Recruitment of components of Polycomb Group chromatin complexes in *Drosophila*

Sylvain Poux, Donna McCabe and Vincenzo Pirrotta*

Department of Zoology, University of Geneva, 30 quai Ernest Ansermet, CH1211 Geneva, Switzerland

*Author for correspondence (e-mail: vincenzo.pirrotta@zoo.unige.ch)

Accepted 11 October; published on WWW 27 November 2000

**SUMMARY**

Polycomb Group complexes assemble at polycomb response elements (PREs) in vivo and silence genes in the surrounding chromatin. To study the recruitment of silencing complexes, we have targeted various Polycomb Group (PcG) proteins by fusing them to the LexA DNA binding domain. When LexA-PC, -PSC, -PH or -SU(Z)2 are targeted to a reporter gene, they recruit functional PcG-silencing complexes that recapitulate the silencing behavior of a PRE: silencing is sensitive to the state of activity of the target chromatin. When the target is transcriptionally active, silencing is not established but when the target is not active at syncytial blastoderm, it becomes silenced. The repressed state persists through embryonic development but cannot be maintained in larval imaginal discs even when the LexA-PcG fusion is constitutively expressed, suggesting a discontinuity in the mechanism of repression. These proteins also interact with other PC-containing complexes in embryonic nuclear extracts. In contrast LexA-PHO is neither able to silence nor to interact with PC-containing complexes. Analysis of pho mutant embryos and of PRE constructs whose PHO-binding sites are mutated suggests that, while PHO is important for silencing in PRE constructs, it is not necessary for embryonic PcG silencing.

Key words: Chromatin silencing, Pleiohomeotic, Polycomb complexes, Homeotic genes, *Drosophila*

**INTRODUCTION**

Chromatin complexes of Polycomb Group (PcG) proteins maintain the repressed state of homeotic genes in embryonic regions in which they were initially repressed by transient regulators such as Hunchback. The target of PcG complexes in vivo is the polycomb response element (PRE), a regulatory region that confers PcG-dependent silencing to the chromatin region surrounding it for many tens of kilobases (Chan et al., 1994). PREs have been identified in several homeotic genes as well as in the regulatory regions of genes such as *engrailed*, *polyhomeotic*, *escargot* (Kassis, 1994; Fauvarque and Dura, 1993). Antibody staining of polytene chromosomes confirms the presence of PcG proteins at the site of insertion of transposons containing PREs (Zink et al., 1991; Franke et al., 1992; Rastelli et al., 1993; Carrington and Jones, 1996), and chromatin immunoprecipitation experiments indicate that PcG proteins are associated with a region of more than 1 kb surrounding the PRE site (Orlando et al., 1998). A detailed analysis of the *bxd* PRE from the *Ultrabithorax* gene shows that it extends over more than 1.5 kb and is composed of multiple sequences that harbor residual PRE function when tested individually (Horard et al., 2000).

Although the PREs are specific sites for the binding of PcG complexes, we know little about the sequences that define them, the process by which the PcG proteins are recruited and the factors that determine whether or not the complex will form at a particular PRE. In general, the known PcG proteins do not have specific DNA-binding activities and do not contain structural domains suggestive of direct interaction with DNA. The exception is PHO (Pleiohomeotic), whose recent cloning has revealed that it is a sequence-specific DNA-binding protein with homology to the mammalian YY1 factor (Brown et al., 1998). Mutations in the *pho* gene produce a variety of phenotypes, including features indicative of homeotic derepression (Girton and Jeol, 1994). Most PREs contain in fact one or more PHO consensus binding sequences and Fritsch et al. have shown that when these sites are mutated in the *bxd* PRE, silencing in larval imaginal discs is lost (Fritsch et al., 1999). It has been proposed, therefore, that PHO binds to PRE sequences and recruits other PcG components. However, specific interactions between PHO and other PcG proteins have not been demonstrated. Furthermore, if PHO acts as a recruiter, it is unlikely to be the sole recruiting protein. Another recruiter might be GAGA factor. This protein binds cooperatively to GAGA consensus sequences present in multiple copies in many, though not all, PREs but also found in the promoter region of many genes, where it stimulates expression. Mutations in *Trl* (*Trithorax*-like), the gene that encodes GAGA factor, decrease the silencing ability of the *Fab-7* PRE (Hagstrom et al., 1997). In vitro, GAGA consensus sequences are a target for binding of PcG complexes present in nuclear extracts and some of these complexes contain GAGA protein (Horard et al., 2000). Still, other recruiting agents are likely to
exist since certain PRE fragments contain neither PHO nor GAGA sites, but still bind in vitro to PcG complexes. An important feature of PRE silencing is that its establishment is dependent on the state of activity of the target gene. This is critical for the role played by the PRE homeotic gene regulation, where the target gene must be repressed in those cells in which it was repressed in the early embryo but it must remain accessible and potentially active in cells in which it had been active at early stages. Thus, some feature of the PcG recruiting or silencing mechanisms must be sensitive to the state of activity of the chromatin.

Starting from the assumption that the assembly of PcG complexes is a stepwise process that involves many components, some interacting directly with DNA and some only with other proteins, we have asked to what extent this process could be abbreviated by providing some PcG proteins with DNA-binding domains, enabling them to act as initial recruiters. This approach was first used with PcG proteins by Bunker and Kingston, who showed that the Drosophila PC, PSC and SU(Z)2 proteins, fused to the LexA DNA binding domain, could repress to some extent a reporter gene in mammalian tissue culture cells (Bunker and Kingston, 1994). Müller showed that PC fused to the GAL4 DNA-binding domain recruited endogenous PcG proteins and efficiently repressed a reporter gene in Drosophila embryos (Müller, 1995). This silencing was not stable and faded in later embryonic development, presumably as the GAL4-PC protein stopped being produced.

Müller’s work led to the conclusion that targeting a PcG protein to a DNA sequence by means of a specific DNA-binding domain, would guarantee the recruitment of a full-fledged PcG silencing complex through protein-protein interactions between PcG components. These results raised many questions that could be addressed by extending the same approach. We were interested in learning which PcG proteins could act as recruiters and which were not directly involved in recruitment but might normally act at some earlier (or later) step. An essential task of PcG complexes in homeotic gene regulation is to determine whether or not to assemble at a given PRE. Since targeting by means of a specific DNA-binding domain would in principle bypass a large part of the assembly process, would the recruited complex mimick a PRE and be able to discriminate between the active and inactive state of the target gene? We find that several PcG proteins can recruit a silencing complex that maintains the repressed state but allows an active gene to remain active. Other proteins, including PHO, cannot. This suggests that PHO might not be a major recruiter of the PcG complex. Furthermore, in vitro immunoprecipitation experiments confirm that, unlike other PcG proteins, PHO is not associated with the repressive complex. Finally, we show that during embryonic development PHO is not essential for PRE function, although it is needed for effective silencing at later stages.

**MATERIALS AND METHODS**

**Fly strains and mutants**

All transgenic flies were produced using the Df[1]w67c23 strain, which is y,w-. The PcG mutations used were pho1 and pho85+. To test for rescue of the pho1 mutation by the hs-LexA-PHO transposon, pho1/IcD flies homozygous for the transposon were established. Larvae were heat shocked daily for 30 minutes in a 37°C water bath, beginning 4 days after egg laying and continuing until the first flies began to eclose.

**Transposon constructs**

The BHL4 construct was first assembled in Bluescript (Stratagene). An oligonucleotide containing a dimeric LexA-binding site with sequence ACTTGTACTGTAGCATAAGTATAACA oligomerized in four copies was inserted in the Smal site of the polylinker, followed by the 500 bp bxI(b) fragment of the BX enhancer (Qian et al., 1993) inserted in the PstI-EcoRI sites, and by the 2.4 kb HindIII 2212H1 enhancer fragment (Pouz et al., 1996) inserted in the HindIII site. The BHL4 assembly was then excised with Eagl + Xhol and cloned in the corresponding sites of the CaSpeR/ubx-lacZ vector (Chan et al., 1994). All LexA fusions were first assembled in Bluescript containing a 700 bp HindIII fragment with the LexA-coding region from the CDM lex construct (Bunker and Kingston, 1994). The PC, PSC and SU(Z)2 LexA fusions were kindly provided by C. Bunker (Bunker and Kingston, 1994) and were first recloned in Bluescript. For LexA-GAGA and LexA-PH, the ATG codon of the respective cDNA was replaced, using PCR, by a linker sequence and then ligated to the 3’ end of the LexA DNA-binding domain. These two fusions contain all the amino acids encoded by the cDNA except for the initiator methionine. The LexA-PH fusion contains the pho cDNA sequence from Actl site and encodes all but the first four amino acids of the PHO protein. The LexA fusions were then cloned in the C4-Yellow hs vector, made by inserting the Xhol-SacI hsp70 cassette from CaSpeR-hs (Thummel and Pirrotta, 1991) in the SalI-SacI sites of C4-Yellow (Sigrist and Pirrotta, 1997). A new polylinker containing unique XhoI, EcoRV, XbaI, SacI cloning sites was inserted in the XbaI-SalI sites of the hsp70 cassette polylinker. The αIT-LexA-PC construct consists of the α-tubulin promoter (O’Donnell et al., 1994) from position –157 (including the first, noncoding exon and first intron) fused, by a PCR-generated fragment linking the ATG initiating the second tubulin exon, to LexA-PC. This construct was then cloned in C4-Yellow. The αIT-LexA-PH transposon was made by replacing the Pmel-NotI LexA-PC fragment from αIT-LexA-PC and replacing it with the corresponding LexA-PH fragment. Details of the constructions and of the C4-Yellow and C4-Yellow hs vectors are available upon request.

**Antibodies**

Rabbit polyclonal antibodies were raised using GST fusion proteins. Anti-LexA was directed against amino acids 5-202 of LexA, amino acids 191-354 of PC, amino acids 819-926 from PSC, amino acids 477-558 of SU(Z)2 and amino acids 87-431 of PH. The fusion proteins were expressed in BL21 bacteria and purified on glutathione-Sepharose columns, washed with PBS and eluted with 0.1 M EDTA. The homogenate was boiled 5 minutes and centrifuged 10 minutes at high speed. 20 μg of the supernatant was loaded on an 8% SDS acrylamide gel. For nuclear extracts, prepared as described (Horard et al., 2000), 10 μg were loaded per lane. The gel was then transferred to a PVDF membrane (Millipore). For western blots, anti-LexA antibodies were used at 1:3000; anti-Fbox1 was used at 1:1000; and anti-PC at 1:1500. The second antibody was a AP-conjugated
Recruitment of Polycomb complexes

anti-rabbit diluted 1:10,000 (Promega). For polytene chromosomes, salivary glands were fixed in 2% formaldehyde, PBS and 2% Triton X-100 for 1 minute, followed by spreading in 45% acetic acid, 2% formaldehyde in PBS. The chromosomes were incubated 1 hour in blocking solution (Boehringer), then overnight at 4°C with anti-LexA antibody at 1:300. After washing three times for 30 minutes each time, they were then incubated with biotinylated anti-rabbit (1:1000) for 3 hours at room temperature. After washing three times for 30 minutes each time, they were stained for 1 hour with streptavidin-FITC plus 5 μg/ml DAPI, washed for 30 minutes and photographed or imaged with a Zeiss fluorescence photomicroscope and a Hamamatsu C5810 CCD camera.

Staining of embryos and discs
To test the repressive effects of the LexA-PcG fusion proteins, flies carrying the BHL4 reporter transposon were crossed with flies carrying the LexA-PcG transposon. Embryos were collected at 1 hour intervals, aged for different times and then heat shocked for 45 minutes at 37°C. After further incubation for 12-18 hours at room temperature, they were fixed and stained. Staining of embryos and imaginal discs was carried out with anti-β-galactosidase antibody as described (Poux et al., 1996). In situ hybridizations of embryos with a lacZ probe followed the procedure of Tautz and Pfeifle (Tautz and Pfeifle, 1989). Embryos were also stained with monoclonal anti-UBX antibody (kindly provided by J. Lopez), followed by goat anti-mouse antibody conjugated with Cy3, and imaged with a Zeiss fluorescence photomicroscope and Hamamatsu C5810 CCD camera.

Embryonic extracts and immunoprecipitation assays
Overnight embryo collections were made from LexA-PC, LexA-SU(Z) and LexA-PHO flies. The embryos were heat shocked 45 minutes at 37°C and allowed to recover 1 hour at 25°C before homogenization. The preparation of the nuclear extracts and the immunoprecipitation assays are described by Horard et al. (Horard et al., 2000).

RESULTS

Expression and localization of LexA fusion proteins
Transposon constructs were designed to express a number of PcG or related proteins as chimeras, fused at the N-terminus to the LexA DNA-binding domain. The constructs were assembled in the C4-Y enhancer transposon vector, which uses the yellow gene as an indicator and the hsp70 promoter to drive the expression of the chimeric gene (Fig. 1A). As an additional control, we prepared a construct expressing the LexA moiety alone. Western blots stained with anti-LexA antibody confirmed that within one hour of the heat shock all constructs produced detectable proteins with the expected molecular weight and stable for several hours after the heat shock treatment (Fig. 2). The bands of chimeric proteins were also recognized by antibodies against the corresponding PcG
protein. For LexA-PHO, we used an antibody against YY1, a mammalian factor that shares a region of homology with PHO (Brown et al., 1998). Commercial anti-YY1 antibody directed against the C-terminal peptide (the region of homology with PHO) recognized several bands in wild-type embryonic extracts but, in LexA-PHO extracts (lane 6), but not in wild type extracts (lane 5). Anti-YY1 antibody recognizes several bands in WT nuclear extracts (lane 7), and also detects the LexA-PHO protein in LexA-PHO extracts (lane 8). (B) Polytene chromosomes stained with anti-LexA antibodies. The LexA protein produced after a 30 minutes heat shock does not bind to euchromatic sites but two spots are present in the chromocenter. (C,D) Heat-shock-induced LexA-PC (C) and LexA-PHO (D) proteins bind to many euchromatic sites.

Silencing of the reporter gene

To test the silencing ability of the LexA-PcG proteins, we constructed transgenic flies carrying BHL4, a reporter construct consisting of the Ubx-lacZ gene under the control of a Ubx embryonic enhancer, BX, and a Ubx imaginal disc enhancer, 2212H1. The enhancers were immediately preceded by four copies of a dimeric LexA-binding sequence and the construct was assembled in a CaSpeR vector, containing the miniwhite gene as a marker (Fig. 1B). Fig. 3A shows that early embryos carrying the reporter construct alone, displayed the typical expression pattern of the BX enhancer. During germ band extension parasegmental expression was seen in broad stripes at PS6, 8, 10 and 12 and was strongly repressed more anteriorly by the Hunchback product (HB). By the end of germ band extension, expression in the thoracic segments was derepressed by the disappearance of the HB repressor and expression dependent on the H1 enhancer set in both even- and odd-numbered parasegments, later becoming confined to a group of dorsolateral cells in each segment (Fig. 4B). In larvae, expression from this enhancer was found primarily in the dorsal imaginal discs: eye, wing and haltere (Fig. 5A).

To test silencing activity, transgenic flies carrying the BHL4 reporter gene were crossed with lines carrying each of the LexA fusion constructs. The embryos were heat shocked at different times to express the chimeric protein, then fixed and stained 12-18 hours later. In embryos that expressed the LexA DNA-binding domain alone, the BHL4 pattern was not appreciably altered by heat shocks administered at any time from 1 hour after deposition. In embryos expressing LexA-PC, the pattern of expression of the reporter gene depended on the developmental stage at which the LexA-PC protein was induced. Very early induction (1-2 hours after deposition) resulted in maintenance of the four stripe, even-numbered parasegment pattern characteristic of the BX enhancer. Repression in the anterior half of the embryo was maintained to the end of embryonic development and expression failed to appear in the odd-numbered parasegments. However, expression in parasegments 6, 8, 10 and 12 continued unimpaired (Fig. 3B), showing that the silencing induced by the LexA fusion protein, just like that produced by a PRE, was not ubiquitous but is sensitive to the state of activity of the target gene. To show that the abdominal expression is not simply a persistence of the β-galactosidase produced at early times, we carried out in situ hybridization with a lacZ probe. This revealed that transcription continued in parasegments 6-12 and that the later activation of the H1 enhancer was restricted to the same domain (Fig. 4C). These results show
that the silencing induced by LexA-PC depends on the state of activity of the reporter gene and results in maintenance of the earlier expression pattern. Active chromatin apparently interferes with the establishment of silencing. Since transcriptional activity is unlikely to prevent the binding of LexA-PC, the interference must occur at a later step in the recruitment of a functional PcG complex by LexA-PC.

Consistent with this, inducing LexA-PC at later times during embryonic development, after the pattern of expression has expanded, has a correspondingly restricted silencing effect. If the heat shock was administered to embryos 2-3 hours old, the reporter gene was expressed in both even and odd parasegments but still limited to the posterior half of the embryo, beginning with PS6 (Fig. 3B). Induction of LexA-PC at 5-6 hours, after derepression has set in, produced an essentially derepressed pattern with expression in all parasegments, including thorax and head (Figs 3B, 4D).

The results obtained with LexA-PSC, -SU(Z), and -PH were essentially identical to those observed with LexA-PC. These four proteins can recruit a silencing complex with similar properties and can be thought of as ‘core’ PcG proteins. In contrast, we saw no silencing or maintained repression after induction of LexA-GAGA or -PHO (Fig. 3C). We conclude that LexA-PHO and -GAGA are unable by themselves to recruit PcG complexes to the reporter gene.

Silencing in larvae

The repression resulting from the induction of LexA-PC in the early embryo is maintained through embryonic development but does not persist in larvae. The H1 enhancer normally activates strong and uniform expression of the reporter gene in the dorsal imaginal discs: halteres, wings and eye-antennae (Fig. 5A). In larvae that had been heat shocked to induce LexA-PC during early embryonic development, these discs continue to express β-galactosidase strongly and uniformly (Fig. 5B), instead of being restricted to the posterior compartment of the haltere (PS6). The same result was obtained with LexA-PSC, -SU(Z), -PH or -PHO. The silencing established by the LexA-PcG protein in the embryo is not self-maintaining but is lost when that protein is diluted or degraded during growth of the imaginal discs.

A single heat shock administered during larval development had little effect on the expression of the reporter gene in imaginal discs (Fig. 5C). This would be expected from the fact that the LexA-PC protein is not effective when induced after the reporter gene is already activated. However, repeated heat shocks, twice daily during the first three days after hatching, resulted in a significant decrease of imaginal disc staining, showing that continued and persistent overexpression of LexA-PC can eventually achieve partial silencing of an active gene (Fig. 5D). This late silencing also affects the expression of the miniwhite marker gene. Fig. 5F shows that four heat shock treatments administered to late third instar larvae and pupae caused a reduction in eye pigmentation of the resulting flies while they had no effect on flies carrying the reporter gene alone. The same results were observed with the other “core” proteins but not with the GAGA or PHO fusion proteins.

We considered the possibility that the distance of the H1 enhancer from the LexA-binding sites might account for the inefficient silencing in imaginal discs. However, in the embryo, H1-directed expression is correctly silenced by LexA-PC in the anterior and in the odd-numbered parasegments (Fig. 4). In the
BHL4 transposon, the LexA-binding sites lie more than 7 kb from the promoter of the miniwhite gene. Might LexA-PHO or -GAGA show some silencing activity if the LexA-binding sites were placed closer to the miniwhite gene? To test this, we inserted the LexA-binding sites 310 bp or 110 bp from the white transcription start (Qian et al., 1992). We then tested the effect of repeated induction of the chimeric protein by heat shocks administered during the late third instar and the first

**Fig. 4.** In situ hybridization with lacZ mRNA from the BHL4 reporter gene. (A) Embryos during germ band extension and (B) after germ band retraction, when dorsolateral expression dependent on the 2212H1 enhancer appears in each parasegment. (C) LexA-PC embryos heat-shocked at 2-3 hours and allowed to develop until germ band retraction: 2212H1 expression is silenced in the thorax but still present in the abdomen. (D) In embryos that had been heat-shocked at 5-6 hours, repression is not maintained and the 2212H1 enhancer is activated in all parasegments. Arrows indicate the spots typical of 2212H1 expression. The arrowhead shows the position of PS6.

**Fig. 5.** The silencing induced by LexA-PC is transient. All larvae and flies shown carry the BHL4 target and either hs-LexA-PC effector (A-D,F), or the α1T-LexA-PC effector (E). In A,C,D,E, the left-hand images are of wing marginal discs and the right-hand images are of haltere discs (plus assorted leg discs). (A) With no LexA-PC induction: 2212H1-dependent expression is present in dorsal discs. (B) Heat-shock induction at blastoderm: the silencing established in the embryo is not maintained in wing and haltere discs. (C) A single heat-shock 12 hours before staining the discs is also insufficient to silence the 2212H1 enhancer. (D) Repeated heat shocks 2 days before staining are necessary to partially silence expression in the discs. (E) Constitutive expression of LexA-PC from the tubulin promoter is not sufficient to repress the 2212H1 expression in the discs. (F) A series of heat shocks leads to partial silencing of the miniwhite gene in the eye, compared with the no heat shock control (RT).
Recruitment of Polycomb complexes

two days of pupation. While this treatment shows a significant decrease in eye pigmentation in the case of LexA-PC, no effect was seen with LexA-PHO or -GAGA (not shown).

Constitutively expressed LexA-PC

Although repression is effective in the embryo, we were unable to prolong it and maintain a continuous PS6 boundary of expression by administering heat shocks twice daily beginning with the early embryo to ensure continued presence of the LexA fusion protein. To provide a more uniform supply of the fusion protein we also used the promoter from the α1-tubulin gene to express LexA-PC (Fig. 1C). This promoter, together with the first intron of the α1-tubulin gene, directs expression in all cells in all tissues of the embryo and larva and can be considered constitutive (O’Donnell et al., 1994). It is also strongly expressed in ovarian nurse cells and therefore accumulates the expressed protein in the oocyte. When females homozygous for the α1-tubulin-LexA-PC transgene are crossed to males homozygous for the BHL4 reporter gene, the resulting embryos showed correct expression of Ubx-lacZ and maintenance of the segmental boundary of expression (Fig. 3B). Interestingly, although the LexA-PC protein is deposited in the egg and is zygotically expressed from embryonic stage 5, it does not initiate repression prematurely, which would prevent the initial expression of the BHL4 reporter, nor does it repress prematurely at the sites of endogenous PREs (which would be lethal). This implies that the assembly of the repressive complex cannot take place before blastoderm and depends on events that occur at that time.

We then looked at larval imaginal discs to see if the continuous presence of LexA-PC could maintain the segmental restriction established in the embryo. Fig. 5E shows that the silencing of the reporter gene did not persist beyond embryonic development. Expression in imaginal discs failed to observe the PS6 segmental boundary and was visible in the entire wing and haltere discs. The failure to silence in larvae was not attributable to insufficient levels of LexA-PC since western blots showed at all stages amounts of LexA-PC comparable with endogenous PC. These results suggest that PcG silencing in larval stages involves additional proteins that cannot be directly or indirectly recruited by LexA-PC.

In vitro binding of LexA-PcG proteins

The expression of the chimeric PcG proteins opens the possibility to analyse in vitro the physical interactions of the LexA-fusion proteins with the endogenous PcG proteins. We prepared extracts from embryos carrying the LexA-PC, LexA-SU(Z)2 or LexA-PHO expression constructs, which had been heat shocked 1 hour prior to collection. When these extracts are incubated with a radioactively labelled DNA probe consisting of four tandem copies of the double LexA recognition sequence, immunoprecipitation with anti-LexA antibodies confirmed that the LexA-fusion proteins bound efficiently and specifically to the DNA probe (Fig. 6).

The polytene chromosome staining experiments indicated that the LexA-fusion protein becomes incorporated in the endogenous PcG complexes visible on the chromosomes within a half hour from the onset of the heat shock. The extracts should then contain PcG complexes tagged with the LexA DNA-binding domain. To test this we incubated extracts made from LexA-PC embryos with the LexA probe and immunoprecipitated using anti-PSC antibody. Fig. 6A,B shows that this antibody immunoprecipitated the LexA probe when the nuclear extracts were made from LexA-PC embryos but not...
when they came from wild-type embryos, indicating that, as expected, PSC and LexA-PC are associated in PcG complexes. In this indirect immunoprecipitation assay, the radioactive LexA DNA serves as a sensitive tag to probe the composition of the complexes in which the LexA-fusion protein can be incorporated in vivo. Anti-SU(Z)2 and anti-PH, as well as anti-PSC antibodies, precipitate the LexA-binding site probe in the presence of extracts from LexA-PC embryos but not with wild-type embryonic extracts (Fig. 6A,B). When extracts from LexA-SU(Z)2 embryos are used, anti-PC, anti-PSC and anti-PH antibodies likewise precipitate the probe (not shown).

For LexA-PHO extracts we used LexA antibody or anti YY1 antibody. Both antibodies precipitated the LexA probe in presence of LexA-PHO extracts but not with wild-type extracts (Fig. 6C), showing that the LexA-PHO protein binds efficiently to LexA sites. In the presence of LexA-PHO extracts, anti-PC, -SU(Z)2 or -PH did not precipitate the LexA probe, indicating that these proteins are not stably associated with LexA-PHO. Similarly, with LexA-PC extracts, anti-YY1 did not precipitate the LexA probe. Anti-YY1 did precipitate PRE fragments containing consensus PHO-binding sites, even in the case of PRE fragments that are not precipitated by anti-PC antibody (not shown). Together these experiments indicate that neither the endogenous PHO nor the transgenic LexA-PHO are directly associated with PcG complexes. In contrast, GAGA factor interacts with at least some PcG complexes. As reported elsewhere (Horard et al., 2000), anti-GAGA factor antibody is able to immunoprecipitate the LexA probe in the presence of LexA-PC extracts but not with wild-type extracts.

A second look at PHO

In view of the inability of LexA-PHO to silence the reporter gene and its lack of detectable association with PC-containing complexes, we re-examined the evidence that PHO is needed for PRE silencing during embryonic development. In fact, larvae homozygous for the strong pho1 allele survive and look quite normal, dying only during late pupal development with only relatively weak homeotic transformations, mostly affecting the antennae and sex combs (Girton and Jeol, 1994). The weakness of these phenotypes is generally attributed to a long-lasting maternal contribution that suffices to carry the mutants through normal embryonic development. A weaker pho allele, pho1, allows occasional survival to adulthood but the surviving pho1 females are sterile. Many of the eggs deposited are unfertilized and those that are produce embryos that fail to hatch (Girton and Jeol, 1994). These embryos, however, appear to suffer primarily from segmentation abnormalities rather than homeotic derepression.

Since the genetic evidence indicated an important maternal PHO contribution, we also tested the activity of a LexA-PHO transgene driven by the constitutively expressed α1-tubulin promoter, which is active also during oogenesis. Like the heat shock-driven construct, this transgene did not silence the BHL4 reporter gene, confirming the inability of LexA-PHO to recruit a repressive complex. The LexA-PHO protein made in both cases was physiologically active, as shown by the fact that it can fully rescue homozygous pho1 mutants. Two copies of the α1-tubulin-LexA-PHO rescued pupal lethality as well as the maternal sterility of pho1 pho1 females. With the heat shock-driven construct, two copies of the transgene produced healthy pho1 pho1 adults with one heat shock daily beginning 4 days after egg laying. The rescued pho1 pho1 males occasionally displayed sex comb bristles on the mesothoracic legs and excess pigmentation in the 4th abdominal tergite but they were fully fertile, while the pho1 pho1 females had severely impaired fertility that could not be rescued by a paternal pho+ allele. When pho1 pho1 females were crossed with pho1 pho1 males, only 10% of the embryos hatched, even when heat shocked before blastoderm to express LexA-PHO, confirming a very early role for maternal PHO. These embryos showed frequent and various segmental abnormalities but never displayed the classical homeotic derepression phenotypes. Antibody staining showed that in all embryos, UBX protein was still confined to parasegments 5-13 and was never detectable more anteriorly (Fig. 7A). The few larvae that hatched from these embryos grew slowly and rarely reached the pupal stage, probably

Fig 7. Role of pho. (A) pho1/pho1 embryos from homozygous mutant mothers rescued by the hs-LexA-PHO construct, stained with anti-UBX antibody. At this resolution, no derepression of Ubx is detectable, compared with wild-type embryos similarly stained (B). (C) In situ hybridization of embryos carrying a PBX+2212H1 transposon, shows 2212H1 expression in all parasegments. (D) When the PBX+2212H1 enhancers are flanked by the PRED (Fritsch et al., 1999), anterior repression of 2212H1 is maintained. (E) Embryos carrying the same construct but with all the PHO sites mutated (PREDpho , Fritsch et al., 1999) maintain anterior repression equally well. Arrows indicate the H1-directed expression. The arrowheads indicates the PS6 boundary.
because evident head skeleton abnormalities precluded normal feeding. Although LexA-PHO protein was not detectable in these embryos, we cannot exclude the possibility that the uninduced expression level was sufficient to permit normal PcG regulation of homeotic genes, but entirely insufficient to alleviate the other developmental phenotypes. These results nevertheless raise the possibility that PHO is not essential for PcG silencing in the embryo.

These results contrast with those of Fritsch et al. who have shown that mutating the PHO binding sites in the bxd PRE causes a loss of silencing ability in larval imaginal discs (Fritsch et al., 1999). The discrepancy between their observations and ours again suggests the possibility that PcG silencing in embryos might differ from silencing in larvae and might not require PHO in the same way. We tested embryos from the same fly lines of Fritsch et al., kindly provided by J. Müller, bearing a transgene containing the PRE with mutated PHO sites together with the PBX embryonic enhancer and the dorsal imaginal disc enhancer (equivalent to our H1 enhancer) driving the Ubx-lacZ reporter (lines PRE D pho mut 1.3, 7.4 and 8.1 of Fritsch et al., 1999). In situ hybridization on embryos from these lines showed that expression remained clearly restricted even after germ band retraction, when the onset of H1-dependent expression was confined to PS6-12. Fig. 7C-E compares these embryos with embryos carrying the parallel construct with the unmutated PRE, and with the derepressed pattern produced by an analogous construct containing PBX and H1 enhancers but no PRE (Poux et al., 1996). In larval imaginal discs, however, the constructs with mutated PHO sites fail to maintain repression anterior to PS6, as described by Fritsch et al. (Fritsch et al., 1999). We conclude that, while PHO plays an important role in PRE function in the late larva and pupa, it may not be required for PcG silencing at the same PRE during embryonic development.

DISCUSSION

Maintenance of the repressed state

Our results show that several PcG proteins, targeted by fusion to a DNA-binding domain, can recruit a repressive PcG complex, as first shown for PC (Müller, 1995). Several new conclusions can be drawn from our experiments. First, the recruitment of the silencing complex cannot occur before blastoderm. The α-tubulin-LexA-PC construct reveals that even when the protein is deposited in the egg during oogenesis, as well as being zygotically expressed, it does not block the initiation of transcription from the Ubx promoter. These results suggest that the LexA-PcG protein cannot establish repression at this early stage, just as the endogenous PcG proteins known to be present in the normal pre-blastoderm embryo do not prevent the initiation of Ubx transcription. Thus, PcG silencing directed by a PRE or by LexA-PC appears only to set in after blastoderm. One possible explanation is that the assembly of a functional PcG complex at the PRE or at the LexA-binding sites is a multistep process that requires time and is not accomplished until after blastoderm. Another interesting possibility is that the state of the chromatin in nuclei whose very rapid nuclear divisions are just beginning to slow down, cannot yet support the establishment of PcG silencing, for example, because the nucleosomes still bear the deposition-associated histone acetylation pattern. A similar argument might explain why centric heterochromatin is not detectable until blastoderm (Hiraoka et al., 1993).

Second, the repression established by the LexA-PcG protein is not unconditional but it is sensitive to the state of activity of the target. Like a genuine PRE complex, the LexA-PcG protein establishes silencing only in cells in which the reporter gene is inactive, thus discriminating between active and inactive chromatin targets. The later-acting H1 enhancer can still function but only in the progeny of cells that were active at early times. The difference between our results and Müller’s probably lies in the fact that the BXD enhancer used in his experiments becomes active only after gastrulation (Poux et al., 1996), after PcG silencing sets in. In contrast, since in our case transcription directed by the BX enhancer begins during syncytial blastoderm, it is already active by the time the LexA-PC protein is produced, and is not silenced in its early expression domain. However, expression initiating in new domains after blastoderm is efficiently repressed.

The fact that, like the endogenous PRE, the action of the LexA-PcG protein distinguishes between active and silent chromatin, indicates that the discrimination occurs after the binding of the first PcG protein. The sensitive step could be the assembly of a sufficient nucleus of PcG proteins or still later, the involvement of other factors that effect the silencing. Shao et al. have shown that a PC-containing complex purified from embryonic nuclear extracts can prevent chromatin remodelling in vitro if it is bound to chromatin before the addition of purified SWI/SNF complex but not if it is added simultaneously or afterwards (Shao et al., 1999). If the activation of the Ubx-lacZ reporter gene by the enhancers involves the recruitment of the Drosophila SWI/SNF complex, this observation could help to understand how the LexA binding sites function as a genuine synthetic PRE possessing at least one aspect of the cellular memory displayed by endogenous PREs.

Third, the LexA-binding sites are not, nevertheless, a fully functional PRE. Silencing by LexA-PcG proteins is much less effective during larval development. It is possible that the activity of the H1 enhancer in imaginal discs is more difficult to repress, e.g. because of very high activator concentration. More likely, once the H1 enhancer is active, it is much more difficult to repress by LexA-PC induced at later times. We cannot explain, however, why neither daily heat shocks, nor constitutive expression of LexA-PC can maintain a continuity between embryonic silencing and larval silencing. Repeated heat shocks do eventually reduce the level of expression of the reporter in imaginal discs but the memory of the early domains of repression is lost. The apparent discontinuity in PcG silencing between the embryo and the larva, suggests that there might be a real mechanistic difference between the two states. In a recent report, Docquier et al. also find differences in the maintenance properties of embryos and larvae (Docquier et al., 1999). One possible explanation is that additional proteins, recruited at a true PRE but not by the LexA fusion proteins, might be necessary for continuous repression at postembryonic developmental stages.

LexA proteins unable to repress

PcG proteins PSC, SU(Z)2 and PH are as effective as PC in recruiting a silencing complex, implying that any one of the
‘core’ PcG proteins can reconstitute a silencing complex. In contrast, LexA-GAGA and -PHO do not silence the reporter gene. That LexA-GAGA does not silence is hardly surprising. Many promoters, including the hsp70 promoter, the α-tubulin promoter or the Ubx promoter itself, contain GAGA-binding sites but are not thereby targets for PcG silencing in vivo. We cannot exclude the possibility that the LexA-GAGA fusion protein is defective in some respect, although it is able to bind to the normal endogenous sites on polytene chromosomes and participate in PcG complexes. Most likely, however, GAGA factor by itself cannot recruit PcG complexes. We suppose that, like many other nuclear factors, GAGA can have either a stimulating or a repressing activity, depending on the binding of other proteins. In the chromatin context of the PRE, however, GAGA interacts with PcG proteins to form a stable complex (Horard et al., 2000).

The PHO protein binds to DNA sequences contained in the bxd PRE and has been proposed as a major recruiter of PcG complexes (Brown et al., 1998). Fritsch et al. have shown that mutating the PHO binding sites in the bxd PRE causes loss of silencing in imaginal discs (Fritsch et al., 1999). In our experiments, however, LexA-PHO is unable to silence the reporter gene. Since LexA-PHO rescues efficiently pho mutants in vivo, we conclude that it is fully functional and that PHO cannot, by itself, recruit PcG complexes able to silence the reporter gene either in the embryo or in imaginal discs.

PHO and PcG complexes

The ability of LexA fusion proteins to assemble PcG complexes that can be targeted to LexA-binding sites allowed us to explore in vitro the composition of the complexes. It is important to note that what is seen in the immunoprecipitation experiments is the bulk of PcG complexes that contain the LexA-Pc protein. We can not distinguish between complexes recruited by the LexA-Pc protein at the LexA-binding site and complexes formed at other PREs, to which the LexA-Pc protein has been recruited. These experiments therefore do not necessarily reflect the nature of the complex formed at the reporter gene, which is limited to what components can be directly or indirectly recruited by the LexA fusion protein. Nevertheless, the in vitro binding experiments reveal the ability of the LexA fusion protein to participate in complexes containing other PcG proteins.

The binding experiments show that the four ‘core’ proteins PC, PSC, SU(Z)2 and PH are recruited to PcG complexes and are themselves able to recruit repressive complexes. We cannot show that all four proteins are simultaneously associated in one complex but it is very likely that at least PC, PSC and PH can participate in the same complex (Kyba and Brock, 1998). Similar experiments have also shown that GAGA factor is associated with at least some PcG complexes (Horard et al., 2000), although it is not itself able to recruit them to the reporter gene. In contrast, PHO neither recruits any of the core PcG proteins nor participates in the majority of PcG complexes present in embryonic extracts. Conceivably, when the LexA-binding domain is bound to DNA, it might hinder the access of other PcG proteins to the PHO moiety. We think this is unlikely because even wild-type PHO does not seem to be associated with PcG proteins. A PRE fragment containing three PHO binding sites but no GAGA sites does not bind PC-containing complexes in vitro (Horard et al., 2000), although it interacts readily with endogenous PHO or LexA-PHO in the same embryonic extracts, indicating that also endogenous PHO is not stably associated with PcG complexes (B. Horard, R. Melfi and V. P., unpublished observations). Similarly, Shao et al. do not detect PHO in their purified PRIC1 complex (Shao et al., 1999).

There remains the possibility that PHO is not the protein that binds in vivo to the PHO consensus binding sites in the PRE. Fritsch et al. (Fritsch et al., 1999) have shown that these sites in the bxd PRE are important for silencing in imaginal discs (Fritsch et al., 1999). Formally, this does not exclude the possibility that these are binding sites for some other protein required for silencing. More likely, PHO does intervene at the PRE but not as a direct recruiter of PcG complexes. Its role might be to alter the chromatin conformation at the PRE and facilitate the interactions between different components necessary for effective silencing. Mammalian YY1 has been shown to act as a repressor as well as an activator, depending on the context (for a review, see Thomas and Seto, 1999). It can interact directly with histone deacetylases and, presumably, contribute directly to repression (Yang et al., 1996). In addition, it may be a component of some mammalian PcG complexes (Garcia et al., 1999). In Drosophila, PHO might make a similar contribution to silencing, at least when it is bound in the context of the PRE. The domain of YY1 that interacts with HDAC is not conserved in PHO but a different sequence motif might preserve the ability to interact with Drosophila histone deacetylase complexes.

Our experiments showing no obvious Ubx derepression in pho"pho" embryos and no derepression when all pho consensus sites in the PRE are mutated support the idea that neither PHO nor its binding sites play an essential role in embryonic PcG silencing. Simon et al. have reported a very limited derepression of abdA and AbdB in pho mutant embryos (Simon et al., 1992). This effect, involving ectopic expression in a few cells of PS5, might also be interpreted as an indirect consequence of segmentation defects frequently observed in pho mutants (Girton and Jeol, 1994) rather than a real derepression. In any case, the available evidence excludes a major role for PHO as a recruiter of PcG complexes. On the other hand, PHO-binding sites are necessary for silencing in larval imaginal discs (Fritsch et al., 1999) and pho function is required for normal imaginal segmental identity. Our results suggest that this function is required to stabilize PcG silencing in late larval and pupal development, and that its essential role in embryonic development is unrelated to homeotic regulation. The establishment and maintenance of silencing complexes at a PRE is clearly a complicated multistage process and the nature of the initial recruiters is still unknown.

We thank J. Kassis and J. Müller for providing the PHO cDNA, mutants and transgenic fly lines; C. Bunker and R. Kingston for some of the LexA fusions; P. Wensink for the α-tubulin promoter; J. Lopez for anti-UBX antibody. We also thank C. Tatout, B. Horard, R. Melfi and J. Guiard for help at different stages. This work was supported by grants from the Swiss National Science Foundation and from the Human Frontier Science Program, and by a contribution from the Georges and Antoine Claraz Donation.

REFERENCES

Brown, J. L., Mucci, D., Whiteley, M., Dirksen, M. L. and Kassis, J. A.