

Polycomb group proteins and heritable silencing of *Drosophila* Hox genes

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SUMMARY

Early in *Drosophila* embryogenesis, transcriptional repressors encoded by Gap genes prevent the expression of particular combinations of Hox genes in each segment. During subsequent development, those Hox genes that were initially repressed in each segment remain off in all the descendent cells, even though the Gap repressors are no longer present. This phenomenon of heritable silencing depends on proteins of the Polycomb Group (PcG) and on *cis*-acting Polycomb response elements (PREs) in the Hox gene loci. We have removed individual PcG proteins from proliferating cells and then resupplied these proteins after a few or several cell generations. We show that most PcG proteins are required throughout development: when these proteins are removed, Hox genes become derepressed. However, we find that resupply of at least some PcG

proteins can cause re-repression of Hox genes, provided that it occurs within a few cell generations of the loss of repression. These results suggest a functional distinction between transcriptional repression and heritable silencing: in at least some contexts, Hox genes can retain the capacity to be heritably silenced, despite being transcribed and replicated. We propose that silenced Hox genes bear a heritable, molecular mark that targets them for transcriptional repression. Some PcG proteins may be required to define and propagate this mark; others may function to repress the transcription of Hox genes that bear the mark.

Key words: Cellular memory, Polycomb group, Repression, *Drosophila*, Hox genes

INTRODUCTION

Cell determination in metazoans has been defined as the process whereby one or more cells become committed to follow a particular developmental pathway (Hadorn, 1965). This process often occurs early in development and the descendent cells of the original founders can maintain their determined state for many cell generations without overtly differentiating (Chan and Gehring, 1971; Garcia-Bellido and Capdevila, 1978). In insects, segmental determination is achieved by the transcriptional activation of particular combinations of homeobox-containing (Hox) selector genes (Garcia-Bellido, 1975; McGinnis and Krumlauf, 1992). Such Hox genes encode transcription factors and the particular combination of such factors present in the cells of each segment specifies their determined state (Lewis, 1978; Struhl, 1982; reviewed in McGinnis and Krumlauf, 1992). Studies in *Drosophila* have shown that Hox proteins are continuously required in determined cells and their descendants. Removal of Hox gene function even late in development usually leads to a switch of the determined state (Lewis, 1963; Morata and Garcia-Bellido, 1976; Sanchez-Herrero et al., 1985). Because Hox genes work combinatorially, it is equally important that those genes that are not initially activated in the founding cells of a segment remain silent in all their descendants. Loss of Hox gene silencing also results in switches in the determined state

(Lewis 1978; Struhl, 1981; reviewed by McGinnis and Krumlauf, 1992; Bienz and Müller, 1995).

In *Drosophila*, the heritable silencing of Hox genes occurs in two steps (reviewed by Bienz and Müller, 1995). First, locally expressed Gap gene products such as Hunchback (Hb), Krüppel (Kr) and Knirps (Kni), directly bind to *cis*-acting regulatory sequences in Hox genes during early embryogenesis and repress transcription, thereby delimiting Hox gene expression domains (Qian et al., 1991; Zhang et al., 1991; Müller and Bienz, 1992; Shimell et al., 1994; Zhou et al., 1998). Second, those Hox genes that are initially repressed in each segment become locked into an inactive state so that they remain silent for the rest of development when the Gap repressors are no longer present. Heritable silencing at these later stages requires the products of the Polycomb Group (PcG) genes (Lewis, 1978; Struhl, 1981; Duncan, 1982; Jürgens, 1985), many of which are conserved in both sequence and function in vertebrates (Brunk et al., 1991; van Lohuizen et al., 1991; Pearce et al., 1992; van der Lugt et al., 1994; Müller et al., 1995; Schumacher et al., 1996). In *Drosophila* embryos that lack the function of any of these PcG proteins, Hox gene expression is initiated within the correct spatial domains but soon spreads outside of these domains, becoming general before the end of embryogenesis (Struhl and Akam, 1985; Soto et al., 1995).

Despite the many PcG proteins that have been identified and

characterized, the molecular basis of heritable Hox gene silencing is poorly understood. Silencing appears to involve the acquisition of some kind of mark by Hox genes that are initially repressed. This mark, sometimes referred to as a cellular memory, both confers transcriptional repression and is faithfully propagated each time a silenced gene replicates and the cell divides. Neither the nature of the mark, nor the mechanisms responsible for its acquisition and propagation are known.

Most PcG proteins do not bind to DNA directly. Instead, they appear to bind to the chromatin of specific *cis*-regulatory sequences in Hox genes that are called Polycomb response elements (PREs) (Strutt et al., 1997; Orlando et al., 1998). PREs were initially identified by virtue of their ability to silence inappropriate activation of Hox reporter genes in a PcG protein-dependent fashion (Müller and Bienz, 1991; Simon et al., 1993; Chan et al., 1994). More recently, the removal of a PRE from a silenced gene has been shown to result in a loss of repression, even if the PRE is removed late in development (Busturia et al., 1997). Hence, PREs must be present continuously to maintain the silenced state and therefore appear to be a crucial part of the memory mechanism.

PcG proteins include components of at least two distinct multimeric complexes that each contain different PcG proteins (Franke et al., 1992; Shao et al., 1999; Ng et al., 2000). One complex, PRC1, contains the Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and Sex combs on midlegs (Scm) proteins (Shao et al., 1999), and appears to be associated physically with the chromatin of PREs in formaldehyde crosslinking experiments (Strutt and Paro, 1997; Orlando et al., 1998). A second complex includes the Extra sex combs (Esc) and Enhancer of zeste (E(z)) proteins (Ng et al., 2000). Neither of these complexes appears to contain proteins with DNA-binding activity. The only known DNA-binding PcG protein is Pleiohomeotic (Pho), a zinc-finger protein that is related to the mammalian transcription factor YY1 (Brown et al., 1998). Studies on a *Ultrabithorax* (*Ubx*) PRE suggest that binding of Pho protein to this PRE is essential to establish PcG protein-mediated silencing in embryos and to maintain it in imaginal discs (Fritsch et al., 1999). However, the relatively mild phenotype of *pho* homozygotes suggests that if Pho tethers PcG complexes to DNA, it is probably not the only DNA-binding protein that provides this tethering function (Fritsch et al., 1999).

Two additional properties of PcG proteins are notable. First, experiments using DNA-tethered PcG proteins such as LexA-Psc or Gal4-Pc have established that these fusion proteins function as potent transcriptional repressors in *Drosophila* embryos as well as in mammalian tissue culture cells (Bunker and Kingston, 1994; Müller, 1995). Hence, their recruitment to PRE-containing portions of Hox genes may suffice to repress transcription. Second, studies on the subcellular distribution of PcG proteins have shown that the bulk of Pc, Ph and Psc protein dissociates from chromatin during mitosis (Buchenau et al., 1998; Dietzel et al., 1999). This finding raises questions about how PcG proteins propagate the silenced state from one cell generation to the next.

Most studies of PcG gene function have been performed in embryos, which limits interpretation of their roles in the long-term propagation of heritable silencing. Exceptions are *Polycomb* (*Pc*), *Polycomblike* (*Pcl*), and *super sex combs* (*sxc*),

which have been shown to be required for repression of Hox genes during subsequent development, and *extra sex combs* (*esc*) and *pleiohomeotic* (*pho*), which are crucially needed in the early embryo but appear to play only a minor role during subsequent development (Struhl, 1981; Struhl and Brower, 1982; Duncan, 1982; Ingham, 1984; Busturia and Morata, 1988; Girton and Jeon, 1994; Fritsch et al., 1999).

We show that several other PcG gene products are also required for the stable silencing of Hox genes throughout development. However, these gene products appear to fall into at least two distinct classes depending on whether their elimination leads to a relatively rapid and general derepression of Hox genes, or a slow and spatially more complex destabilization of Hox gene silencing that occurs over many cell generations. We also test whether the loss of silencing caused by eliminating particular PcG gene products can be reversed by resupplying these proteins at later times. Surprisingly, we observe that such reversal can occur, provided that resupply takes place within a few cell generations of the Hox genes becoming derepressed. These last results provide a functional distinction between transcriptional repression and the inheritance of the repressed state. In particular, they suggest that the *cis*-acting mark that confers heritable silencing can persist for at least a few cell generations following transcription activation of a previously silenced gene, allowing the silenced state to be re-established upon resupply of the depleted PcG gene product.

MATERIALS AND METHODS

Transgenes

hs-Pc: a transformant line carrying a P[yhsPc] insert (Fauvarque et al., 1995) on the second chromosome was kindly provided by J.-M. Dura.

hs-Psc and *hs-Su(z)2* transformant lines carrying inserts (Rastelli et al., 1993) on the X chromosome were kindly provided by Vincenzo Pirrotta. A recombinant chromosome carrying *hs-Psc* and *hs-Su(z)2* was then generated.

hs-Scm: an *Eco47III-NotI* fragment from an Scm cDNA clone (Sc9 pNB40; Bornemann et al., 1996) was inserted into CaSpeR-hs to obtain CaSpeR-hs-Scm. The transgene was injected into yw embryos and several *hs-Scm* transformant lines were isolated; an insert on the second chromosome was used.

Drosophila strains

The following mutant alleles were used: *ph*⁵⁰⁴ (called *ph*⁰ in the text) carries mutations in both *ph-d* and *ph-p* and behaves like a null mutation (Dura et al., 1987); *Asx*^{XT129}, *Psc*^{e24}, *Su(z)2*^{1.b7}, *Pcl*^{D5}, *E(z)*⁶³ are either protein-null mutations or behave like null mutations; *Su(z)2*^{1.b8} is a deficiency that removes both *Psc* and *Su(z)2* (see Soto et al., 1995 for details); *Pc*^{XT109} is a protein-null allele (Franke et al., 1995); *Scm*^{D1} is a frameshift mutation that genetically behaves as a null allele (Bornemann et al., 1998); *E(z)*⁶³ is a protein-null mutation (Carrington and Jones, 1996); and *Sce*^{D1} is an apparent null mutation (Breen and Duncan, 1986).

The following strains were used in this study:

*ph*⁵⁰⁴ w *FRT101/FM7c*
 yw; *FRT42D Su(z)2*^{1.b8}/*SM6b*
 yw; *FRT42D Su(z)2*^{1.b7}/*SM6b*
 yw; *FRT42D Psc*^{e24}/*SM6b*
 yw; *FRT42D Pcl*^{D5}/*CyO*
 yw; *FRT42B sca Asx*^{XT129}/*CyO*
 w; *E(z)*⁶³ *FRT2A/TM6B*

w; hs-CD2 ri Pc^{XT109} FRT2A/TM6B
w; FRT82B Scm^{D1}/TM6B
w; FRT82B Sce¹/TM6B
y w hs-Psc hs-Su(z)2; FRT42D Su(z)2^{1.b8}/SM6b
w; hs-Scm; FRT82B Scm^{D1}/TM3
w; hs-Pc; hs-CD2 ri Pc^{XT109} FRT2A/ TM3
w flp122 hs-nGFP FRT101
yw flp122; FRT42D hs-nGFP
yw flp122; FRT42B hs-nGFP
yw flp122; hs-nGFP FRT2A
yw flp122; M(3)ⁱ⁵⁵hs-nGFP FRT2A
yw flp122; FRT82B hs-nGFP

Analysis of clones and heat shock regimes

Clones were generated by crossing the appropriate fly strains listed above and heat-shocking the F1 larvae. Heat shock treatment to induce clones was done in vials for 1 hour in a 37°C water bath; larvae were then allowed to develop for the appropriate time at 25°C. Prior to dissection larvae were subjected to another 1 hour heat shock followed by a 1 hour recovery period to induce expression of the GFP marker protein. For the rescue experiments shown in Fig. 3, 1 hour heat shocks were applied every 12 hours over a 96 hour period; for the resupply experiments in Figs 4-6, 1 hour heat shocks were given every 6 hours over a 24 or 48 hour period as indicated.

Staining procedures

Inverted larval carcasses were fixed and double-labeled with antibodies against Ubx or Abdominal-B or Caudal and GFP, followed by incubation with fluorescently labeled (Cy3 and DTAF) secondary antibodies. Discs were mounted in MOWIOL containing Dabco.

RESULTS

Requirements for PcG genes for the heritable silencing of Hox genes in the imaginal wing disc

Homozygotes for most PcG mutations die as embryos. To test the long-term requirements for the PcG genes *Pc*, *ph*, *Psc*, *Suppressor of zeste 2* (*Su(z)2*), *Scm*, *Pcl*, *Sex combs extra* (*Sce*), *E(z)* and *Additional sex combs* (*Asx*), we used Flp-mediated mitotic recombination (Golic, 1991) to generate clones of cells that lack the function of these genes. To assay Hox gene silencing in such clones, we monitored the expression of three Hox genes *Ubx*, *Abdominal-B* (*Abd-B*) and *caudal* (*cad*) in the imaginal wing disc, where they are normally stably repressed using antisera against their protein products. For each PcG locus, we used mutant alleles that do not produce a protein or are at least genetically defined as null alleles (see Materials and Methods). Two of these PcG loci (*Psc* and *ph*) each contain two neighboring transcription units that encode closely related proteins that can partially substitute for each other (Dura et al., 1987; Brunk et al., 1991; van Lohuizen et al., 1991; Wu and Howe, 1995; Soto et al., 1995). Therefore, in the case of *Psc*, we used a deficiency that removes both *Psc* and the related *Su(z)2* gene (Wu and Howe, 1995); we shall refer to this double mutant as *Psc-Su(z)2*. Similarly, for *ph* we used an allele that carries lesions in both the proximal and the distal *ph* transcription unit and we shall refer to the double mutant as *ph⁰* (Dura et al., 1987). In all experiments, PcG mutant cells were identified by the absence of a GFP-expressing marker gene (see Materials and Methods).

In a first set of experiments, we analyzed clones 24, 36, 48, 72 and 96 hours after clone induction. We found that the clones of individual PcG mutations showed profound differences with

respect to both misexpression of Hox genes as well as the general phenotype of the clones. Based on these two criteria we subdivide the different PcG genes into three groups and present the results obtained with members of each group in turn. Group 1 includes *Psc-Su(z)2* and *ph*, group 2 includes *Pc*, *Scm*, *Sce* and *Pcl*; and group 3 is represented by *E(z)* and *Asx*.

Psc-Su(z)2 and *ph⁰* mutant clones

The group 1 mutants *Psc-Su(z)2* and *ph⁰* show the most severe phenotypes of all PcG mutants (Figs 1, 2). First, both *Psc-Su(z)2* and *ph⁰* mutant clones show strong misexpression of all three Hox genes, although the timing of misexpression differs for each Hox gene. High levels of *Ubx* misexpression are already apparent within 24 hours of clone induction (Fig. 2). Misexpression of *Abd-B* is also detectable within 24 hours of clone induction and accumulates to high levels by 48 hours (Fig. 2). *cad* misexpression is first detected at 48 hours and accumulates to high levels by 72 hours (the late accumulation of high levels of *cad* in these clones is also correlated with a late reduction in the level of *Ubx* misexpression, possibly reflecting downregulation of *Ubx* by *cad* (Fig. 2). Second, a striking phenotype of *Psc-Su(z)2* and *ph⁰* mutant clones is the large size and rounded shape of the clones (Figs 1, 2), reminiscent of clones of mutations that cause disc tumors (Xu et al., 1995; Justice et al., 1995). This phenotype is not observed for clones of any of the other PcG mutants. We find that in the absence of *Psc-Su(z)2* or *ph* function, imaginal disc cells are larger than wild-type cells (data not shown) but additional studies will be needed to establish how cell growth and cell division rates are changed in these mutant clones. Finally, we note that for all three Hox genes, misexpression is observed first in clones located within the central 'wing pouch' part of the disc (destined to give rise to wing blade), and only later in clones located in more peripheral portions of the disc (destined to form the prospective wing hinge and notum).

Psc and *Su(z)2* are adjacent genes that encode related proteins that can partially substitute for each other in embryos (Soto et al., 1995). To test the independent contributions of these genes in later development, we also monitored *Ubx* and *Abd-B* expression in *Psc* single mutant clones or in discs of *Su(z)2* homozygous larvae (Fig. 1). We did not observe misexpression of either gene, suggesting that each of these two functions can fully substitute for the other in imaginal disc cells.

Pc, *Pcl*, *Scm* and *Sce* mutant clones

Clones of the group 2 mutants *Pc*, *Scm*, *Sce* and *Pcl* also show misexpression of *Ubx* and *Abd-B* (Figs 1-3). However, in contrast to *Psc-Su(z)2* and *ph⁰*, clones that are mutant for any one of these four genes show a considerable delay in the onset of *Ubx* and *Abd-B* misexpression (Fig. 1). We first detect *Ubx* and *Abd-B* misexpression between 36 and 48 hours after clone induction. However, misexpression at this time is generally confined to clones in the wing pouch and, in the case of *Abd-B*, the level of misexpression is relatively low (Fig. 2). By 96 hours after clone induction, most group 2 mutant clones show strong expression of *Ubx* and *Abd-B*; but we often find that both genes are still repressed in some clones located in the prospective wing hinge and notum (Fig. 1). We also assayed *cad* expression in these clones, but failed to detect any evidence for *cad* misexpression even 96 hours and more after clone

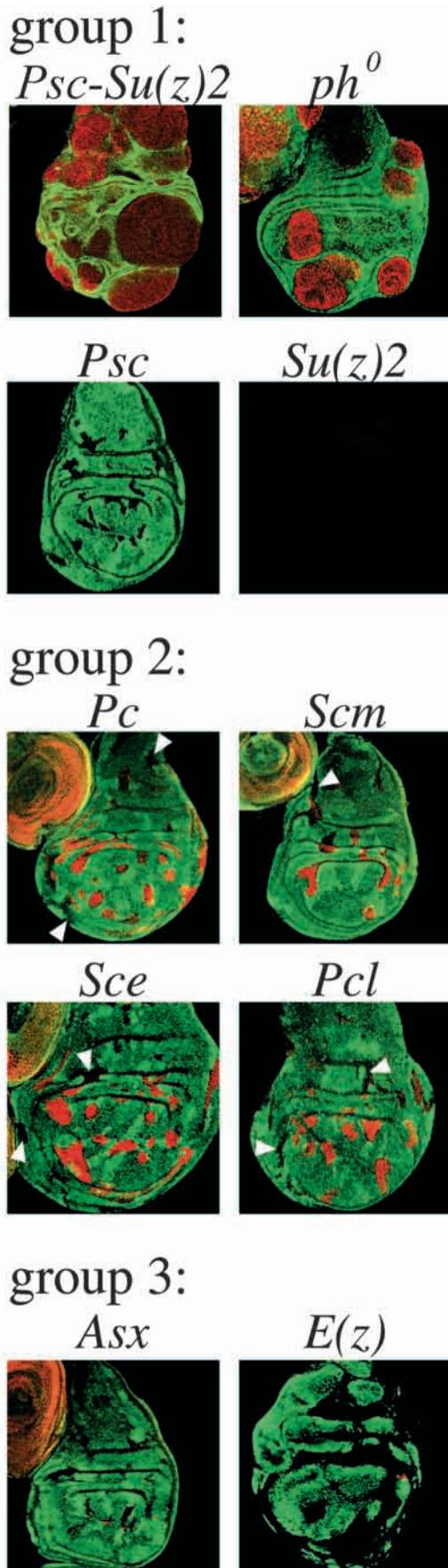


Fig. 1. PcG genes function throughout development to repress Hox genes. Wing imaginal discs with clones of cells that are homozygous for the indicated PcG mutations were stained with antibodies against GFP (green) and Ubx (red; *Pc*, *Scm*, *Sce*, *Pcl*, *Asx*, *E(z)*) or Abd-B protein (red; *ph⁰*, *Psc*, *Su(z)2*, *Psc-Su(z)2*), respectively. Neither Ubx nor Abd-B protein are normally expressed in the wing imaginal disc. In each case, homozygous PcG mutant cells are marked by the absence of GFP protein (green); in the case of *Su(z)2*, an imaginal disc from a *Su(z)2* homozygous larva is shown, in all other cases clones were induced 96 hours prior to analysis. In the *E(z)* experiment, the Minute technique was used (see text); without the Minute technique, *E(z)* mutant clones are eliminated by 96 hours after clone induction. Strong misexpression of Ubx and Abd-B is detected in almost all PcG mutant clones but note that in the group 2 mutants Ubx is still repressed in certain parts of the disc (white arrowheads, see text). No misexpression is seen in *Asx* or *E(z)* mutant clones except for some rare Ubx-positive cells near the presumptive wing margin; *Psc* and *Su(z)2* single mutant clones also show no misexpression but strong misexpression of *Abd-B* is seen in *Psc-Su(z)2* mutant clones. Note the relatively larger size of the *ph⁰* and *Psc-Su(z)2* mutant clones.

induction. Nevertheless, we presume that *cad* is eventually misexpressed, at least in the case of *Pc* mutant clones, because these clones differentiate ectopic analia-like structures in the adult (Struhl, 1981), an outcome that should require *cad* gene activity (Moreno and Morata, 1999).

The longer lag period between clone induction and the loss of silencing observed for group 2 mutant clones could, in principle, be due to the perdurance of PcG gene product in mutant cells. However, we consider this unlikely, at least in the case of *Pc*, for two reasons. First, we have assayed Pc protein expression in *Pc* mutant clones and find that it is undetectable by 48 hours, when *Ubx* is strongly misexpressed in some clones but not in others. Second, we also performed the Pc experiment using the Minute technique to generate *Pc⁻/Pc⁻* clones that carry two copies of a wild-type Minute allele (i.e. *Pc⁻M⁺/Pc⁻M⁺*), which gives them a growth advantage relative to their *Pc⁻M⁺/Pc⁺M⁻* neighbors. Although these clones are indeed larger than *Pc* mutant clones generated without using the Minute technique (compare Fig. 1 with Fig. 2), we nevertheless observe a similar delay in the onset of *Ubx* and *Abd-B* misexpression in both cases (data not shown; Fig. 2 shows the results of the analysis using the Minute technique). Moreover, some clones fail to show *Ubx* and *Abd-B* misexpression, even after 96 hours, when they can form large portions of the disc.

Thus, group 2 mutant clones differ from group 1 mutant clones specifically in the timing of misexpression, which is significantly delayed in all respects. Nevertheless, we note that both classes of mutant clones show the same rank order in Hox gene misexpression, with *Ubx* being misexpressed earlier than *Abd-B*, and *cad* being misexpressed later, if at all. They also show similar regional differences in the timing of misexpression: in particular, *Ubx* and *Abd-B* are first misexpressed in the wing pouch, and only later in the presumptive wing hinge and notum.

Asx and *E(z)* mutant clones

Asx and *E(z)* mutant clones show no misexpression of either *Ubx* or *Abd-B* even 96 hours after clone induction, except near the presumptive wing margin where we occasionally find some

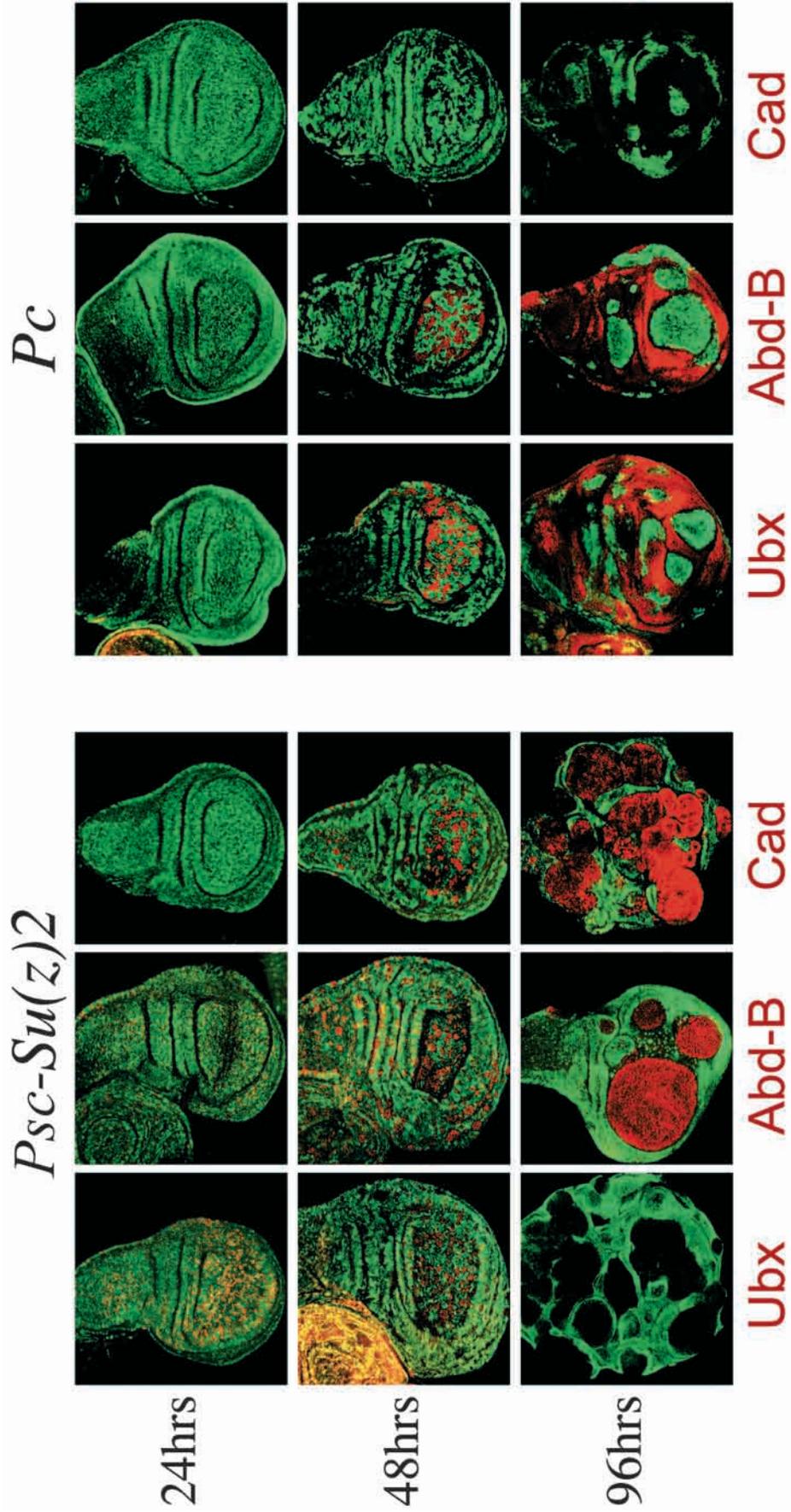


Fig. 2. Kinetics of derepression of the Hox genes *Ub*, *Abd-B* and *cad* in group 1 and group 2 mutant clones. Wing imaginal discs with *Psc-Su(z)2* (left) and *Pc* (right) mutant clones: 24, 48 and 96 hours after clone induction, respectively; the Minute technique was used in the *Pc* experiments. Discs were stained with antibodies against Ub, Abd-B or Cad protein as indicated (red in each case), and clones are marked by the absence of GFP protein (green). Repression of each target gene is lost significantly faster in *Psc-Su(z)2* mutant clones than in *Pc* mutant clones: 24 hours after clone induction, *Psc-Su(z)2* mutant clones show strong misexpression of *Ub* and moderate misexpression of *Abd-B* whereas both genes are still repressed in *Pc* mutant clones. 48 hours after clone induction, *Psc-Su(z)2* mutant clones show strong misexpression of *Abd-B* throughout the disc and *cad*

starts to become misexpressed in the wing pouch and notum, whereas Ub signal is becoming downregulated (see text). In contrast, in the case of *Pc*, 48 hours after clone induction only clones in the pouch show strong misexpression of Ub and weak misexpression of *Abd-B*, whereas both Hox genes are still repressed in clones in other regions of the disc; no misexpression of *cad* is detected. 96 hours after clone induction, strong misexpression of *Abd-B* and *cad* is present in all *Psc-Su(z)2* mutant clones and Ub signal is no longer detectable; *Pc* mutant clones show strong expression of *Ub* and *Abd-B* but both Hox genes are still repressed in parts of the notum and in the wing hinge region; no Cad protein is detected. See text for further details.

Ubx-expressing cells (Fig. 1). The *E(z)* experiment was performed using the Minute technique, as it appears that *E(z)* mutant clones are eliminated by cell competition if not given a proliferation advantage (Fig. 1 and data not shown). In the haltere, the wild-type expression pattern of *Ubx* is unaffected in *E(z)* mutant clones, suggesting that the lack of misexpression is not because the cells are defective in cellular processes such

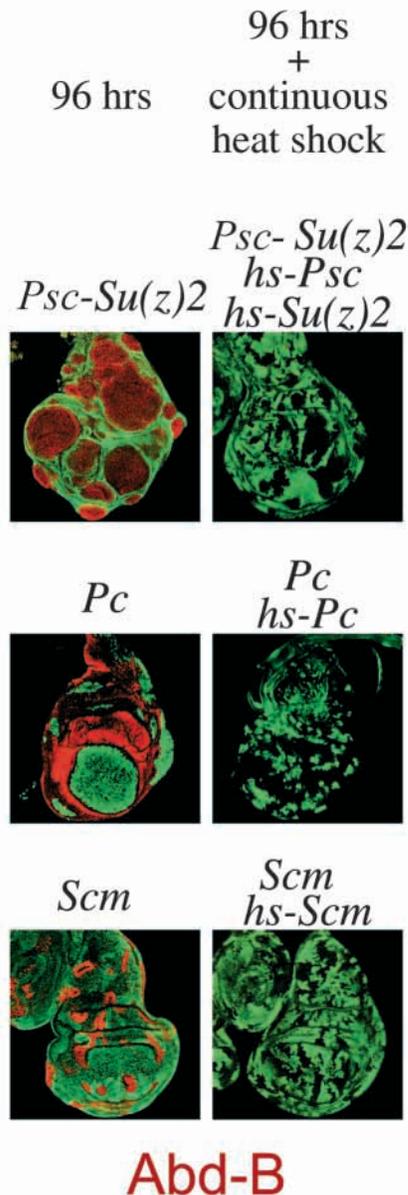


Fig. 3. Rescue function of *hs-Psc*, *hs-Su(z)*, *hs-Pc* and *hs-Scm* transgenes. 96 hours after clone induction, *Psc-Su(z)2*, *Pc* and *Scm* mutant clones in wing imaginal discs all show strong misexpression of Abd-B protein (left column); in the case of *Pc*, the Minute technique was used. Animals carrying the indicated *hs-PcG* transgene(s) (right column) were repeatedly heat-shocked for 1 hour every 12 hours beginning at the time of clone induction. *Abd-B* stays tightly repressed and the tumorous phenotype of *Psc-Su(z)2* mutant clones is rescued. Owing to the repeated heat shocks, additional clones are induced each time, but note that even the biggest clones that were induced by the first round of clone induction show no Abd-B signal.

as transcriptional regulation. Thus, although both *Asx* and *E(z)* are needed to repress Hox genes in the embryo (Jones and Gelbart, 1990; Simon et al., 1992; Soto et al., 1995; Sinclair et al., 1998), their functions appear to be largely dispensable for the maintenance of repression in imaginal disc cells. In this respect, both *Asx* and *E(z)* are similar to *esc* and *pho*, which play relatively minor roles in Hox gene silencing during imaginal disc development. Hence, we put all four genes in a separate group, group 3.

We note that our results with *E(z)*, obtained using clones of cells homozygous for a protein-null allele, differ from those obtained using temperature shifts to manipulate the function of a temperature-sensitive mutant allele (LaJeunesse and Shearn, 1996). In contrast to our findings, such temperature-shift experiments suggest that late loss of *E(z)* function can cause *Ubx* misexpression. This difference could be attributed to an exceptionally long perdurance of the wild-type gene product in our experiments, or to suboptimal levels of gene function throughout development in the temperature shift experiments. The failure of *E(z)* null mutant cells to survive unless given a proliferative advantage using the Minute technique argues against the first explanation. Nevertheless, our placement of *E(z)* in group 3 is tentative.

Resupply of some PcG proteins can restore heritable silencing

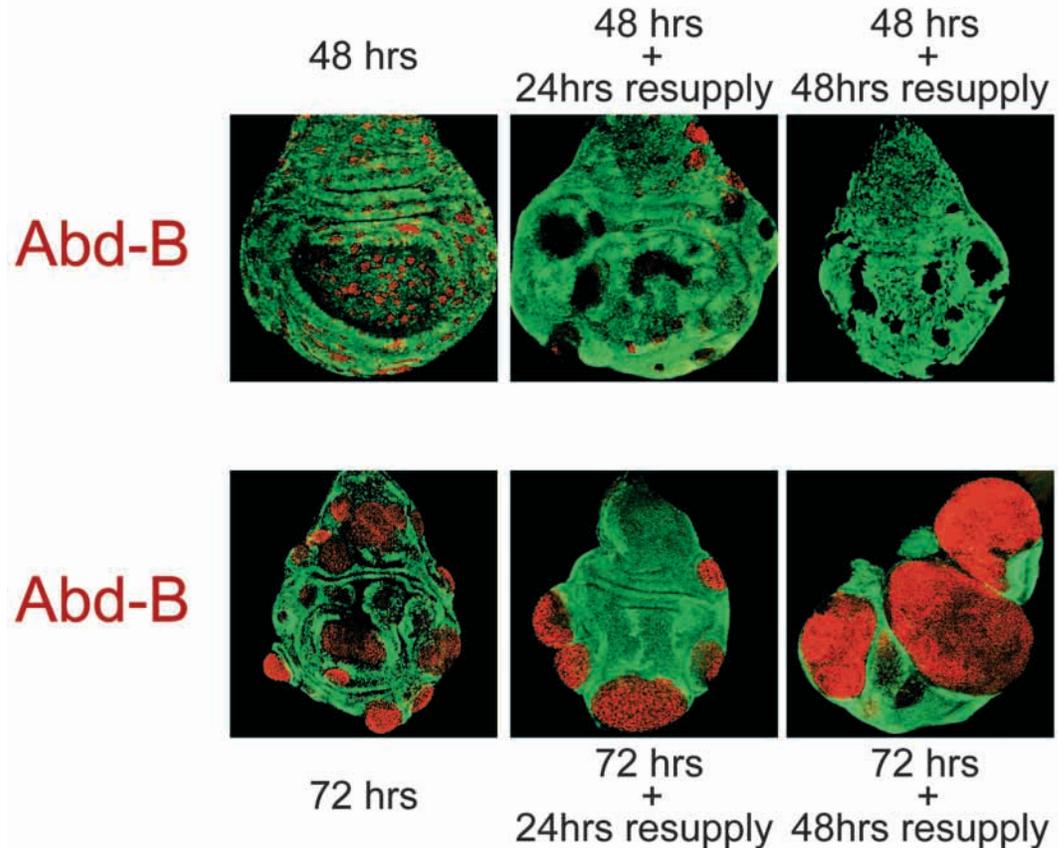
The relationship between transcriptional repression and the stable inheritance of Hox gene silencing is poorly understood. As noted in the introduction, it is generally assumed that silenced Hox genes are marked in some way that targets them for transcriptional repression and that this mark is stably inherited each time the gene replicates. However, it is not known whether transcriptional repression is required to maintain the mark. To examine whether transcriptional repression is required for the stable inheritance of the silenced state, we asked whether the loss of silencing caused by removing particular PcG gene products can be reversed by resupplying the same products at later times.

As a first step towards performing this experiment, we asked whether a regular supply of PcG protein expressed under heat-shock control from an *hsp70-PcG* gene transgene can rescue Hox gene silencing in corresponding PcG mutant clones. Three PcG mutant conditions were examined: *Psc-Su(z)2*, *Pc* and *Scm*. In each case, mutant clones were induced in first instar larvae that also carried the appropriate *hsp70-PcG* transgene(s) (both *hsp70-Psc* and *hsp70-Su(z)2* transgenes were used together for the supply to *Psc-Su(z)2* mutant clones). Beginning at the time of clone induction, these larvae were then repeatedly heat-shocked every 12 hours over a 96-hour period, and *Ubx* and *Abd-B* expression assayed after the last heat shock. For all three mutant conditions, no ectopic *Ubx* or *Abd-B* expression was observed (Fig. 3 and data not shown). We note that for all three cases, heat shock-induced expression of these PcG gene products does not alter the expression of either *Ubx* or *Abd-B* in their normal domains (e.g., in the haltere disc for *Ubx* or in the central nervous system for both genes; data not shown).

Thus, for each mutant condition, we find that a continuous supply of PcG gene product is sufficient to maintain Hox gene silencing in the absence of endogenous gene function. We next resupplied PcG proteins to PcG mutant clones in which

Psc-Su(z)2 hs-Psc hs-Su(z)2

Fig. 4. Resupply of Psc and Su(z)2 proteins to *Psc-Su(z)2* mutant clones. *Psc-Su(z)2* mutant clones in wing discs from larvae carrying a *hs-Psc* and a *hs-Su(z)2* transgene, stained with antibodies against Abd-B (red) and GFP (green). Before the resupply is started, Abd-B signal is comparably strong 48 hours (top left) and 72 hours (bottom left) after clone induction. Psc and Su(z)2 proteins were then resupplied over a 24 hour (middle) or a 48 hour period (right) by giving 1 hour heat shocks every 6 hours. *Abd-B* becomes completely re-repressed and the tumorous phenotype of the clones is rescued if the resupply is started 48 hours after clone induction. No re-repression is observed if the resupply is started 72 hours after clone induction and the overgrowth phenotype of the clones is also no longer rescued.



repression had been lost. In all experiments, clones of mutant cells were induced early in larval life and resupply was achieved by heat-shocking larvae every six hours over a 24 hour or 48 hour period, beginning 48 or 72 hours after the initial clone induction.

In a first set of experiments, we analyzed *Psc-Su(z)2* mutant clones. Because derepression of *Ubx* is transient in *Psc-Su(z)2* mutant clones, possibly owing to downregulation by derepressed Cad protein (Fig. 2), we analyzed only the expression of *Abd-B* after resupply of *Psc* and *Su(z)2* gene function. 48 hours after clone induction, *Psc-Su(z)2* mutant cells show robust *Abd-B* expression (Fig. 4). If the resupply of *Psc* and *Su(z)2* gene function is started 48 hours after clone induction, *Abd-B* expression becomes undetectable in most clones after a 24 hour resupply period and is absent from all clones after a 48 hour resupply period. Thus, resupply of *Psc* and *Su(z)2* function to these clones results in complete re-repression of *Abd-B* (Fig. 4). However, if the resupply is started 72 hours after clone induction, even extensive resupply of *Psc* and *Su(z)2* function over a 48 hours period does not result in a reduction of *Abd-B* expression (Fig. 4). Thus, it appears that *Abd-B* can no longer become re-repressed after a period of prolonged depletion of *Psc-Su(z)2* gene function. We also note that the tumor-like phenotype of *Psc-Su(z)2* mutant clones, like the misexpression of *Abd-B*, is rescued if the resupply is started 48 hours after clone induction but not if the resupply is started after 72 hours (Fig. 4).

We next analyzed *Pc* mutant clones. As described above, *Pc* mutant clones show a longer delay in the appearance of *Ubx* and *Abd-B* misexpression than *Psc-Su(z)2* mutant clones, even if the Minute technique is used. In particular, 48 hours after clone induction, misexpression of *Ubx* and *Abd-B* is confined to clones in the wing primordium, and both Hox genes are still repressed in clones at the periphery of the wing disc (Fig. 5). Further, *Abd-B* expression at this time is significantly weaker than that observed after 72 or 96 hours (Figs 2, 3, 5), suggesting that *Abd-B* is only partially derepressed after 48 hours. If we start to resupply *Pc* protein 48 hours after clone induction, we find that *Abd-B* expression is no longer detected after a 24 hour resupply period (Fig. 5); this suggests that *Abd-B* becomes completely re-repressed. In contrast, *Ubx*, which is already strongly derepressed at the time of resupply, does not appear to be re-repressed (Fig. 5). The same results are obtained if *Pc* is resupplied over a 48 hour period (data not shown). Similar results were obtained when we started the resupply 72 hours after clone induction (Fig. 5): both *Ubx* and *Abd-B* appear to be re-repressed only in clones located in peripheral portions of the disc, where they were only partially derepressed when resupply began, but not in clones in the center of the wing disc, where they were already strongly expressed (Fig. 5). We note that it is unlikely that re-repression of *Ubx* and *Abd-B* is masked in these experiments by the persistence of their protein products generated prior to resupply because we can readily detect re-repression of *Abd-B* within 24 hours of resupply in

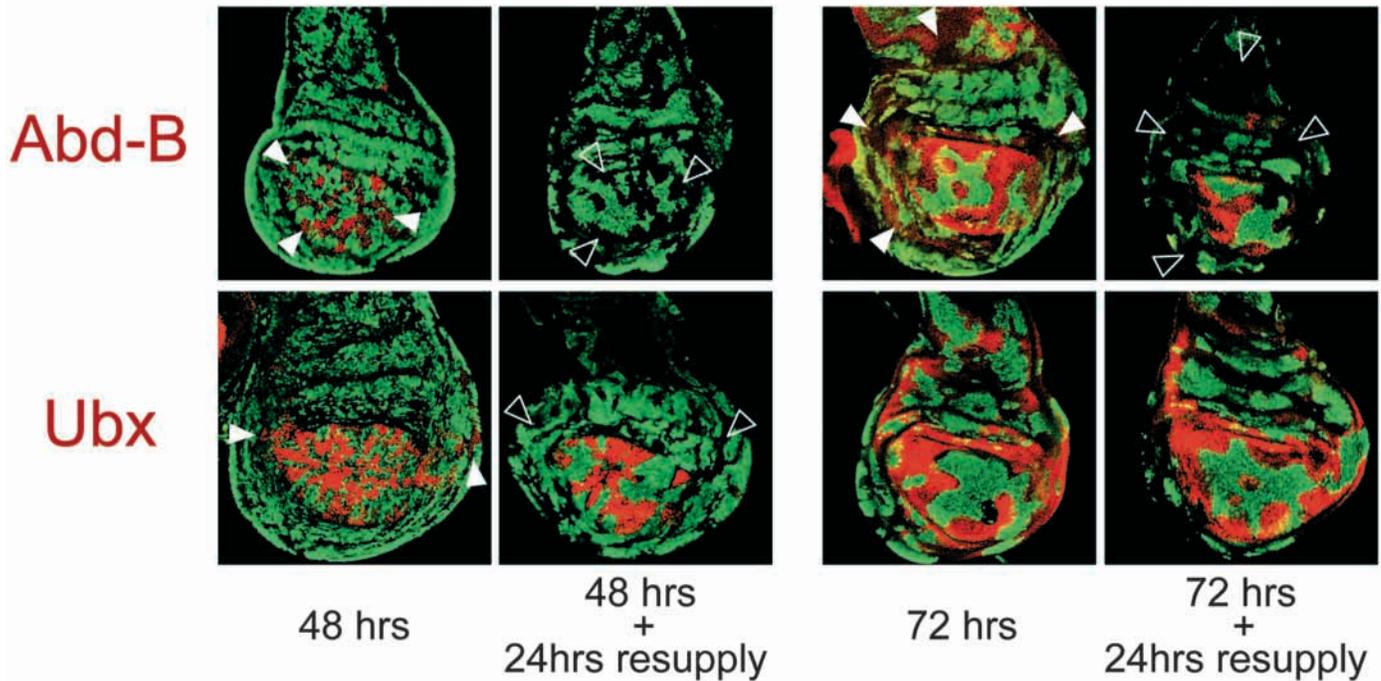
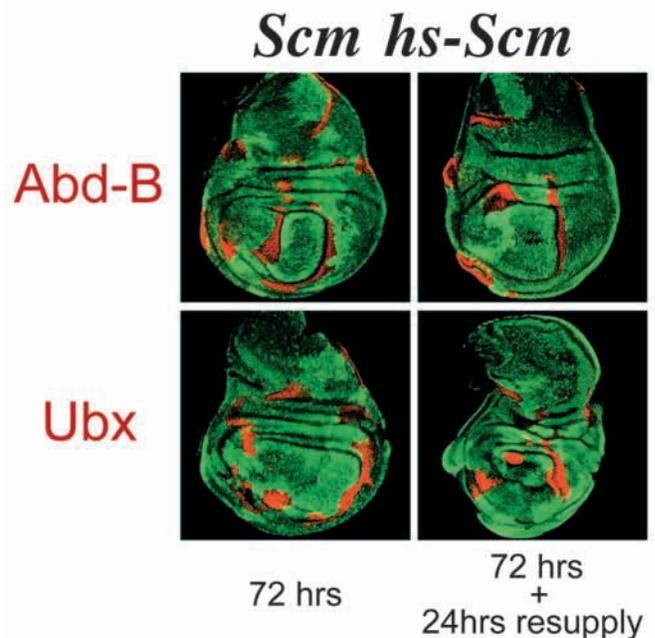
Pc hs-Pc

Fig. 5. Resupply of Pc protein to *Pc* mutant clones. *Pc* mutant clones in wing discs from larvae carrying a *hs-Pc* transgene, stained with antibodies against GFP (green) and Abd-B (top row) or Ubx (bottom row), in each experiment the Minute technique was used. The resupply was started 48 hours (left) or 72 hours (right) after clone induction and the heat shock regime described in Fig. 4 was used. (Left) 48 hours after clone induction, weak misexpression of *Abd-B* is seen in clones in the center of the wing discs (white arrowheads), note that *Abd-B* is completely re-repressed in all clones after the resupply (empty arrowheads). 48 hours after clone induction, strong misexpression of *Ubx* is seen in clones in the pouch, whereas clones in more peripheral parts of the disc show weaker misexpression (white arrowheads); after the resupply, *Ubx* is only re-repressed in clones that showed weak *Ubx* expression before the resupply. (Right) 72 hours after clone induction, strong misexpression of *Abd-B* is seen in clones in the center of the wing disc whereas clones in peripheral parts of the disc show weaker misexpression (white arrowheads). Note that after the resupply, *Abd-B* is re-repressed, but only in clones in peripheral parts of the disc (empty arrowheads) and not in clones in the center of the disc. Note that the strong *Ubx* signal present 72 hours after clone induction is not significantly reduced after the resupply.

Psc-Su(z)2 mutant clones (Fig. 4) and downregulation of strong *Ubx* expression in group 1 mutant clones occurs over a period of 24 hours (Fig. 2 and data not shown). Thus, resupply of *Pc* appears to restore repression in cells in which *Ubx* and *Abd-B* are only partially derepressed but not in cells in which these genes are fully derepressed.

Finally, we performed resupply experiments on *Scm* mutant clones. Only a fraction of *Scm* mutant clones examined 48 hours after clone induction show misexpression of *Ubx* and *Abd-B*, and the level of misexpression appears low. However, after 72 hours, most clones show robust misexpression of both *Ubx* and *Abd-B* (Fig. 6). When we resupplied *Scm* gene product for 24 hours beginning 48 hours after clone induction, we observed that some clones now expressed high levels of

Fig. 6. Resupply of *Scm* protein to *Scm* mutant clones. *Scm* mutant clones in wing discs from larvae carrying a *hs-Scm* transgene, stained with antibodies against GFP (green) and Abd-B (top) or Ubx (bottom). 72 hours after clone induction, most clones show strong misexpression of *Abd-B* and *Ubx*. Neither *Abd-B* nor *Ubx* are re-repressed after resupplying *Scm* protein over a 24-hour period using the heat shock regime described in Fig. 4.



Ubx and *Abd-B*, but the fraction and location of clones that show misexpression remained similar to that before resupply began (data not shown). When resupply was begun 72 hours after clone induction, we failed to find evidence for repression (Fig. 6).

Thus, in the case of *Psc-Su(z)2* mutant clones, resupply of wild-type gene function can restore silencing of both *Ubx* and *Abd-B*, provided that resupply occurs within a window of opportunity of approx. 24 hours after the genes are fully derepressed. For *Pc* and *Scm* mutant clones, resupply either fails to restore silencing, or can do so only if it occurs before *Ubx* and *Abd-B* are fully derepressed. Finally, for all three PcG mutant conditions, repression can no longer be restored after prolonged absence of the wild-type function.

DISCUSSION

Heritable silencing of Hox genes is thought to depend on a common *cis*-acting property of the silenced genes that both marks them for transcriptional repression and is replicated together with the genes themselves, allowing the silenced state to be propagated to both daughter cells following cell division. The nature of this *cis*-acting property remains mysterious, although it is generally assumed to be an aspect of chromatin structure, such as the degree of compaction, the state of histone acetylation or the association of particular accessory proteins. Even more mysterious is the mechanism by which such a state might be stably inherited each time a silenced Hox gene replicates and the cell divides. Although our present results do not resolve either mystery, they do provide evidence for a functional distinction between transcriptional repression and the inheritance of the repressed state. Specifically, we find that depletion of some PcG proteins can lead to the relatively rapid derepression of silenced Hox genes, without disrupting their ability to be repressed subsequently upon resupply of the depleted protein. Hence, the *cis*-acting mark that targets silenced Hox genes for transcriptional repression can be retained, at least for a few cell divisions, even if the gene is derepressed. Our results also indicate that the stability of Hox gene silencing varies for different Hox genes in the same segment, and for the same Hox gene in different regions within a segment. As we discuss below, these results constrain possible models that have been put forward to explain the heritability of the silenced state.

Different requirements for PcG gene products

Previous analyses suggested that all known PcG genes were needed to repress inappropriate Hox gene expression in the embryo, but that PcG genes fell into two groups with respect to their requirements in imaginal discs. In particular, members of one group, epitomized by *esc* and *pho*, appear to play crucial roles only early in development, when heritable silencing is first established (Struhl, 1981; Struhl and Brower, 1982; Breen and Duncan, 1986; Girton and Jeon, 1994; Fritsch et al., 1999). In contrast, members of the other group, such as *Pc* and *Pcl*, are required throughout subsequent development to ensure that heritable silencing is maintained (Struhl, 1981; Duncan, 1982).

The results of this study suggest that the majority of known PcG genes falls into the class of continuously required PcG genes and that this class can itself be further subdivided into

at least two distinct groups. The first group, which includes *Psc-Su(z)2* and *ph*, appears to be required continuously for the transcriptional repression of Hox genes. Removal of these gene products results in a relatively rapid and complete derepression of all Hox genes. By contrast, the second group, which includes *Pc*, *Scm*, *Pcl* and *Sce*, appears to be required for the long-term stability of the silenced state. Removal of these gene products leads to Hox gene misexpression, but only after relatively long lag periods during which some Hox genes can remain repressed for several, and in some cases many, cell generations.

The distinction between group 1 and group 2 genes might, in principle, be a trivial reflection of differences in the half-lives of their gene products: group 2 products might persist much longer than group 1 products and hence rescue the silenced state of Hox genes over a longer period. Although we cannot eliminate this possibility, we think it unlikely for at least two reasons. First, in the case of *Pc*, a group 2 gene, we find that some Hox genes can remain repressed for extended periods of time after we can no longer detect residual Pc protein in mutant clones (e.g. *Abd-B* in ventral portions of the prospective wing hinge and *cad* in the entire wing disc). Second, maternally supplied products of some group 1 and group 2 genes (e.g. *ph*, *Psc-Su(z)2*, *Pc*) fail to rescue the absence of zygotic gene function during embryogenesis (McKeon and Brock, 1991; Simon et al., 1992; Soto et al., 1995). Because such mutant embryos show a general loss of Hox gene silencing by 12 hours postfertilization, we infer that their products have similarly limited half lives that are less than 12 hours. Thus, we favor the view that group 1 and group 2 PcG proteins have different properties because they perform different roles in heritable gene silencing. As we discuss below, the results of our resupply experiments lead us to suggest that group 2 PcG proteins are required to maintain or propagate the *cis*-acting mark that targets silenced Hox genes for transcriptional repression, whereas group 1 PcG proteins are required to repress Hox genes that bear this mark.

Resupply experiments: the role of transcriptional repression in maintaining the silenced state

A major uncertainty in understanding Hox gene silencing is the causal relationship between transcriptional repression and inheritance of the silenced state. Here, we have addressed this uncertainty by first depleting specific PcG gene products and then resupplying them at varying time intervals after Hox genes become derepressed. The most informative results were obtained for resupply experiments involving *Psc-Su(z)2*, a group 1 gene locus. Removal of *Psc-Su(z)2* gene function causes robust mis-expression of Hox genes within 48 hours. However, this misexpression can be reversed, and silencing re-established, by resupply of the wild-type gene function, provided that it occurs within a window of opportunity between 48 and 72 hours after the initial removal of wild type gene function. Resupply after this time is no longer effective in re-establishing silencing.

At a minimum, these results suggest that transcription per se does not immediately remove the mark that targets Hox genes for silencing. Instead, this mark appears only to be lost when transcription persists for a period of up to 24 hours, during which the cells probably undergo two or more division cycles. One interpretation of this finding is that *Psc-Su(z)2* function is specifically involved in mediating the transcriptional

repression of Hox genes that are marked for silencing. In the absence of *Psc-Su(z)2* function, repression is lost, but the mark can be retained for at least a few cell generations. This mark could be residual PcG proteins aside from Psc and Su(z)2 that remain associated with Hox genes or it could be some chromatin modification, such as the state of acetylation, that is created by the action of PcG gene products. We imagine that this mark is destabilized by persistent transcriptional activity, perhaps because this activity reduces the efficiency with which the mark can be propagated when the gene is replicated, and hence is irreversibly lost following prolonged depletion of *Psc-Su(z)2* gene function. We also imagine that Psc and Su(z)2 proteins, if resupplied in time, recognize this mark, bind there and re-establish the fully silenced state. A precedent for a protein mark that is needed for inheritance of a repressed state and that stays associated with the chromatin of the target gene throughout the cell cycle is the fission yeast protein Swi6 (Nakayama et al., 2000). However, we do not know at present whether the re-establishment of PcG silencing requires that the cells pass through S-phase, a step that is needed for re-establishing silencing in budding yeast (Miller and Nasmyth, 1984).

Our finding that Hox genes can remain marked for silencing even after being derepressed constrains possible mechanisms for heritable silencing. For example, it is not compatible with mechanisms which posit that silenced genes replicate during a distinct phase of the cell cycle which commits them to reassembling the silenced state (e.g. late in S phase, when heterochromatin is replicated). In such a model, Hox genes that are misexpressed because of *Psc-Su(z)2* depletion would now replicate together with actively transcribed genes and hence be irreversibly derepressed.

Similarly, the trithorax group (trxG) proteins Brahma (Brm), Osa and Moira (Mor), components of the *Drosophila* SWI/SNF-like Brm complex, are needed for the activation of Hox gene transcription (Tamkun et al., 1992; Brizuela and Kennison, 1997; Collins et al., 1999; Crosby et al., 1999, Kal et al., 2000). Chromatin remodeling assays using PRC1 and a human SWI/SNF complex suggest that these two complexes compete with each other for interaction with the nucleosomal template (Shao et al., 1999). Moreover, order-of-addition experiments have led to the proposal that PRC1 can only interfere with chromatin remodeling by the SWI/SNF complex if PRC1 is associated with the nucleosomal template before SWI/SNF can access the template (Shao et al., 1999). According to this view, Hox gene derepression caused by *Psc-Su(z)2* depletion should be accompanied by remodelling of the Hox gene chromatin by the SWI/SNF-like Brm complex, and this remodelling should then become irreversible and would be heritably maintained. Thus, our evidence that the mark that confers heritable silencing can be maintained even when a Hox gene is derepressed appears to argue against such a mechanism.

We also performed resupply experiments with the PcG genes *Pc* and *Scm*. In contrast to the results we obtained with the *Psc-Su(z)2* resupply experiment, we found that once Hox genes were fully derepressed, we could no longer re-establish silencing by resupply of either *Pc* or *Scm*. However, at least for the case of *Pc*, resupply could restore silencing in cells where Hox gene transcription was not yet fully derepressed. These results are compatible with an interpretation in which both of these group 2 PcG gene functions are normally required for

maintaining the stability of the silenced state, for example, by providing a component that is part of the heritable mark that targets Hox genes for repression by group 1 PcG gene products. Hence, depletion of either protein might cause a gradual destabilization of the mark, resulting in full derepression and the loss of the capacity for re-establishing the silenced state when the mark is abolished.

Hox gene- and tissue-specific differences in the stability of the silenced state

In general, loss of group 1 and group 2 PcG gene products results in misexpression of Hox genes. However, for any given PcG gene product, we observe significant differences in the timing of the loss of silencing that depend on the Hox gene examined and the position of cells within a tissue. For example, the loss of group 2 PcG functions such as *Pc* cause *Ubx* and *Abd-B* misexpression in the presumptive wing blade within 48 hours of removing the endogenous gene. However, misexpression of both genes is not observed in the presumptive notum and wing hinge regions of the wing disc until 72-96 hours after gene removal. Moreover, no misexpression of *cad* is observed within the entire imaginal wing disc up until the onset of pupation, although the adult phenotypes of *Pc* mutant clones suggest that *cad* is eventually misexpressed. Thus, the stability of the silenced state appears to vary for different Hox genes within the same cells, and for the same Hox gene in different cells.

The fact that the loss of group 1 and group 2 PcG genes eventually cause most or all Hox genes to be expressed in most or all cells suggests that Hox genes contain enhancers and/or promoters that are targets for ubiquitous transcriptional activators. Products of the trithorax group (trxG) of genes are required for maintaining Hox gene activity in most or all embryonic and imaginal disc cells (reviewed by Kennison, 1995) and might therefore function as such ubiquitous activators (Garcia-Bellido and Capdevila, 1978; Ingham and Whittle, 1980; Ingham, 1981; Biggin and Tjian, 1988; Soeller et al., 1988; Mazo et al., 1990; Tamkun et al., 1992; Farkas et al., 1994; Collins et al., 1999; Crosby et al., 1999, Kal et al., 2000). However, the spatial variation in Hox gene misexpression caused by the loss of PcG gene functions suggests that the timing of derepression also correlates with the activities of position-specific enhancer elements in Hox genes. For example, previous analyses showed that transcriptional activation by ABX, an imaginal disc enhancer from *Ubx*, depends on *vestigial* (*vg*) (Christen and Bienz, 1994), a gene encoding a nuclear protein that is expressed in the pouch of the wing and haltere discs (Williams et al., 1991). Thus, it is likely that locally expressed transcription factors such as Vg protein co-operate with more generally distributed factors such as trxG proteins to activate transcription of homeotic genes in PcG mutant clones, perhaps accounting for the consistent spatial variations in Hox gene misexpression.

Variations in the stability of the silenced state of different Hox genes within the same segment is more difficult to explain. However, we note that rank order of stability appears to correlate with the number of segments that fall between the second thoracic segment, which gives rise to the wing disc, and the segment in which the derepressed Hox gene is normally expressed. Thus, *Ubx*, which is normally active in segments T3-A8, is derepressed first, followed by *Abd-B*, which is active

in segments A5-A9, followed by *cad*, which is active in segment A10. Curiously, Hox gene misexpression in PcG mutant embryos shows a similar correlation: in general, misexpression is first detected in segments close to the normal domain of expression of the Hox gene in question and then spreads progressively to segments located further away (Struhl and Akam, 1985; Simon et al., 1992). One possible interpretation of this conserved relationship is that it reflects the extent of repressive interactions that operate on a given Hox gene during early embryogenesis, when the domains of Hox gene expression are delimited by the Gap gene repressors. Cells located far away from the normal domain of expression of a particular Hox gene may be subjected to a greater number of repressive interactions than cells located closer to the same domain. The strength of these repressive interactions might then determine the stability of the silenced state of the gene that is propagated during subsequent development.

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