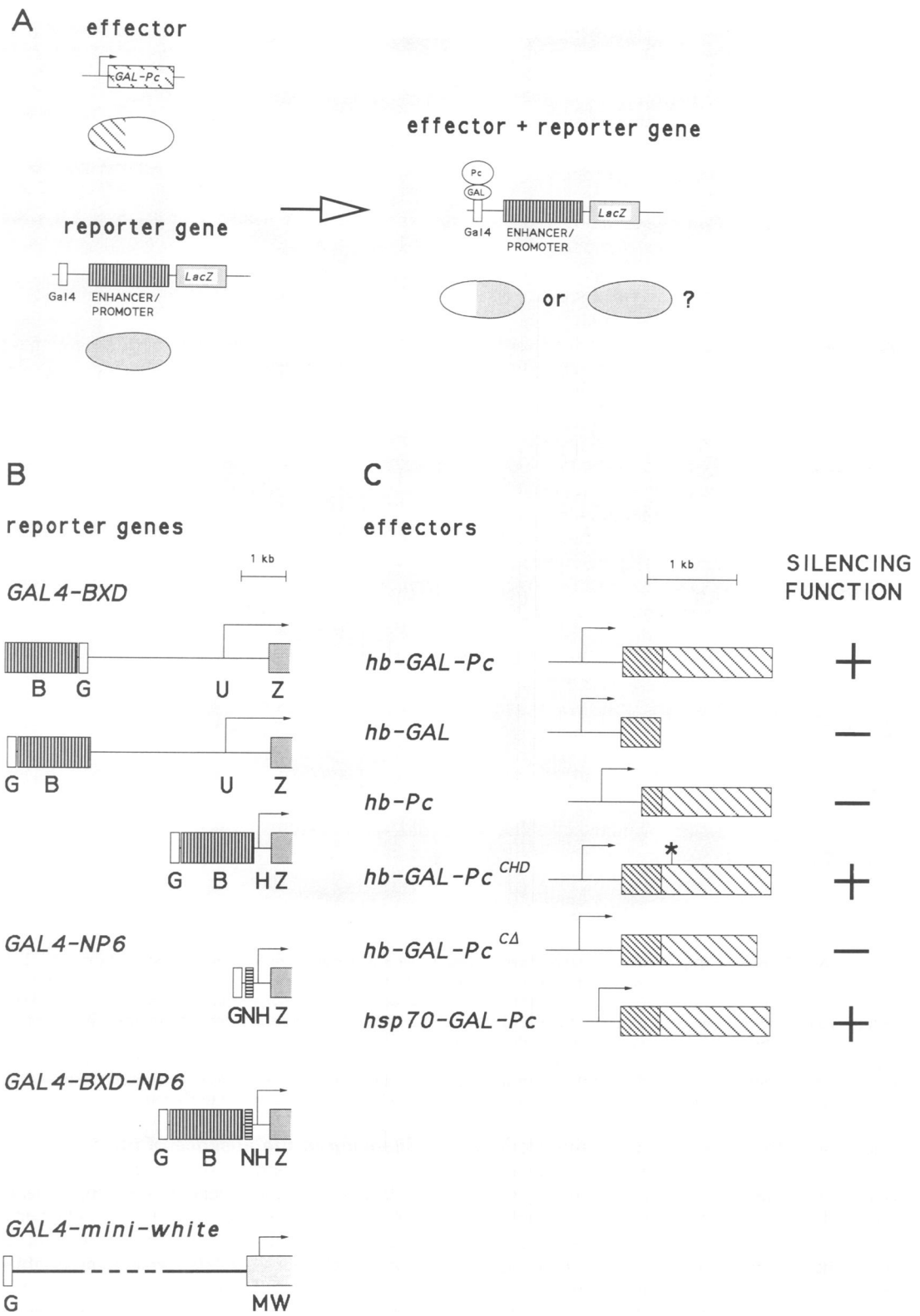

Fig. 1. (**A**) Schematic description of the assay. An enhancer/promoter in a *LacZ* reporter gene directs a head-to-tail expression pattern in the embryo (below). GAL−Pc protein, produced from the effector construct in the anterior part of the embryo, is tested for its ability to bind to the *GAL4* binding sites in the reporter gene and silence its expression anteriorly. (**B**) *GAL4−BXD* reporter genes contain the *BXD* enhancer ('B', vertically striped box), the proximal *Ubx* promoter ('U', thin line) or the *hsp70* minimal promoter ('H', thin line), *LacZ* ('Z', stippled box) and synthetic *GAL4* binding sites ('G', white box) either upstream ('GBUZ' and 'GBHZ') or downstream ('BGUZ') of the *BXD* enhancer. The *GAL4−NP6* reporter gene contains the synthetic enhancer *NP6* (horizontally striped box; see text for details); the *GAL4−BXD−NP6* reporter contains the *BXD* and *NP6* enhancers. The *GAL4−mini-white* reporter gene contains *GAL4* binding sites ~4.5 kb upstream of the *mini-white* gene ('MW', lightly stippled box); it is the *UAS−lacZ* transposon of Brand and Perrimon (1993). The 4.5 kb 'spacer' (thick black line) is a *hsp70* minimal promoter−*LacZ* fusion gene not relevant to this study. Arrows indicate transcription start sites. (**C**) Effector constructs. The wild-type GAL−Pc fusion protein contains the whole Pc coding region (lightly hatched box) fused to the GAL4 DNA binding domain (densely hatched box), which is linked to either a fragment of the *hb* promoter or the heat-inducible *hsp70* promoter (thin lines), as indicated. Asterisk indicates the position of the point mutation in the chromobox. Arrows indicate transcription start sites.

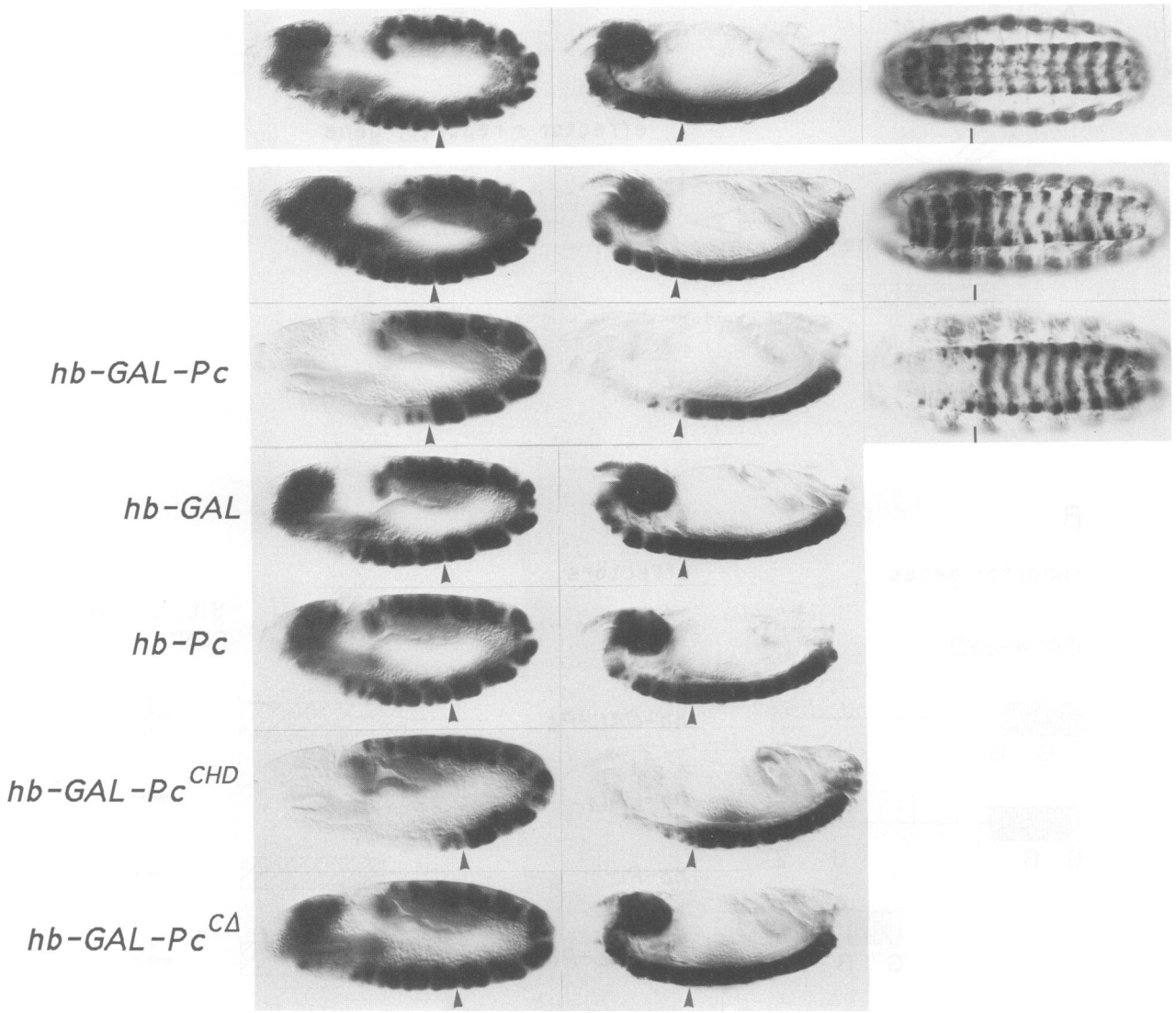

Fig. 2. Silencing by GAL−Pc protein. Transformant embryos carrying reporter or reporter and effector constructs stained for β-gal. Left and middle columns: side views of ~6 and ~12 h-old embryos. Right column: ventral view (ventral nerve cord) of 14–16 h-old embryos. Row 1: *BXD* transformants (Müller and Bienz, 1991) show the head-to-tail *BXD* pattern in the ectoderm of parasegments 2–14. Row 2: *GAL4−BXD* transformants show the same pattern as in row 1. Rows 3–7: progeny of crosses between *GAL−BXD* and various effector transformants. β*gal* expression in the head and thorax is suppressed by the *hb-GAL−Pc* or *hb-GAL−Pc*^{CHD} effector constructs (rows 3 and 6), but not by *hb-GAL*, *hb-Pc* or *hb-GAL−Pc*^{CΔ}. Anterior limits of parasegment 6 are marked by arrowheads; in the third column the anterior limits of parasegment 6 in the ventral nerve cord are indicated by small vertical bars. The *BXD* transformants in row 1 carry BHZ; the *GAL−BXD* transformants in rows 2–7 carry GBHZ (see Figure 1). The same transformant line shown in row 2 was used for all crosses in rows 3–7. Anterior to the left.

~30 amino acids also found in a mouse chromodomain protein (Pearce *et al.*, 1992). Although several lethal *Pc* alleles consist of C-terminal truncations (Franke *et al.*, 1995), deletions that remove this C-terminal stretch do not affect Pc binding to chromosomes (Messmer *et al.*, 1992). To test whether the C-terminal portion of Pc is required for silencing, the C-terminal end of GAL−Pc (*hb-GAL−Pc*^{CΔ}, Figure 1C) was deleted. GAL−Pc^{CΔ} protein did not silence any of the *GAL4−BXD* reporter genes (Figure 2). Most probably, this is due to neither altered stability of the truncated protein nor masking of the GAL4 DNA binding domain: the nuclear distribution of GAL−Pc^{CΔ} protein is indistinguishable from that of wild-type GAL−Pc protein (Figure 3). Also, GAL−Pc^{CΔ} binds to *GAL4* binding sites *in vitro* (data not shown). Most likely, GAL−Pc^{CΔ} protein binds to the reporter gene, but fails to silence it.

Silencing in the absence of hb

Previous studies have shown that hb binding sites linked to *BXD* initiate *Pc*-dependent silencing (Zhang and Bienz, 1992; see above). Therefore, I asked whether silencing by GAL−Pc protein bypasses the requirement for hb. First, I tested whether GAL−Pc protein can establish silencing in the absence of *hb* function. I found that GAL−Pc suppresses reporter gene expression in *hb* mutant embryos (Figure 4A−D). In contrast, in reporter genes in which silencing of *BXD* is mediated by hb binding sites, silencing is abolished in *hb* mutants, as described previously (Figure 4E and F; cf. Zhang and Bienz, 1992; Busturia and Bienz, 1993). Thus, silencing by GAL−Pc is independent of *hb* function.

Second, I tested whether GAL−Pc protein can establish silencing in a body region where hb protein is not present. I substituted the *hb* promoter in *hb-GAL−Pc* with the

Table I. Silencing in the progeny of crosses between reporter (top row) and effector transformant lines (left-hand column)

Effector	Reporter *BXD*	*GAL4−BXD*			*GAL4−NP6*	*GAL4− mini-white*
		BGUZ	GBUZ	GBHZ		
hb-GAL−Pc	no	yes	yes	yes	no	no
hb-GAL	n.d.	no	no	no	n.d.	n.d.
hb-Pc	n.d.	no	no	no	n.d.	n.d.
hb-GAL−Pc^{CHD}	n.d.	yes	yes	yes	n.d.	n.d.
hb-GAL−Pc$^{C\Delta}$	n.d.	no	no	no	n.d.	n.d.
hsp70-GAL−Pc	n.d.	n.d.	n.d.	yes	yes	yes

Abbreviations for the three *GAL4−BXD* reporter genes are as in Figure 1. Silencing (yes) or no silencing (no) was observed in each cross tested; n.d., not done.

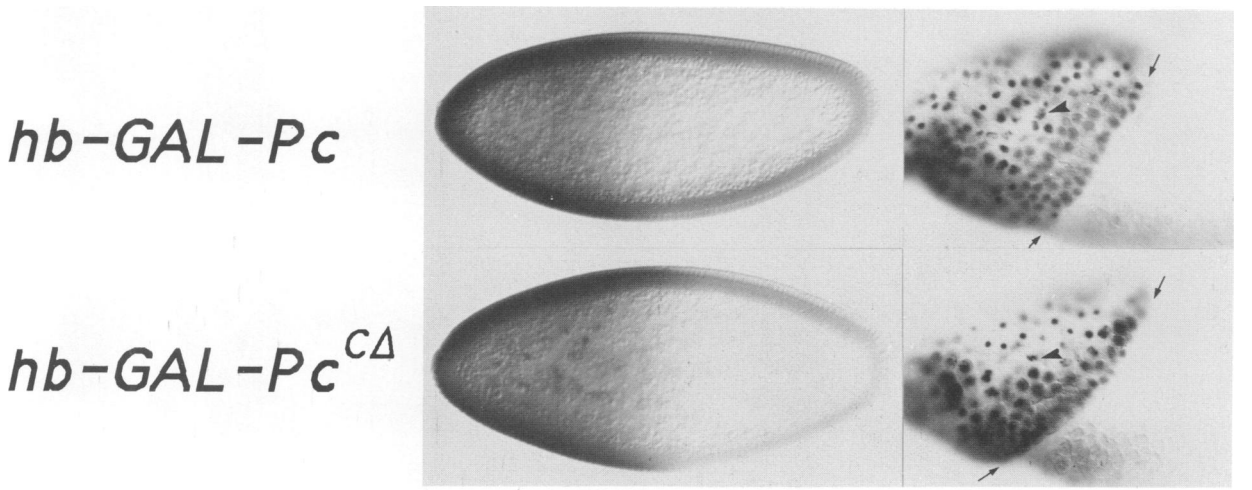

Fig. 3. Distribution of GAL−Pc and GAL−Pc$^{C\Delta}$ protein. *hb-GAL−Pc* and *hb-GAL−Pc$^{C\Delta}$* transformant embryos were stained with anti-Pc antibody. 2.5 h-old blastoderm embryos (left) and the head region of 3.5 h-old embryos (right). Staining reactions were kept short, such that only GAL−Pc fusion protein but not the endogenous Pc protein is shown. In some nuclei the stained structures are irregularly shaped (arrowheads) and might correspond to condensed chromosomes. Note that the staining patterns in the embryo and in the nuclei are indistinguishable between the two constructs. Small arrows indicate the position of the cephalic furrow. Anterior to the left.

heat-inducible *hsp70* promoter (*hsp70-GAL−Pc*, Figure 1C). The *hsp70* promoter allows the expression of GAL−Pc at any time and ubiquitously. *hsp70-GAL−Pc* and *GAL4-BXD* transformants were crossed and their progeny heat-shocked at the blastoderm stage. In these heat-shocked embryos, β*gal* expression was almost completely suppressed along the whole body axis throughout embryogenesis (Figure 5B and D). Heat-shocked control embryos (*GAL4−BXD* transformants without *hsp70-GAL−Pc*) showed the normal BXD pattern (Figure 5A and C). Thus, GAL−Pc can silence the BXD enhancer along the whole body axis.

Silencing of other enhancers

In all experiments described so far, reporter genes contained the *BXD* enhancer and either the proximal *Ubx* promoter or a minimal *hsp70* promoter. I therefore asked whether GAL−Pc can also silence a reporter gene containing no *Ubx* sequences. I constructed a reporter gene which contains *GAL4* binding sites upstream of a synthetic enhancer, *NP6*, linked to the *hsp70* minimal promoter and *LacZ* (Figure 1C). *NP6* consists of six copies of an oligomerized homeoprotein binding site (Desplan *et al.*, 1988). Linked to a reporter gene, *NP6* confers expression at late embryonic stages in a pattern, called *NP6* pattern, which extends from head to tail (Vincent *et al.*, 1990). Several *GAL4−NP6* transformant lines were isolated, all of which showed this *NP6* pattern (Figure 5E).

To test whether GAL−Pc protein can silence the *GAL4−NP6* reporter gene, *GAL4−NP6* and *hb-GAL−Pc* (or *hsp70-GAL−Pc*) transformants were crossed. Embryos from the *hb-GAL−Pc* cross did not show any suppression of the *NP6* pattern (Figure 5F). Similarly, embryos from the *hsp70-GAL−Pc* cross did not show suppression of the *NP6* pattern at late stages if they were heat-shocked at the blastoderm stage (Figure 5H). However, if these embryos were heat-shocked once at a late embryonic stage and analysed shortly thereafter, the *NP6* pattern was almost completely suppressed (Figure 5J; see figure legend for details). This suggests that GAL−Pc protein can repress the *GAL4−NP6* reporter gene, but that repression of *GAL4−NP6* is transient, probably lasting as long as there is sufficient GAL−Pc protein (produced by the heat-shock) to bind to the reporter gene. Consequently, the activity of the *NP6* enhancer is probably restored once the GAL−Pc protein levels drop below a critical threshold concentration insufficient for DNA binding. In a reporter gene in which *GAL4* binding sites are placed 1.6 kb upstream of *NP6*, GAL−Pc protein (produced by a late heat-shock) does not silence the *NP6* enhancer, suggesting that GAL−Pc cannot silence *NP6* at a distance (results not shown; see Materials and methods).

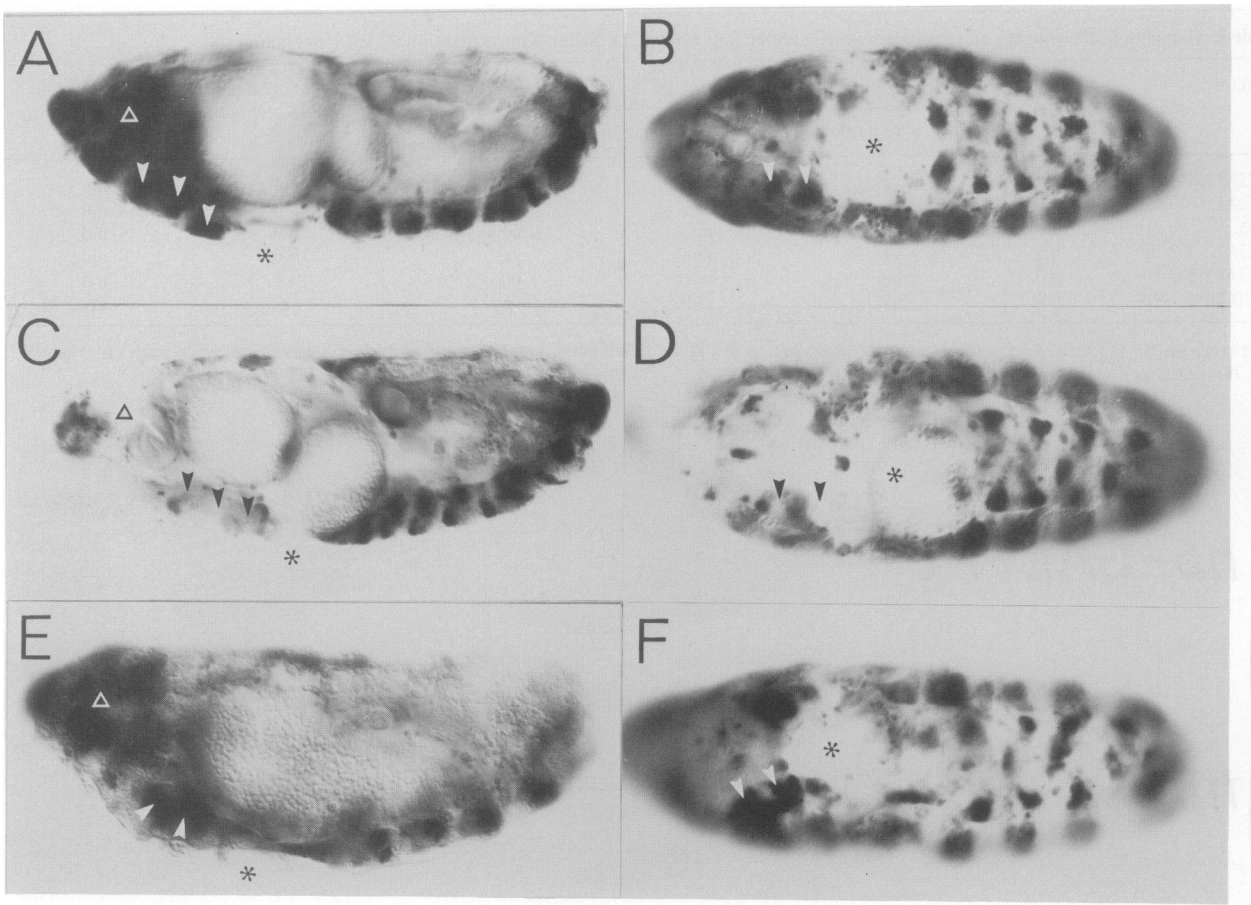

Fig. 4. Silencing by GAL−Pc is independent of *hb* function. Side views (**A**, **C** and **E**) or ventral views (**B**, **D** and **F**) of *hb* mutant embryos, 10–15 h old, stained for β-gal. The asterisks mark the gaps due to the *hb* mutation. (A and B) *GAL4−BXD* transformant showing the head-to-tail *BXD* pattern with expression in the nerve cord anterior to the gap (white arrowheads) and in the head (white triangle in A). (C and D) Embryos of the same *GAL4−BXD* line carrying a *hb-GAL−Pc* construct. Expression anterior to the gap is silenced in the nerve cord (black arrowheads) and in the head (black triangle in C) due to GAL−Pc protein. (E and F) *hb* transformants carrying a reporter gene in which hb protein binding sites are linked to the *BXD* enhancer (Zhang and Bienz, 1992). In wild-type embryos β-*gal* expression is silenced anterior to parasegment 6, but in *hb* mutants silencing is abolished and β-*gal* staining is present in the nerve cord (white arrowheads) and in the head (white triangle in E) anterior to the gap. Note the lack of strong expression anterior to the gap in (C) and (D) compared with (A), (B), (E) and (F) where β-*gal* staining anterior to the gap is as strong as posterior to the gap. Heads to the left.

I next tested whether GAL−Pc can silence a *mini-white* gene containing *GAL4* binding sites (*GAL4−mini-white*, Figure 1C). The *white* gene product is required for eye pigmentation, and *white* null-mutants have completely unpigmented eyes. The *GAL4−mini-white* gene partially restores pigmentation in flies which lack endogenous *white* function. This requires *white* expression for only a short period late in development (Steller and Pirrotta, 1985). If *GAL4−mini-white* transformants which also carry the *hsp70-GAL−Pc* effector construct are heat-shocked during this period, eye pigmentation is uniformly reduced or even completely abolished, suggesting that GAL−Pc silences the *GAL4−mini-white* gene (results not shown; for details see Materials and methods). However, if GAL−Pc protein is produced only early in development (e.g. by heat-shock during the first larval instar), eye pigmentation is not affected. Thus, as in the case of *GAL4−NP6*, GAL−Pc represses the *GAL4−mini-white* gene only transiently (i.e. once GAL−Pc protein is no longer available the reporter gene becomes transcriptionally active). A third reporter gene, containing *GAL4* binding sites linked upstream and downstream of an α*Tubulin−LacZ* fusion gene, was not even transiently silenced by the GAL−Pc protein (see Materials and methods).

The results with *GAL4−NP6* and *GAL4−mini-white* contrast with those with *GAL4−BXD* in which an early pulse of GAL−Pc protein was sufficient to silence the *BXD* enhancer stably throughout embryogenesis (Figures 2 and 5B and D). It appears that silencing of *BXD* is maintained even if GAL−Pc protein is no longer bound to DNA.

I tested whether this maintenance of silencing of *GAL4−BXD* reporter genes is dependent on *Pc-G* gene function: indeed it is, as silencing is lost in *Pc*, *Polycomb-like*, *Additional sex combs*, *Sex combs on midleg*, *Sex combs extra* and *Enhancer of zeste* [*E(z)*] mutant embryos (Figure 6A and results not shown). In contrast to these *Pc-G* gene products, another, the *extra sex combs* (*esc*) product, is critically required only early in development and becomes dispensable thereafter (Struhl, 1981; Struhl and Brower, 1982; Busturia and Morata, 1988). This early action of *esc* suggests that it might help the transition between *hb*-dependent repression and *Pc-G*-dependent maintenance of silencing. If so, bringing the Pc protein directly to the DNA (using GAL−Pc) might bypass not

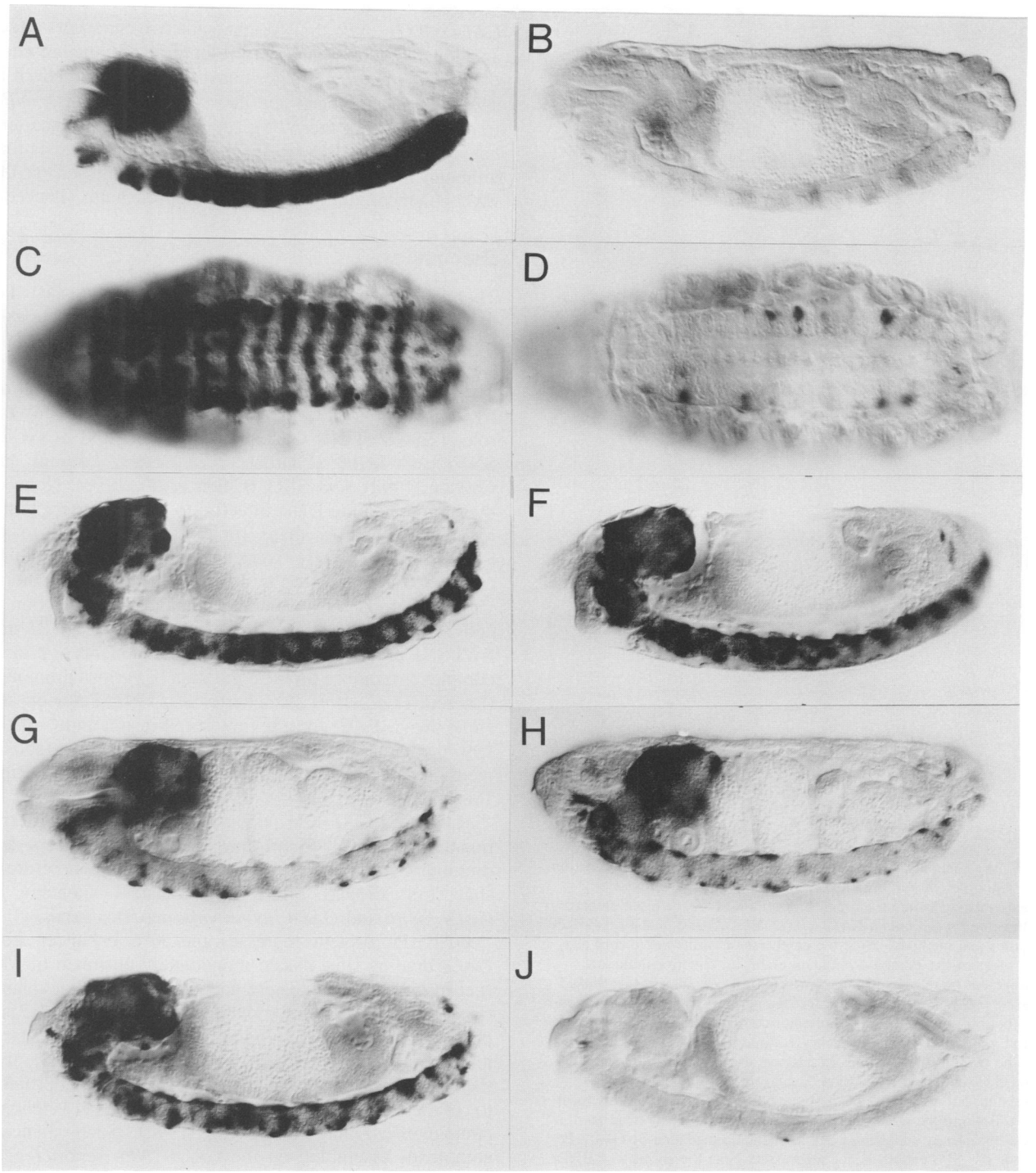

Fig. 5. Transient versus heritable silencing. *GAL4−BXD* (**A−D**) and *GAL4−NP6* (**E−J**) transformant embryos carrying various effector constructs and stained with β-*gal* antibody. Side and ventral (C and D) views of 11–16 h-old embryos. (B) and (D) carry one copy of the *hsp70-GAL−Pc* construct; (A)−(D) were heat-shocked at the blastoderm stage. The *BXD* pattern is almost completely suppressed throughout embryogenesis, due to GAL−Pc. (E−J) *GAL4−NP6* transformant (E), showing the head-to-tail *NP6* pattern in the nervous system. (F) A *GAL4−NP6* transformant carrying the *hb-GAL−Pc* construct shows the full *NP6* pattern, indistinguishable from (E). (G and H) *GAL4−NP6* transformants carrying the *hsp70-GAL−Pc* construct, (H) heat-shocked at the blastoderm stage and (G) not heat-shocked. Both show the same *NP6* pattern. [The embryos in (G) and (H) are older than in (E) and (F), and the staining is not so strong in this focal plane]. (I and J) *GAL4−NP6* transformant with (J) and without (I) the *hsp70-GAL−Pc* construct; both were heat-shocked at 8–9 h of development and fixed 4 h afterwards. Note that the *NP6* pattern is almost completely suppressed (J).

only the need for hb but also the need for the esc protein. This is not the case, as silencing is also abolished in embryos lacking the *esc* gene product (Figure 6A). In summary, it appears that *Pc-G* genes maintain silencing established by GAL−Pc even when the GAL−Pc protein is no longer present. As this process occurs on *GAL4−BXD* reporter genes but not on *GAL4−NP6* and *GAL4−mini-white*, this implies that the maintenance of *Pc-G*-dependent silencing requires specific *cis*-regulatory sequences present in *BXD*.

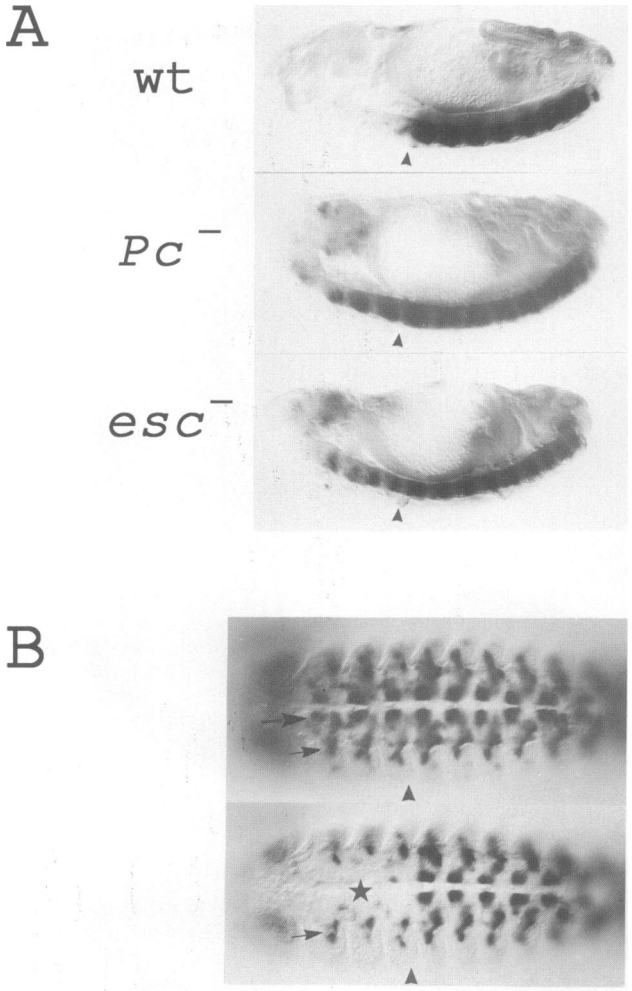

Fig. 6. (**A**) Silencing due to GAL−Pc depends on *Pc-G* gene function. Top, wild-type embryo; middle, *Pc* mutant embryo; bottom, *esc* mutant embryo stained for β-*gal*. Side views of 10–12 h-old embryos. All three embryos contain *GAL4−BXD* (BGUZ) and *hb-GAL−Pc* (see Materials and methods). Note the derepression in the nerve cord and brain lobes of the *Pc* and *esc* mutant embryos. Anterior limits of parasegment 6 are marked by arrowheads. (**B**) *Pc* silencing on linked enhancers. *GAL4−BXD−NP6* transformant embryos without (top) and with (bottom) the *hb-GAL−Pc* effector construct stained for β-*gal*. Ventral view of 10–12 h-old embryos. Anterior to the left in all cases. Top: the two enhancers direct an additive expression pattern (large arrow, *BXD*-activated expression; small arrow, *NP6*-activated expression; not all aspects of the two patterns are visible in this focal plane). Bottom: the *BXD* pattern in the thorax and in the head is almost completely suppressed due to silencing mediated by GAL−Pc, whereas the NP6 pattern is not suppressed. Note the absence of BXD-directed expression in the thorax (black asterisk), whereas the NP6 pattern in the thorax is present at normal levels (small arrow). Arrowheads mark the boundary between thorax and abdomen.

The results described above imply that, at least in body regions where the GAL−Pc protein establishes silencing, Pc-G proteins are probably associated with the *BXD* control region in the form of a 'silencing complex' (see Discussion). GAL−Pc protein silences the *GAL4−NP6* reporter gene, provided that GAL−Pc is bound to the construct at the time when the *NP6* enhancer is active. Therefore, one might expect that in a combination construct where *BXD* and *NP6* are linked (*GAL4−BXD−NP6*, Figure 1C), the proposed silencing complex associated with *BXD* would also silence *NP6* activity. In *GAL4−BXD−NP6* transformants the two enhancers direct an additive expression pattern (Figure 6B). In the presence of the *hb-GAL−Pc* effector construct, the *BXD* pattern is suppressed in the head and thorax of *GAL4−BXD−NP6* transformants but the *NP6* pattern is not suppressed and extends from head to tail (Figure 6B). This suggests that although *Pc-G*-dependent silencing is established on the *BXD* enhancer, the linked *NP6* enhancer is not silenced.

Discussion

The experiments described here demonstrate that Pc, tethered to DNA as a GAL−Pc fusion protein, acts as a transcriptional silencer and establishes *Pc-G*-dependent silencing of reporter genes with homeotic gene sequences. The method used to analyse the mechanism of repression by Pc is analogous to methods used in yeast to study the role of SSN6−TUP1 (Keleher *et al.*, 1992), SNF2 (SWI2), SNF5 and SNF6 (Laurent *et al.*, 1991; Laurent and Carlson, 1992), and SIR1 (Chien *et al.*, 1993). None of these proteins bind DNA directly; to act as transcriptional regulators, each of these proteins is presumed to be tethered to DNA by sequence-specific DNA binding proteins. When tethered to a promoter as fusion proteins via heterologous DNA binding domains, they function to activate or repress transcription. The SSN6−TUP1 and SWI/SNF proteins are thought to be components of protein complexes required for the regulation of various promoters (Laurent *et al.*, 1991; Keleher *et al.*, 1992; Peterson and Herskowitz, 1992; for a review see Winston and Carlson, 1992), whereas SIR1 protein is required for the establishment of silencing at the mating-type loci (Pillus and Rine, 1989; Chien *et al.*, 1993).

How does GAL−Pc fusion protein act as a transcriptional silencer? I found that different enhancers and promoters respond differently to GAL−Pc. Therefore, I shall first discuss briefly the transient silencing of *GAL4−NP6* and *GAL4−mini-white* reporter genes.

GAL−Pc protein represses the *NP6* enhancer from *GAL4* binding sites placed immediately upstream of *NP6* (i.e. in *GAL4−NP6*). There are several possible explanations of how GAL−Pc protein acts in this case. Pc and ph proteins have been found previously to be part of large multimeric protein complexes (Franke *et al.*, 1992). It is therefore conceivable that GAL−Pc recruits other proteins to DNA to form such complexes (putative 'silencing complexes'; Zhang and Bienz, 1992). Such silencing complexes assembled on the *GAL4−NP6* reporter gene might (unspecifically) interfere with the binding of transcription factors to the *NP6* enhancer and/or the TATA-box. Another possibility is that transcriptional activators and factors of the general transcription machinery still bind, but that GAL−Pc protein, perhaps via complexed proteins, somehow interferes with the transcriptional activation process *per se* (e.g. by 'quenching'; Levine and Manley, 1989). Note, however, that GAL−Pc does not silence *NP6* from *GAL4* binding sites placed 1.6 kb upstream of the enhancer.

Interestingly, transient silencing of the *GAL4−mini-white* gene acts at a considerable distance, as the *GAL4* binding sites are located >4 kb upstream of the *mini-white* promoter. The *white* promoter is relatively weak and lacks a canonical TATA-box, but it contains promoter

sequences mediating long-range interactions (Qian *et al.*, 1992). The *white* promoter thus appears to be very sensitive and might therefore be susceptible to silencing by GAL−Pc at a distance.

Heritable silencing

If the GAL−Pc protein is provided only transiently, the *GAL4−NP6* and *GAL4−mini-white* reporter genes can still be activated once the GAL−Pc protein is no longer available. In contrast, reporter genes containing *Ubx* sequences remain stably silenced in a *Pc-G* gene-dependent fashion, even when the GAL−Pc protein is no longer present. From this I conclude that the maintenance of *Pc-G*-dependent silencing requires specific *cis*-regulatory sequences. I shall refer to these sequences as *Pc-G* response elements (PREs) and suggest that the *BXD* enhancer contains PREs. I imagine that PREs are *cis*-regulatory elements through which Pc-G proteins are anchored to DNA and thereby confer stable and heritable silencing throughout development (see below). The existence of PREs in the *Ubx* upstream region was first postulated by Simon *et al.* (1993). Orlando and Paro (1993) found that the immunoprecipitation of crosslinked chromatin with anti-Pc antibodies enriches for a fragment containing the *BXD* enhancer, providing further evidence for an interaction of Pc with *BXD*.

How does GAL−Pc protein establish stable and heritable silencing? A possible model for this is shown in Figure 7. Among the proteins recruited to DNA by GAL−Pc could be DNA binding members of the Pc-G, e.g. Posterior Sex Combs (Psc) or E(z) (both of which are putative DNA binding proteins; Brunk *et al.*, 1991; van Lohuizen *et al.*, 1991; Jones and Gelbart, 1993). I imagine that such DNA binding proteins bind to the PREs in *BXD*. Although they might not bind to these PREs on their own, they would bind in this case due to the cooperativity provided by GAL−Pc. Cooperative interactions between different Pc-G proteins in the silencing complex might facilitate and stabilize their binding to PREs. Once anchored to PREs, the silencing complex would be stably and heritably maintained even in the absence of GAL−Pc protein. It has been suggested that silencing complexes might 'linger on' during replication due to protein−protein interactions, and thereby become redistributed and reassembled on daughter strands without ever completely detaching from DNA (Zhang and Bienz, 1992). Recently Chan *et al.* (1994) discovered independently another fragment in the *Ubx* upstream region which appears to contain PREs. I imagine that in the *Ubx* gene, interactions between distant PREs further stabilize the binding of silencing complexes to provide the ultimate stability required during cell proliferation (i.e. by a form of 'cooperative DNA binding at a distance'). Silencing complexes anchored to PREs would thus constitute a memory for the molecule which recruited them. In the case of a gene lacking PREs, i.e. *GAL4−NP6*, silencing complexes would not be anchored and would therefore decay as soon as the tethering molecule disappears.

Note that a silencing complex assembled on one enhancer, i.e. *BXD*, does not silence a linked enhancer and promoter lacking a PRE (i.e. the *NP6* enhancer and the *hsp70* minimal promoter in the *GAL4−BXD−NP6* reporter gene). It appears that in this case it is the *BXD* enhancer, not the promoter, which is silenced, although it could also be that in cells where *NP6* is active, transcriptional activators which bind to the *NP6* enhancer disrupt a silencing complex formed between *BXD* and the promoter (recall that *NP6* is placed immediately upstream of the *hsp70* TATA-box). Thus, it does not seem that silencing complexes assembled on PREs 'spread' over long distances to block indiscriminately the activity of linked enhancers and promoters. This result rather suggests that promoters and/or enhancers might have to be equipped with PREs to respond to silencing.

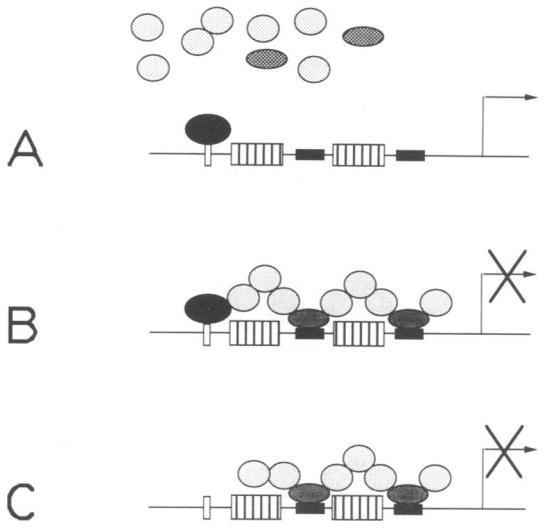

Fig. 7. Model of GAL−Pc action. (**A**) A *GAL4−BXD* reporter gene (thin line) with activator binding sites (vertically striped boxes), PREs (small black boxes) and *GAL4* binding sites (small white box); arrow indicates transcription start site. (**B**) The model suggests that GAL−Pc protein (black oval) binds to the *GAL4* binding sites and directly or indirectly recruits endogenous Pc and other Pc-G proteins (stippled circles) to DNA to form a silencing complex. Among these proteins are also DNA binding members of the *Pc-G* (stippled ovals), which become tethered to the PREs; binding is stabilized by cooperativity between Pc-G proteins and perhaps by multiple PREs. (**C**) Eventually, when GAL−Pc protein decays, the silencing complex has become stably anchored to PREs and can be heritably maintained in the absence of GAL−Pc. Note that in the absence of PREs (i.e. on the *GAL4−NP6* reporter gene), silencing complexes would decay once GAL−Pc is no longer present. For simplicity, activator proteins were omitted, but note that the model does not imply that silencing complexes prevent activator proteins from binding to DNA. Although not shown here, it is conceivable that the silencing complex directly contacts the basal transcription machinery at the proximal promoter and thereby blocks it.

Functional domains in the Pc protein

Several Pc mutations located in the chromodomain abolish chromosome binding. In contrast, GAL−Pc protein bearing one of these mutations (GAL−Pc^{CHD}) retains normal silencing function. This suggests that chromatin binding and silencing might be conferred by separate portions of Pc. Evidently, in GAL−Pc^{CHD} the requirement for a functional chromodomain is bypassed by the tethering function of GAL4 (but recall that the subsequent maintenance of silencing requires the function of the endogenous wild-type Pc protein).

Pc−βgal fusion proteins which lack the C-terminal portion of Pc show a normal binding pattern on salivary gland chromosomes (Messmer *et al.*, 1992), suggesting that this portion of Pc is dispensable for chromosome

binding. However, several lethal *Pc* alleles consist of C-terminal truncations (Franke *et al.*, 1995), implying that chromatin binding alone is insufficient for *Pc* function. The results reported here show that GAL−$Pc^{C\Delta}$ protein fails to silence, although it is present at wild-type levels in the nuclei, binds to *GAL4* binding sites *in vitro* and therefore presumably binds to reporter genes *in vivo*. This shows that the C-terminal portion of Pc is essential for silencing activity. Bearing in mind the model suggested above, a possible explanation might be that this portion contains a domain by which GAL−Pc recruits other proteins to DNA. However, it is also possible that the deletion of the C-terminal portion has an indirect effect by disrupting other parts of the protein. Sequence conservation of the C-terminal portion between Pc and a mouse homologue of Pc (Pearce *et al.*, 1992) argues for the first possibility. It was shown recently that repressor activity of the *Drosophila* Pc protein in a mammalian tissue culture cell assay also requires the C-terminal portion of Pc (Bunker and Kingston, 1994).

Thus, it appears that the Pc protein contains at least two separable functional domains: (i) the chromodomain, by which native Pc recognizes and binds to its target, e.g. to a PRE binding protein or perhaps even to DNA; and (ii) the C-terminal portion, which appears to be a domain required for the recruitment of other non-DNA binding Pc-G proteins into silencing complexes.

The natural 'tether'

I propose that, in the case of *Ubx*, hb acts as the natural 'tether' to recruit Pc-G proteins to DNA for two reasons: (i) hb protein binding sites linked to the *BXD* enhancer initiate *Pc*-dependent silencing (Zhang and Bienz, 1992); and (ii) heritable silencing due to GAL−Pc is established independently of *hb*.

How could hb protein recruit Pc-G proteins? A possible model is that hb protein interacts with factors bound to the proximal *Ubx* promoter to form a negative transcription complex. This postulated negative complex then directly or indirectly promotes the occupancy of PREs by DNA binding Pc-G proteins. Bound to PREs, these proteins would tether other Pc-G proteins to DNA, assembling stable silencing complexes that become independent of hb. Scenarios in which hb protein by direct physical interaction recruits a Pc-G protein to DNA are also possible. Early activators, such as the fushi-tarazu protein (Ingham and Martinez-Arias, 1986; Müller and Bienz, 1992; Qian *et al.*, 1993) or trithorax (Garcia-Bellido and Capdevila, 1978; Ingham, 1983, Mazo *et al.*, 1990), might compete with hb and Pc-G proteins and prevent the establishment of silencing complexes.

Materials and methods

Drosophila strains and transformation

Embryos of a *cn;ry*^{42} strain were injected with the various constructs and transformants were isolated (Bienz *et al.*, 1988). The following mutant alleles were used: *hb*^{9K57} (Lehmann and Nüsslein-Volhard, 1987); *Pc*^{3} (Lewis, 1978); *esc*^{6} (Struhl, 1981); *E(z)*^{s2} (Jones and Gelbart, 1990); *Pcl*^{XM3}, *Sce*^{1} and *Scm*^{D1} (Breen and Duncan, 1986); and *Asx*^{XF23} (obtained from J.Castelli-Gair). *hb*^{9K57} is a null allele exhibiting loss of zygotic *hb* function as well as an antimorphic effect with respect to maternal *hb* function (Lehmann and Nüsslein-Volhard, 1987). This minimizes any functional contribution of maternal hb protein which perdures anteriorly in early *hb*$^{-}$ embryos.

Plasmid constructions and transformants

Reporter genes. *BXD* reporter genes (based on a Carnegie 20 transformation vector), containing the minimal *BXD* enhancer linked to either the proximal *Ubx* promoter or a *hsp70* minimal promoter, and the *LacZ* gene have been described previously (Müller and Bienz, 1991). A modified *BXD* construct, containing a linker with unique *Kpn*I and *Not*I sites between the *BXD* enhancer and the proximal *Ubx* promoter, has also been described previously (Zhang and Bienz, 1992). A *Hin*dIII−*Xba*I fragment containing five optimized *GAL4* binding sites (the '*Sca*I' site 17mer; Webster *et al.*, 1987) was isolated from pF91 (a gift from A.Brand) and inserted (i.e. via a subcloning step in Bluescript) into the *Kpn*I−*Not*I linker of the modified *BXD* construct to obtain 'BGUZ'. 'GBUZ' was obtained by the insertion of five *GAL4* binding sites upstream of the *BXD* enhancer (i.e. via a subcloning step in Bluescript). GBHZ was made by the substitution of the proximal *Ubx* promoter and the *LacZ* gene in GBUZ with the *hsp70* minimal promoter and *LacZ* gene from HZ50 (Hiromi and Gehring, 1987). *GAL4−NP6* was obtained by the insertion of five *GAL4* binding sites upstream of the *NP6* enhancer in NP6−HZ50 (a gift from J.-P.Vincent; Vincent *et al.*, 1990; i.e. *GAL4* and *NP6* were linked via a subcloning step in Bluescript). Similarly, *GAL4−BXD−NP6* was assembled in Bluescript and then inserted into HZ50.

GAL4−mini-white is the *UAS−lacZ* vector (Brand and Perrimon, 1993). To construct the *GAL4−αTubulin−LacZ* reporter gene, an *αTubulin−LacZ* reporter gene containing a 2.5 kb fragment of the *αTubulin* promoter was used, which drives constitutive expression in most cells throughout embryogenesis (J.Müller, unpublished results), fused to the *LacZ* coding region and *αTubulin* 3′ sequences (kindly provided by B.Holmgren). Five *GAL4* binding sites were placed upstream and downstream of this *αTubulin−LacZ* reporter gene to create *GAL4−αTubulin−LacZ*, which was then inserted into a modified C20 vector (J.Müller, unpublished results). *BGUZ* and *GAL4−αTubulin−LacZ* contain 10 *GAL4* binding sites, whereas all other reporter genes contain five *GAL4* binding sites.

Effector constructs. The *hb-GAL−Pc* fusion gene was assembled in Bluescript via various subcloning steps from the following starting plasmids: pE8-B100A, a genomic subclone containing the *hb* gene (Tautz *et al.*, 1987); pMA424, containing the DNA binding domain of GAL4 (Ma and Ptashne, 1987); and Pc12c, containing a *Pc* cDNA (Paro and Hogness, 1991). Convenient restriction sites were engineered by PCR or site-directed mutagenesis to link the different fragments isolated from these plasmids and to obtain hb-GAL−Pc bs [*Kpn*I−*TCGAC-*(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT-*(GAL4 codons 4–147)-*CCGGAATTCCCATCGGGG-*(Pc codons 3–390)-***TGA****GC−Sal*I]. This *hb-GAL−Pc* fusion gene was then subcloned as a *Kpn*I−*Sal*I fragment into the backbone of the transformation vector 'pry', containing RNA trailer sequences from the *Drosophila hsp70* gene downstream of the *Sal*I site, to obtain hb-GAL−Pc pry (i.e. by substitution of the −3.1*Ubx−βgal* fusion gene in 'pry', a modified transformation vector containing a unique *Kpn*I site upstream and a *Sal*I site downstream of the −3.1*Ubx−βgal* fusion gene; Bienz *et al.*, 1988; Müller and Bienz, 1991).

The other effector constructs were made via subcloning steps in Bluescript and then insertion into the pry backbone as *Kpn*I−*Sal*I fragments. They are as follows: hb-GAL bs, *Kpn*I−*TCGAC-*(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT-*(GAL4 codons 4–147)-*CCGGAATTC****TAGT****−Bam*HI−Bluescript polylinker−*Sal*I; hb-Pc bs, *Kpn*I−*TCGAC-*(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT-*(GAL4 codons 74–147)-*CCGGAATTCCCATCGGGG-*(Pc codons 3–390)-***TGA****GC−Sal*I; hb-GAL−Pc^{CHD} bs, *Kpn*I−*TCGAC-*(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT-*(GAL4 codons 4–147)-*CCGGAATTCCCATCGGGG-*(Pc codons 3−*−390)-***TGA****GC−Sal*I [* Pc codon 31 mutated from *ATC* (Ile) to ***TTC*** (Phe), as present in *Pc*^{106}, a *Pc* null allele; Messmer *et al.*, 1992]; and hb-GAL−$Pc^{C\Delta}$ bs, *Kpn*I−*TCGAC-*(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT-*(GAL4 codons 4–147)-*CCGGAATTCCCATCGGGG-*(Pc codons 3–344)-***TAG****TCTA−Sal*I.

The hsp70-GAL−Pc pry construct [*Kpn*I/*Xho*I−*hsp70* promoter−*Xba*I−*GCCGCCAACATGAAGCTT-*(GAL4 codons 4–147)-*CCGGAATTCCCATCGGGG-*(Pc codons 3–390)-***TGA****GC−Sal*I] was made by substitution of the *hb* promoter with a 0.45 kb *Xho*I−*Xba*I fragment from the heat-inducible *hsp70* promoter (derived from pCaSpeR-hs, a gift from C.Thummel; i.e. an mRNA leader fusion was made using the unique *Xba*I site in the *hb* mRNA leader sequence 15 nucleotides upstream of the *hb* initiation codon).

Two further effectors, *hb-GAL-Hb* and *hb-GAL-Su(Hw)*, were made

by substitution of the *Pc* coding region with the coding region of *hb* (derived from pE8-B100A) or *su(Hw)* [a cDNA subclone encoding su(Hw) (Parkhurst *et al.*, 1988) was kindly provided by V.Corces]. hb-GAL-Hb bs: *Kpn*I−*TCGAC*-(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT*-(GAL4 codons 4–147)-*CCGGAATTC*-(hb codons 2–758)-***TAA***−*Sal*I. hb-GAL-Su(Hw): *Kpn*I−*TCGAC*-(*hb* promoter nucleotides 3646–3683)-***ATG****AAGCTT*-(GAL4 codons 4–147)-*CCGGAATTC*-[su(Hw) codons 2–944]-***STOP***−*Sal*I. Both, *hb-GAL-Hb* and *hb-GAL-Su(Hw)* did not confer any silencing when crossed to BGUZ transformants.

Several independent transformant lines were obtained with each construct: BGUZ (eight), GBUZ (11), GBHZ (15), *GAL4−NP6* (eight), *GAL4−BXD−NP6* (12), *GAL4−αTubulin−LacZ* (eight), *hb-GAL−Pc* (five), *hb-GAL* (five), *hb-GAL* (five), *hb-Pc* (four), *hb-GAL−Pc*^{CHD} (six), *hb-GAL−Pc*$^{C\Delta}$ (five), *hsp70-GAL−Pc* (three), *hb-GAL-Hb* (five) and *hb-GAL-Su(Hw)* (five). *GAL4−mini-white* transformant lines were a gift from A.Brand. Independent transformant lines of a given effector consistently gave the same result: either all lines conferred silencing or none of them did.

Only reporter lines showing the normal *BXD* pattern (parasegments 2–14) were included in the analysis; two BGUZ and two GBUZ lines, which showed additional patterns due to position effects, were excluded. In addition to the reporter lines with the normal *BXD* pattern, lines were obtained which showed abnormal *BXD* patterns, not seen with the basic *BXD* construct, with all three types of *GAL4−BXD* reporter construct (Müller and Bienz, 1991). Two types of abnormal *BXD* pattern were observed, (A) The *BXD* pattern is present in parasegments 2–14 but is (often strongly) reduced, with pattern elements erratically missing in several parasegments along the whole body axis. The second type of line (B) shows a very strong *BXD* pattern with ectopic stripes in parasegments 0 and 1. Of the eight *BGUZ*, 11 *GBUZ* and 15 *GBHZ* lines: five, three and four lines, respectively, show the normal *BXD* pattern (in all cases complete silencing in the head and thorax in the presence of *hb-GAL−Pc*); three, six and six lines show a reduced *BXD* pattern (A) (in all cases complete silencing in the head and thorax in the presence of *hb-GAL−Pc*); and none, two and five lines show a *BXD* pattern with ectopic stripes (B) (in all cases only partial silencing in the presence of *hb-GAL−Pc*).

All 12 *GAL4−BXD−NP6* transformants showed the head to tail *BXD* and *NP6* patterns superimposed on each other. The *BXD* pattern was completely (six lines), partially (four lines) or not silenced (two lines) in the presence of *hb-GAL−Pc*, whereas the *NP6* pattern was never suppressed. Eight *GAL4−αTubulin−LacZ* transformant lines showed ubiquitous *LacZ* expression from the blastoderm stage onwards. In none of the eight lines could suppression of *LacZ* expression be detected in the presence of *hb-GAL−Pc*.

Detailed maps of the plasmids used are available on request.

Staining procedures and mutant analysis

Embryos were fixed and stained with a monoclonal antibody against β*gal* (Promega), as described by Lawrence and Johnston (1989).

To analyse silencing by GAL−Pc in *hb* homozygous embryos, *GAL4−BXD* and *hb-GAL−Pc* transformants were separately crossed to flies from a *hb*^{9K57}*/TM3* strain. Their progeny (F_1) were crossed (*GAL4−BXD/+*; *hb*^{9K57}*/+*×*hb-GAL−Pc/+*; *hb*^{9K57}*/+*) and the F_2 progeny were analysed. Embryos homozygous for *hb*^{9K57} were easily identified because of their phenotype. *hb* mutant embryos carrying *hb-GAL−Pc* were identified due to their lack of strong expression anterior to the gap, whereas in the control cross (*GAL4−BXD/+*; *hb*^{9K57}*/+* ×*hb*^{9K57}*/+*) all β*gal*-positive *hb* mutant embryos showed strong ectopic expression anterior to the gap.

For the analysis of GAL−Pc-mediated silencing in *Pc-G* mutant embryos, a recombinant chromosome was constructed on which BGUZ and *hb-GAL−Pc* were linked (i.e. in genetic crosses where this chromosome is transmitted through the male germline, β*gal*-positive progeny in all cases also carry the effector construct). For each *Pc-G* mutation (abbreviated as *M* in the following), homozygous mutant embryos were generated by first generating males BGUZ hb-GAL−Pc/Y; *M/+* and then crossing these with females *M*/Bal from the balanced mutant stock in the case of *Pc*, *Asx*, *Pcl*, *Sce* and *Scm*, or with homozygous females *M/M* in the case of *esc* and *E(z)*. In the case of the *E(z)* cross, embryos were collected at 29°C [i.e. the restrictive temperature for the temperature-sensitive *E(z)*^{s2} allele used (Jones and Gelbart, 1990)]; all other collections were performed at 25°C. Mutant embryos were identified by their ectopic expression and by their mutant phenotype in the gut; in the case of *esc* and *E(z)*, all progeny lack the maternal product and paternally rescued embryos (*M/+* zygotes) show less dramatic ectopic expression compared with their homozygous siblings; the *esc*$^{-}$ embryo shown in Figure 6 is most probably an *esc*$^{-}$*/esc*$^{-}$ zygote.

Heat-shocks

Embryos were heat-shocked in a water bath at 36°C for 20 min. Embryos were collected for 1 h and then heat-shocked once at 1.5–2.5 h of development (blastoderm heat-shock) or at 8–9 h of development (late embryonic heat-shock). Note that heat-shocked *hsp70-GAL−Pc* transformant embryos developed into normal fertile flies. To test whether GAL−Pc can transiently silence the *NP6* enhancer at a distance, the *GAL4−BXD-NP6* reporter gene was used, in which GAL4 binding sites and *NP6* are separated by the 1.6 kb *BXD* enhancer. *GAL4−BXD−NP6* and *hsp70-GAL−Pc* transformants were crossed and their progeny heat-shocked at 8–9 h of development and fixed 3 h later. All embryos showed the *NP6* pattern as indistinguishable from non-heat-shocked control embryos. Under the same conditions, GAL−Pc almost completely silences *NP6* activity in *GAL4−NP6* transformants. To test for transient silencing of the *GAL4−mini-white* gene, transformant females *w/ w*; *GAL4−mini-white/GAL4−mini-white* were crossed to transformant males *w*$^{+}$/Y; *hsp70-GAL−Pc/hsp70-GAL−Pc* (four independent *GAL4−mini-white* transformant lines were tested). If their progeny were heat-shocked during the first and second day after puparium formation [a total of three to six 30 min heat-shocks (36°C) were given over a period of 42 h], the males (*w*/Y; *hsp70-GAL−Pc/GAL4−mini-white*) had completely unpigmented eyes (i.e. indistinguishable from *w*/Y males, one line), showed a strong uniform reduction of pigmentation (i.e. to very pale orange, one line), or showed a weak uniform reduction (i.e. to light orange, one line); one line showed no reduction of pigmentation. If the same series of heat-shocks was applied during the first larval instar, males showed the same orange eye color as males from non-heat-shocked control larvae. If *hb-GAL−Pc* was used to test for the establishment of stable silencing of *GAL4−mini-white*, males also showed the normal orange eye color.

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