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The *Drosophila* Trithorax protein is a coactivator required to prevent re-establishment of Polycomb silencing

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SUMMARY

Polycomb group (PcG) and Trithorax (TRX) complexes assemble at Polycomb response elements (PREs) and maintain respectively the repressed and active state of homeotic genes. Although PcG and TRX complexes are distinct, their binding to some PRE fragments in vitro depends on GAGA motifs. GAGA factor immunoprecipitates with both complexes. In presence of a PRE, TRX stimulates expression and prevents the return of repression at later stages. When TRX levels are reduced, repression is re-established in inappropriate regions of imaginal discs, suggesting that TRX insufficiency impairs the epigenetic memory of the active state. Targeting a GAL-TRX fusion shows that TRX is a coactivator that stimulates expression of an active gene but cannot initiate expression by itself. Targeting a histone acetylase to a PRE does not affect embryonic silencing but causes a loss of memory in imaginal discs, suggesting that deacetylation is required to establish the memory of the repressed state.

Key words: Chromatin silencing, Polycomb, Coactivator, Homeotic genes, Epigenetic memory, *Drosophila melanogaster*

INTRODUCTION

In *Drosophila*, the activation of the homeotic genes depends on the products of transiently expressed segmentation genes. When these products disappear, the homeotic expression patterns are maintained in their appropriate domains by the action of the Polycomb group genes (PcG), which preserve the repressed state, and of the trithorax group genes (trxG), which promote the active state (Paro and Harte, 1996; Pirrotta, 1997). The *bxd* Polycomb response element (PRE), from the upstream regulatory region of the *Ultrabithorax* (*Ubx*) gene, is a target for PcG protein complexes (Chan et al., 1994). When inserted in a reporter gene construct, this PRE can recruit PcG complex and maintain the repressed state where the reporter gene was initially inactive while allowing continued expression where the gene was active at the blastoderm stage. Increasing evidence suggests that the recruitment of PcG proteins to PREs is mediated by multiple DNA binding proteins acting in concert (Horard et al., 2000; Hodgson et al., 2001; Poux et al., 2001a; Poux et al., 2001b; Busturia et al., 2001). Two of these proteins are GAGA factor and Pleiohomeotic (PHO), whose binding sequences are present in most PREs. The recent purification of a PcG complex revealed that, in addition to PcG proteins, it also includes general promoter factors such as TAFs (TBP-associated factors) and TBP (TATA-binding protein), suggesting relationships between the PRE complex and promoters (Shao et al., 1999; Saurin et al., 2001).

Most, if not all, known PREs are associated with a Trithorax response element (TRE). The relationship between PcG complexes and TRX complexes and whether TRE and PRE are necessarily linked is not known, but in the case of the *bxd* PRE both response elements are contained within the same region (Tillib et al., 1999). It is generally thought that the function of Trithorax (TRX) is to maintain expression in cells that are not repressed by the PcG complex. Mutations in *trx* genes reduce the expression of the homeotic genes, and may induce segmental transformations reminiscent of homeotic loss-of-function phenotypes (Ingham, 1985). A number of other proteins often grouped together with TRX have a general role in transcription not directly related to PREs. Some of these proteins, for example, are components of the Brahma complex, the *Drosophila* SWI/SNF chromatin remodeling complex that facilitates the binding of other transcription factors (Kingston et al., 1996). However, no interaction between TRX and any of these proteins has been reported and TRX is absent from a purified Brahma complex (Papoulas et al., 1998; Kal et al., 2000). In contrast to the PcG complex, little is known about the composition of the TRX complex except for the fact that ASH1 appears to function as a partner of TRX (Rozovskaia et al., 1999).

Formally, the PRE displays two kinds of memories: one is the memory that maintains the repressed state in the appropriate cells; the other maintains the derepressed state. The repressive memory is illustrated by the fact that, once repression is established in an embryonic cell, it persists in the cellular progeny throughout development. The memory of the

derepressed state has been documented by Cavalli and Paro (Cavalli and Paro, 1998; Cavalli and Paro, 1999). If the target gene is active in the early embryo, or if derepression is forced by massive doses of activator, the derepressed state is inherited by the progeny cells. This memory is affected by *trx* mutations but it remains unclear if TRX is involved in gene activation, in the ensuing expression or in the memory mechanism itself.

The results presented here analyse the role of Trithorax at a PRE. We show that, depending on the context, a reporter gene containing the *bxd* PRE will either act predominantly as a PRE or as a TRE, showing that PcG and TRX complexes have antagonistic functions. We find that, in vitro and in vivo, the interaction of PcG and TRX complexes with a PRE subfragment is dependent on GAGAG sequences and that GAGA factor is associated with a trxG complex. We show that, in the presence of a PRE, TRX is required to prevent the re-establishment of PcG silencing of genes that had been activated at earlier stages. By targeting TRX to reporter genes in the presence or absence of PcG silencing, we find that TRX stimulates expression only in cells where the reporter gene is activated by appropriate enhancers, but does not induce ectopic expression. Experiments targeting activators or histone acetylation provide evidence for two kinds of epigenetic memory that maintain, respectively, the repressed or the derepressed state by mechanisms that are sensitive to acetylation. We propose that, although TRX binding to the PRE stimulates expression, its critical function is to prevent the formation of a repressive complex during later development.

MATERIALS AND METHODS

Transposon constructs

The YGfPfMG construct is the same as the YGPMG construct described in Sigrist and Pirrotta (Sigrist and Pirrotta, 1997), except for the presence of FRT sites surrounding the *Pst*I-*Nde*I fragment of the PRE. It was first assembled in Bluescript (Stratagene). The PRE was inserted in the *Bam*HI-*Pst*I sites between two FRT sequences, the targets for the FLP enzyme. The FRT-PRE-FRT fragment is inserted next to the 406 bp *Su(Hw)* insulator (G). The FRT-PRE-FRT-G fragment was excised with *Not*I-*Xho*I and assembled together with a *miniwhite-G* fragment in the C4-Yellow transposon vector (Sigrist and Pirrotta, 1997) to produce YGfPfMG.

The BHL4G4 and BHL4G4PRE constructs were first assembled in Bluescript. An oligonucleotide containing one GAL4 binding site with sequence (CGGAGTACTGTCCTCCG) oligomerized in four copies was inserted in the *Bam*HI-*Pst*I site of the BHL4 assembly in Bluescript (Poux et al., 2001a). These constructs were then excised with *Eag*I and *Xho*I and cloned in the CaSpeR *Ubx-lacZ* transposon (Chan et al., 1994). To make BHL4G4PRE, the 1.6 kb *Eco*RI-*Sty*I PRE fragment was excised with *Eag*I and *Hin*cII from a Bluescript subclone and inserted in the BHL4G4 cut by *Eag*I and *Xba*I, the latter filled with Klenow DNA polymerase.

The BPx6 S2 *Ubx-lacZ* construct was made by inserting six tandem copies of the BP fragment of the PRE in the S2 *Ubx-lacZ* reporter construct (Horard et al., 2000). The BP subfragment of the PRE contains four GAGA binding sites. Three of these were mutated as described previously (Horard et al., 2000). The fourth site was mutated by a similar procedure, changing AAAGAGAGAGGG to AAAGAAATAGGG. Six tandem copies of the BP fragment with all four sites mutated were inserted in the S2 *Ubx-lacZ* reporter construct.

GAL-TRX and GAL-GCN5 were first assembled in Bluescript containing a 450 bp *Hin*dIII-*Eco*RI fragment with the GAL4-coding region from the pGBT9 construct (Fields and Song, 1989). The ATG codon of the TRX and GCN5 cDNAs were replaced, using PCR, by a linker sequence and then ligated to the 3′ of the GAL4 DNA binding domain. The fusions contained all the amino acids encoded by the cDNAs except for the initiator methionine. The fusions were then inserted in the C4-Yellow hs vector. The hs-LexA-PC and α1T-LexA-PC constructs are described in Poux et al. (Poux et al., 2001a). Details of the constructions are available upon request. The hs-GAL4 lines are those of Cavalli and Paro (Cavalli and Paro, 1998).

Fly strains and mutants

All transgenic lines were produced using the $Df(1)w^{67c23}\ y^{-}w^{-}$ host. The mutations used were Pc^{3}, trx^{1} and trx^{E2}. To test the effect of the *trx* mutations, the BHL4 reporter insertion and the hs-LexA-PC effector transposon insertion were recombined on the same chromosome and the BHL4G4PRE insertion was recombined with the *Pc* or *trx* mutation. To identify homozygous mutant embryos, the mutations were balanced with a TM3 *hb-lacZ* chromosome. Homozygous mutant embryos lack *lacZ* expression in the head region.

To excise the PRE from the YGfPfMG construct, the transgenic flies were crossed at 18°C with a line expressing the FLP recombinase under the control of the heat-shock promoter. The progeny were heat shocked at 37°C for 2 hours during the first 64 hours of development, then again 1 day later for 2 hours. In the next generation, the ΔPRE flies were identified by the change in eye pigmentation and the excision was verified by Southern analysis. To photograph the eyes, flies were raised at 25°C and aged 2 days.

Antibodies

Rabbit polyclonal antibodies were raised using GST fusion proteins containing amino acids 2419-2704 of TRX, 149-425 of GAGA and 93-276 of PHO. The production of the fusion proteins and the affinity purification of the antibodies have been described previously (Horard et al., 2000).

Staining of embryos and discs

To test the effect of the LexA or GAL4 fusion proteins, flies carrying the target were crossed with flies expressing the fusion proteins under control of the α1-tubulin or *hsp70* promoter. Embryos were collected at 1 hour intervals, aged for different times. Heat shock induction was for 45-60 minutes at 37°C. After further incubation at room temperature, embryos or larvae were fixed and stained as described previously (Poux et al., 2001a). For comparisons, stainings were done in parallel and developed for the same length of time.

Immunoprecipitations assays

Immunoprecipitation assays using a LexA probe are described in Poux et al. (Poux et al., 2001a). The subfragments of the PRE and protein immunoprecipitation procedures are described by Horard et al. (Horard et al., 2000).

RESULTS

The *bxd* PRE contains a stimulating activity

In a series of experiments to determine the functions of the *bxd* PRE, we constructed the YGfPfMG reporter gene in which the PRE is flanked by FRT sites and placed close to the *miniwhite* gene. These elements are enclosed by *Su(Hw)* insulators to minimize position effects and to protect the *yellow* marker gene from the action of the PRE (Fig. 1A). The FRT sites allow us to excise the PRE, by crossing to flies expressing the FLP recombinase under control of a heat shock promoter, and to determine the effects of the presence or absence of the PRE at the same insertion site.

Ten independent YGfPfMG lines display different levels of *miniwhite* gene expression and strong pairing effects when homozygous for the transposon insertion. Independently of the level of eye pigmentation, induction of FLP recombinase results in very efficient excision of the *bxd* PRE, leading to one of two effects depending on the line. Lines with weak eye pigmentation are sensitive to PcG mutations (Fig. 2B) and become darker when the PRE is removed (Fig. 2A). In contrast, lines with stronger *white* expression in the presence of the PRE strongly decrease in pigmentation when the PRE is excised (Fig. 2C), demonstrating that, in some cases, the PRE has a stimulating effect. Genetic experiments showed that the stimulatory effect of the PRE is due to *trx* products: in the presence of a heterozygous trx^{E2} mutation, the eye pigmentation of the PRE-containing lines decreases to a level comparable to that of the corresponding ΔPRE line in a wild-type background. Conversely, no effect of *trx* dosage on eye pigmentation was seen in the ΔPRE lines, confirming that the *trx* effect is mediated by the PRE (Fig. 2D). These results show that the *bxd* PRE is also a TRE, and confirm the role of TRX in the stimulation of expression. They also demonstrate that PRE and TRE have independent and antagonistic effects. Depending on the insertion site, the PRE or the TRE function will predominate.

In vitro binding of PC and TRX is dependent on GAGAG sequences

The antagonistic functions of PcG and TRX complexes led us to ask whether they might share some common binding sequences on the *bxd* PRE. Immunoprecipitation experiments using anti-TRX antibodies show that TRX-containing complexes bind in vitro to many subfragments of the *bxd* PRE, which also interact with PC complexes (Fig. 3A). Tillib et al. (Tillib et al., 1999) showed by deletion experiments that PRE and TRE activities require certain distinct sequences but their experiments did not exclude the possibility that they might also share some motifs in the PRE. Binding sites for the GAGA factor would be good candidates for such a dual role because GAGA factor interacts with PcG complexes and competing GAGAG oligonuclotide or mutations of GAGA sites on the PRE subfragment BP abolish the binding of PC complexes in vitro (Horard et al., 2000). Fig. 3C shows that, while the BP fragment of the *bxd* PRE is immunoprecipitated by PC and TRX antibodies, competition with GAGAG oligonucleotide or mutating the GAGA sites abolishes the binding of TRX as well as that of PC. The absence of GAGA sites does not prevent the binding of PHO, showing that this fragment is still able to bind other proteins. These results suggest that PcG and TRX complexes share some DNA-binding determinants, at least in vitro. They also raise the question of a possible participation of GAGA factor in the TRX complex. Co-immunoprecipitation experiments show, in fact, that anti-TRX antibody immunoprecipitates GAGA protein from embryonic extracts (Fig. 3D) indicating that GAGA is associated with at least some trxG complexes.

We then studied the importance of the GAGA binding sites for TRE function in vivo. We previously reported that 5 out of 13 lines of a S2 *Ubx-lacZ* reporter gene containing the BP subfragment of the PRE maintained the correct pattern of expression in the embryo (Horard et al., 2000). When the BP fragment of this reporter gene was replaced with the corresponding fragment in which all GAGAG sequences were mutated, 12 of 13 lines displayed strong ectopic expression and only 1 of 13 showed partial repression in the thorax. In the 12 derepressed lines, the ability to respond to Pc^3 and trx^{E2} mutations was strongly reduced (Table 1), confirming the importance of the GAGAG sites for both PRE and TRE functions in vivo as well as in vitro.

Table 1. Effect of mutating the GAGA binding sites in BP

	Mutations‡			
Construct*	Var†	Pc^3	trx^{E2}	Maintenance§
BPx6 S2 *Ubx-lacZ*	5/13	7/13	6/13	5/13
BPmutx6 S2 *Ubx-lacZ*	1/13	1/13	1/13	1/13

*BPx6 has six tandem copies of BP and BPmutx6 has six tandem copies of BPmut.
†Number of lines that variegate or repress when homozygous for the transposon.
‡Number of lines that respond to mutation/total number of lines.
§Number of lines in which repression was maintained/total number of lines.

Function of TRX in the context of a PRE

It is often said that the function of TRX is to maintain the expression of homeotic genes initiated in the early embryo by the segmentation gene products. However, we have previously shown that the expression of a PRE *Ubx-lacZ* reporter gene initiated by embryonic enhancers is not maintained in imaginal discs in the absence of specific imaginal enhancers (Chan et al., 1994). To investigate the function of TRX, we constructed two new reporter genes, BHL4G4 and BHL4G4PRE, parallel to the BHL4 reporter described by Poux et al. (Poux et al., 2001a), whose expression of the *Ubx-lacZ* gene is driven by two *Ubx* enhancers: the embryonic BX enhancer and the 2212H1 imaginal disc enhancer (Fig. 1C, Fig. 4B). These two constructs contain both LexA and GAL4 binding sites and BHL4G4PRE also contains the *bxd* PRE. We first studied the function of TRX in the context of a PRE.

Out of five lines obtained with the BHL4G4PRE, three showed a well-maintained pattern of expression both in embryos and in larvae, with repression anterior to parasegment 6 (PS6) (Fig. 4A). In older embryos, in situ hybridization showed that the expression initiated by the BX segmental enhancer ceases, as expected, and that 2212H1-directed expression is confined to a series of small cell clusters in the abdomen, confirming that TRX does not maintain continuous expression but rather enhances the expression driven by the appropriate enhancers. In larvae, the weak expression driven by the 2212H1 enhancer in leg discs (Pirrotta et al., 1995), was strongly enhanced in the presence of the PRE, resulting in strong expression in the posterior part of the third leg (PS6). This demonstrates again the stimulatory activity of the *bxd* PRE.

To examine the effect of *trx* mutations, two third-chromosome lines of BHL4G4PRE that maintained the PS6 boundary were recombined with the trx^{E2} mutation. In both lines, a clear decrease in expression was observed in homozygous *trx* embryos. In heterozygous *trx* larvae, however, in addition to a general decrease in expression, we observed ectopic repression. Although the posterior compartment of the haltere disc (PS6) should show strong and uniform expression, it now contained patches of repressed

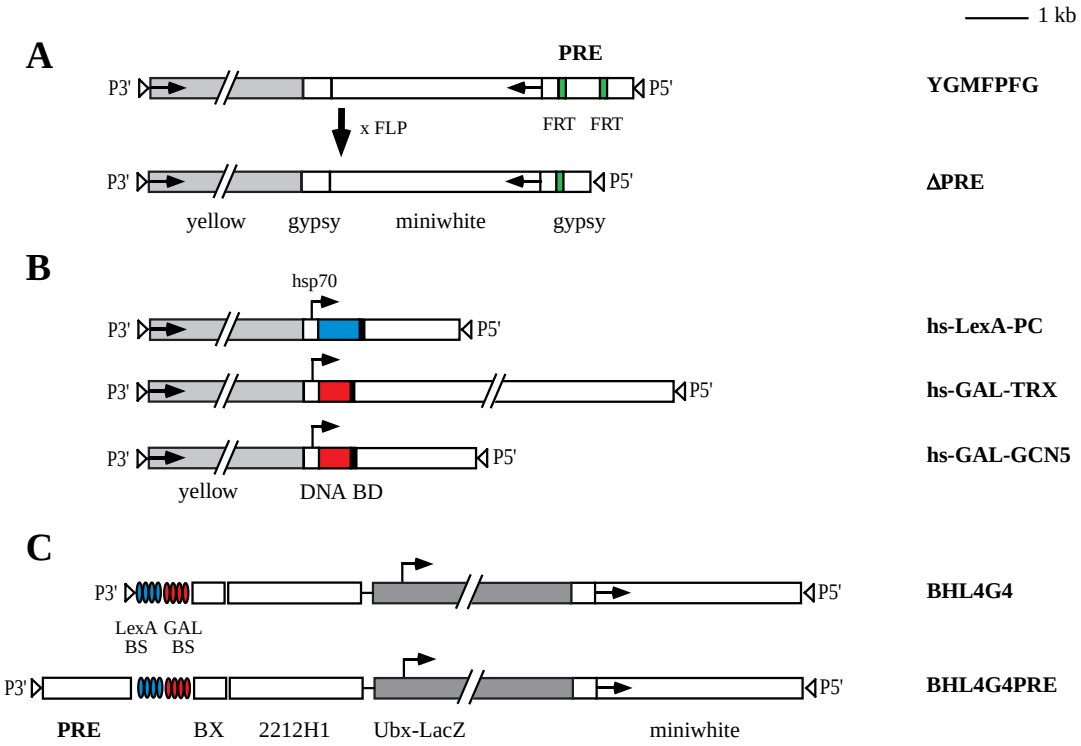

Fig. 1. Transposon constructs. (A) The YGfPfMG construct is shown schematically with the PRE, *gypsy*, *miniwhite* and the *yellow* gene as a marker. FRT sites flanking the PRE allow its excision in the presence of the FLP enzyme, to give YGfMG. (B) The LexA-PC and the GAL-TRX expression constructs: the expression is driven by the *hsp70* promoter and the *yellow* gene serves as a marker. (C) BHL4G4 contains LexA and GAL4 binding sites. The expression of the *Ubx-lacZ* gene is directed by the embryonic BX enhancer and the 2212H1 imaginal disc enhancer of *Ubx*. The marker gene is *miniwhite*. In BHL4G4PRE, the *bxd* PRE is added. The arrows indicate the direction of transcription. The *yellow*, *lacZ* and *trx* genes are not shown to scale.

cells. In addition, expression in the third leg disc was almost completely silenced, with residual patches of weak expression in its posterior part (Fig. 4B). A similar hyperrepression in imaginal discs was also observed when flies carrying a similar reporter gene in a homozygous trx^1 background were raised at 27°C, a restrictive temperature for this temperature-sensitive allele, or when they were transferred to 27°C 1 day after egg deposition (results not shown). These results indicate that when TRX levels are insufficient, silencing may be re-established at later stages, particularly in leg discs, where the expression driven by the 2212H1 enhancer is weak. These results suggest that the role of TRX is to prevent the re-establishment of silencing. The clonal appearance of the patches of ectopic repression in these experiments suggests that there may be a critical stage when an adequate level of TRX is important.

Function of a synthetic Trithorax response element

What might then be the action of TRX independently of a PRE? To answer this question, we constructed a synthetic TRE by fusing the GAL4 DNA binding domain to the N terminus of TRX. The construct was assembled in the C4-Yellow hs transposon, which uses the *yellow* gene as a marker and the *hsp70* promoter to drive expression of the GAL-TRX gene (Fig. 1B). As a target reporter, we used BHL4G4, whose pattern of expression is similar to BHL4 construct (Poux et al., 2001a), and contains four GAL4 binding sites adjacent to four LexA binding sites, allowing us to target simultaneously an activator and a repressor. We first tested the effect of targeting TRX alone. Transgenic flies containing the BHL4 or the BHL4G4 transposon were crossed with flies carrying the hs-GAL-TRX transposon and the resulting embryos were heat

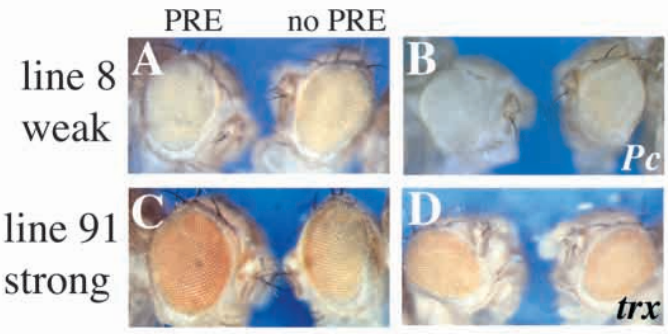

Fig. 2. Eye pigmentation in presence or absence of the *bxd* PRE. (A) Eye pigmentation is repressed by the PRE in line 8 (left) and is increased by deleting the PRE (right). (B) PRE repression is decreased by a heterozygous *Pc* mutation (right) compared to the wild type (left). (C) In line 91, eye pigmentation is stronger in presence of the PRE (left) than in its absence (right). (D) The stimulating effect of the PRE is due to TRX. In flies heterozygous for a *trx* mutation, eye pigmentation decreases (left) to a level comparable to lines without the PRE (right). Eye pigmentation in absence of the PRE is much stronger in line 91 compared to line 8 (compare A, C, right).

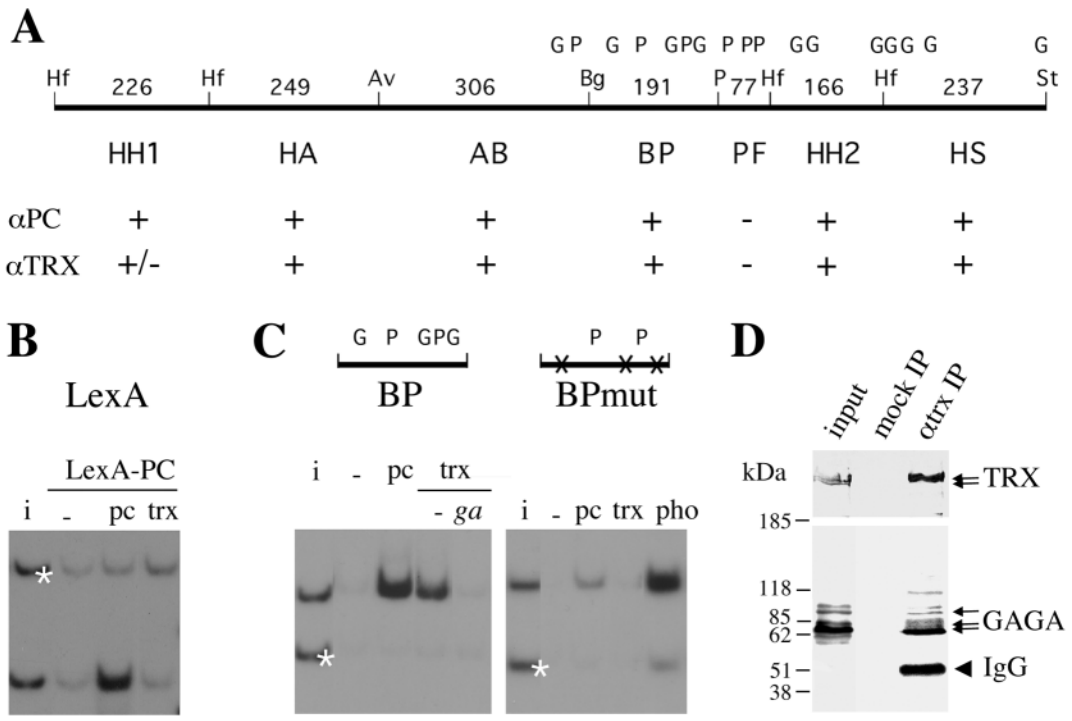

Fig. 3. Immunoprecipitation of PRE fragments. (A) Map of the *bxd* PRE with subfragments produced by *Hin*fI (Hf), *Ava*II (Av), *Bgl*I (Bg), *Pst*I (P) and *Sty*I (St) and their sizes in bp. GAGA and PHO binding sites are indicated by G and P. Immunoprecipitation of a fragment with anti-PC or anti-TRX is indicated by +, no precipitation by –. (B) LexA binding assays. Anti-PC immunoprecipitates the LexA probe from nuclear extracts containing LexA-PC but anti-TRX does not, showing that LexA-PC does not recruit TRX. A control fragment present in the binding reaction is indicated by *. Lanes : –, no antibody; pc, anti-PC; trx, anti-TRX. The immunoprecipitated fragments were analysed on an acrylamide gel together with an aliquot of the input mixture (i). (C) The BP subfragment (left) is precipitated by anti-PC, anti-TRX and anti-PHO but TRX binding is lost in the presence of GAGAG oligonucleotide competitor (*ga*). Mutation of all GAGAG sequences in this fragment (BPmut) impairs the binding of both proteins (right) while PHO still binds the mutated fragment. A diagram of BP and BPmut is shown above each panel. (D) Co-precipitation of GAGA and TRX. Nuclear extracts were immunoprecipitated with no antibody (mock IP) or with anti-TRX (αtrx IP). Western blots of the IPs were probed with anti-GAGA or anti-TRX. The arrowhead indicates the IgGs in the immunoprecipitate.

shocked at different times during development to express the chimeric protein. After further incubation at room temperature for 2-18 hours, the embryos were fixed and stained. GAL-TRX induction is not lethal and does not induce any phenotype even with strong and repetitive heat shocks, showing that the overexpression does not interfere with the normal functioning of endogenous PREs (results not shown). The induction of GAL-TRX had no effect on the BHL4 reporter, which lacks GAL4 binding sites, but it enhanced the expression of the BHL4G4 reporter. Significantly, GAL-TRX did not induce expression where the target gene is normally inactive, since in germ band elongation embryos that were heat shocked at the blastoderm stage, expression was enhanced in PS6, 8, 10 and 12 but not in the odd-numbered parasegments,

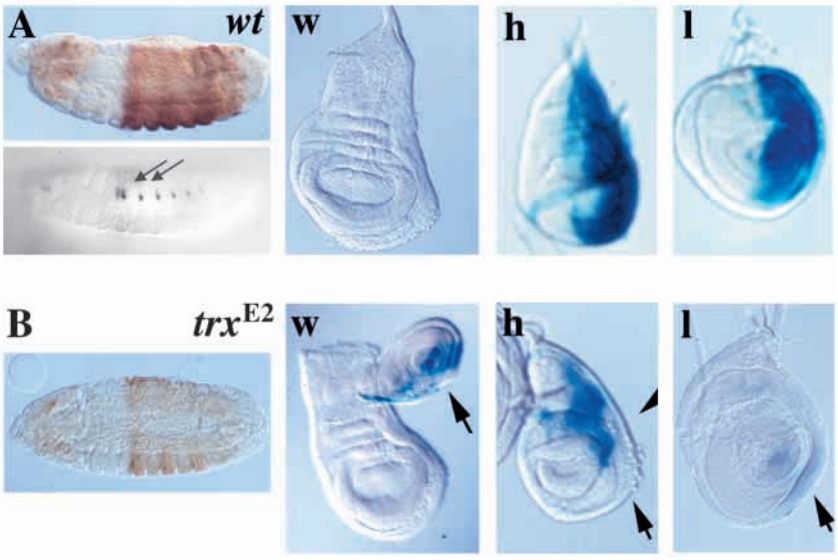

Fig. 4. TRX prevents re-establishment of repression. (A) BHL4G4PRE expression is maintained anterior to PS6 throughout embryonic development. In situ hybridization in late embryos shows that BX-driven expression ends in the ectoderm and H1-driven expression is limited to dorsolateral spots (arrows). In larvae, expression is repressed in the wing disc (w) and limited to the posterior haltere (h) and third leg (l) discs. (B) In homozygous *trx* embryos, the level of expression is reduced. In heterozygous *trx* larvae, expression in the posterior haltere is variegated and almost absent in the third leg disc. The arrows show the repressed domains in the haltere disc and the weak residual expression in the leg disc.

where expression is normally absent at that time (Fig. 5B). The results were the same when GAL-TRX was expressed at any time during embryonic development: the pattern of expression was the same but the level of expression of the BHL4G4 reporter was increased. These results show that GAL-TRX cannot initiate expression by itself and only stimulates expression in the cells where the gene is active, either by facilitating the action of enhancer factors or by stimulating the promoter complex. GAL-TRX therefore acts as a coactivator that enhances expression where transcription is initiated. The stimulation of expression is not permanent and transient induction of GAL-TRX in embryos did not enhance expression in larval tissues. However, repeated heat shocks during larval development significantly increased the weak expression in leg discs, whereas the already strong expression in dorsal imaginal discs was not visibly increased (Fig. 5C), consistent with the results obtained with BHL4G4PRE.

We have previously reported that LexA binding sites behave as a genuine synthetic PRE, at least during embryonic development (Poux et al., 2001a). When LexA-PC is produced, a repressive complex containing other PcG proteins is recruited to the LexA binding sequences of the BHL4 reporter gene, resulting in maintenance of repression in inactive cells, whereas expression was allowed in cells that were initially active. The expression of BHL4 in the presence or absence of LexA-PC is not affected by *trx* mutations (Fig. 5A), implying that the recruitment of a silencing complex by LexA-PC does not involve TRX, which requires other recruiting mechanisms. Immunoprecipitation experiments using a radioactive LexA DNA probe as a tag confirm that the LexA-PC protein is not associated with TRX in extracts containing the LexA-PC protein (Fig. 3B).

We then looked at the effect of targeting both LexA-PC and GAL-TRX to the BHL4G4 reporter gene. We first recombined, on the same chromosome, the hs-LexA-PC and the BHL4G4 construct marked with the *yellow* and *miniwhite* gene, respectively. Recombinant males were then crossed with females carrying the hs-GAL-TRX construct. Induction of both chimeric proteins at 2-3 hours of development resulted in the recruitment of a repressive complex by LexA-PC in the thorax of the embryo, while expression in the abdomen was stimulated by GAL-TRX (Fig. 5D). Therefore, either the transcriptional state determines whether PC complexes or TRX complexes can be recruited, or the two complexes can coexist without interfering with one another. In these experiments, however, LexA-PC has a distinct advantage because it is a much smaller protein than GAL-TRX and, although its expression is induced at the same time, it will be available several minutes before the GAL-TRX protein.

Effect of TRX on the memory of the *bxd* PRE

Cavalli and Paro (Cavalli and Paro, 1998; Cavalli and Paro, 1999) proposed that the epigenetic memory of the *Fab-7* PRE might be mediated by TRX because the heritable derepressed state induced by a pulse of GAL4 activator was lost in flies heterozygous for a *trx* mutation. Our results suggest alternative but not mutually exclusive interpretations. TRX

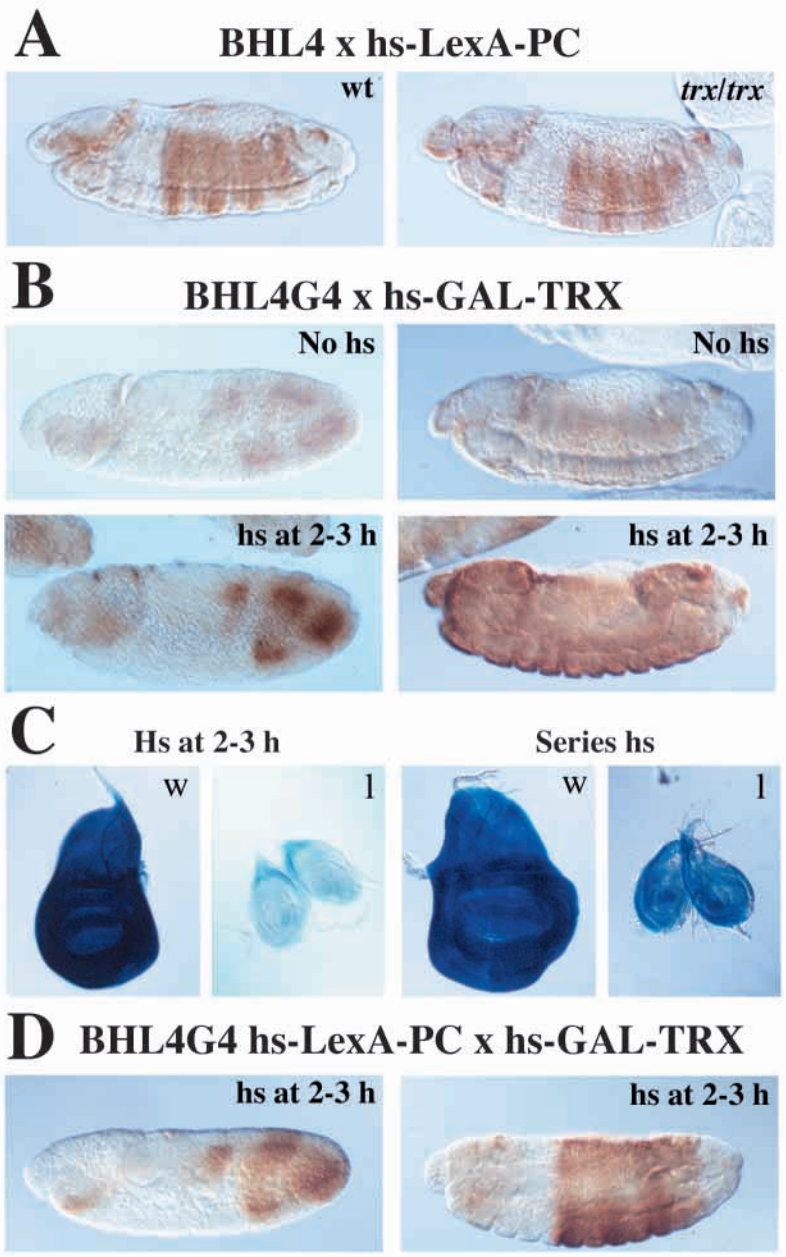

Fig. 5. Effect of targeted TRX. (A) The pattern of expression of embryos carrying both BHL4 and hs-LexA-PC heat shocked at 2-3 hours is the same in a wild-type and in a homozygous *trx* background (*trx*). (B) BHL4G4 expression in early embryos (left) and after germ band retraction (right) with no induction of GAL-TRX (no hs). When GAL-TRX is induced at 2-3 hours, expression is enhanced only in regions where the gene was active (parasegments 6, 8, 10 and 12). In older embryos, after ectopic expression appears in the thorax, expression is stimulated everywhere. (C) The effect of GAL-TRX is transient and its induction at blastoderm does not stimulate expression in larvae (hs at 2-3 hours). A series of heat shocks 2 days before staining strongly enhances expression in leg discs (series hs). D) When both GAL-TRX and LexA-PC proteins are induced, LexA-PC recruits a repressive complex in the thorax (left), while GAL-TRX stimulates expression in the abdomen. Embryos heat shocked as in (B).

function might be needed for effective induction of expression. Or, TRX stimulation of expression might be necessary for sufficient levels of transcription to effect changes in the chromatin state. Thirdly, TRX might preserve the memory of derepression by maintaining an epigenetically inherited chromatin state. To determine the role played by TRX in the epigenetic memory of the PRE, we targeted to the BHL4G4PRE either GAL-TRX or the GAL4 activator, with the same hs-GAL4 line used by Cavalli and Paro (Cavalli and Paro, 1998). As a control, we tested the effect of the two activators on the BHPRE reporter gene, which lacks both LexA and GAL4 binding sites. Induction of these proteins at different times during development had no detectable effect on the expression of the BHPRE reporter. No effect was observed even in the case of GAL-TRX, although the *bxd* PRE contains a TRE.

In embryos containing the BHL4G4PRE reporter, induction of GAL-TRX at the blastoderm stage interfered with the formation of the repressive complex at the PRE and ectopic expression appeared in the thorax at later embryonic stages. The derepressed state was maintained throughout development, long after the disappearance of the GAL-TRX protein induced by the heat shock. As a result, strong expression occurred in all larval imaginal discs (Fig. 6A) and a clear increase in eye pigmentation was visible in adult flies. However, no derepression or other visible effects were noticed when GAL-TRX was induced in 5- to 6-hour-old embryos (Fig. 6B). Even a series of daily heat shocks during larval development failed to induce derepression in imaginal discs (Fig. 6C). This suggests that, once established, the repressive complex is stable and cannot be antagonized by GAL-TRX. We interpret these results to mean that, although endogenous TRX is maternally supplied in the embryo, it is not recruited or does not act before blastoderm but targeting GAL-TRX to the BHL4G4PRE reporter gene at this time antagonizes the formation of the repressive complex. The striking difference between this result and the apparent compatibility of GAL-TRX with LexA-PC silencing suggests that GAL-TRX may cooperate with the TRE contained within the PRE of BHL4G4PRE.

When the GAL4 protein was induced in early embryos, the results were essentially similar although weaker than those obtained with GAL-TRX: ectopic expression appeared in embryos and larval imaginal discs (Fig. 6E). In contrast to Cavalli and Paro (Cavalli and Paro, 1998), we found that GAL4 must be produced no later than the blastoderm stage to give this effect in our system, since no derepression was observed when GAL4 was induced after 5-6 hours, even by repeated heat treatments (results not shown). A second difference in our results with respect to those of Cavalli and Paro concerns the meiotic inheritance of the derepressed state. These authors reported that the GAL4-induced derepression was maintained in a quarter of the progeny flies, implying that a chromatin state stable through meiosis prevented the re-establishment of repression in the progeny embryos. With our two activators, we tested whether our BHL4G4PRE reporter gene can mediate a meiotic effect. We crossed flies in which the activators had been induced at the blastoderm stage either inter se or with wild-type males but detected no sign of derepression in the progeny embryos, larvae or flies. With both activators, the repressed pattern of expression was still maintained in the appropriate regions of the embryo or imaginal discs and eye pigmentation was not altered, even with the GAL4 activator line used by Cavalli and Paro. This difference may be accounted for by the fact that our reporter gene includes an enhancer element that restores the normal pattern of both repression and expression in the early embryo.

To test whether mitotically stable derepression requires *trx* function, flies carrying the GAL-activator construct were crossed with the recombined BHL4G4PRE *trx*^{E2} mutant flies and the resulting embryos were heat shocked at the blastoderm stage. The results with GAL-TRX (Fig. 6D) and GAL4 were essentially similar (Fig. 6F). Ectopic expression was evident during embryonic development but derepression was only partial, compared to wild-type embryos. The derepressed state persisted

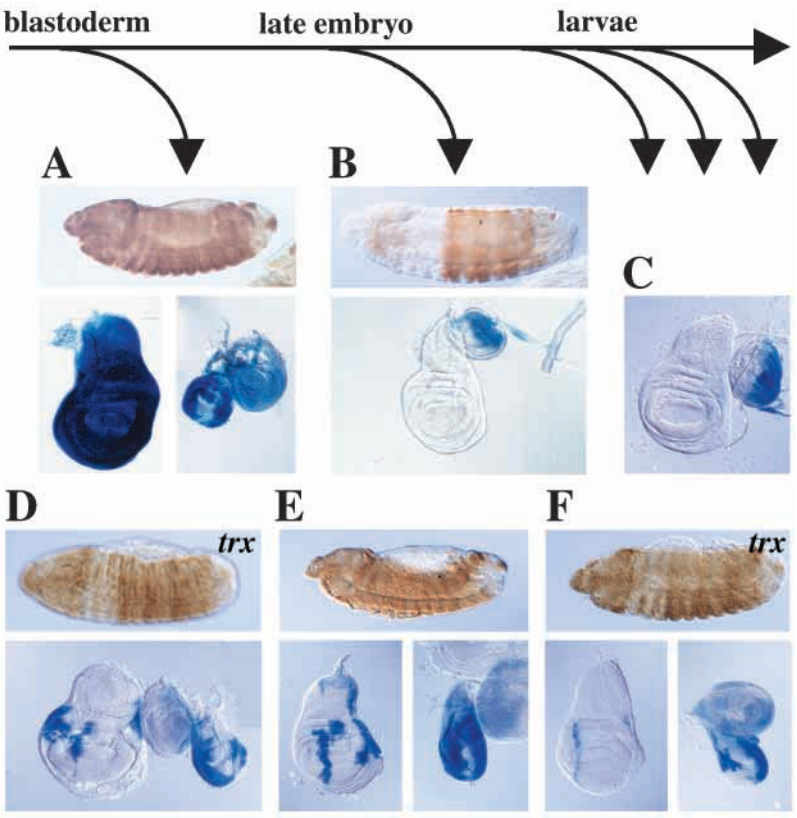

Fig. 6. PRE silencing is destabilized by TRX or GAL4. GAL-TRX (A-D) and GAL4 (E-F) counteract silencing when targeted to the BHL4G4PRE reporter gene. When GAL-TRX is induced at the blastoderm stage, it interferes with the formation of the repressive complex in old embryos as well as in larvae (A). When GAL-TRX is induced after 5-6 hours (B) or during larval development (C), repression is not affected. When the TRX level is reduced, targeting GAL-TRX (D) at blastoderm to the BHL4G4PRE leads to a partial derepression in embryos and larvae. However, the residual repression is well maintained throughout development. Targeting GAL4 to the BHL4G4PRE gave similar results (E), with a partial derepression in a *trx* mutant background (F).

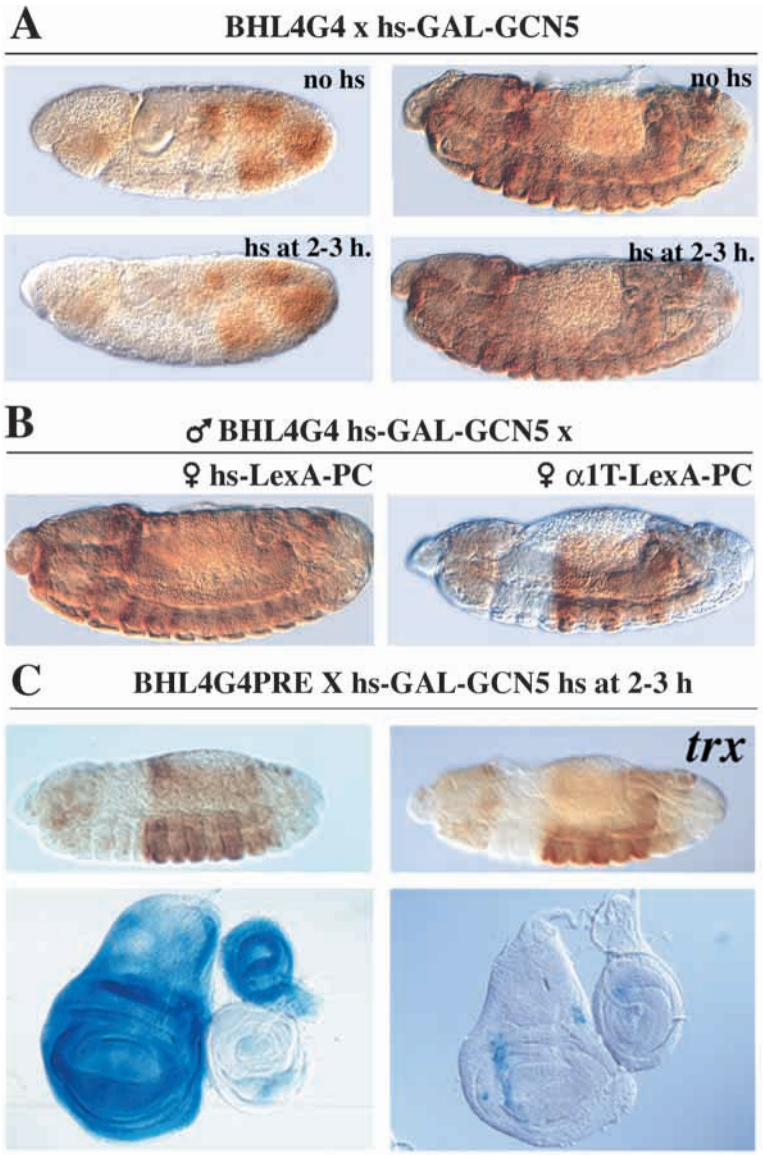

Fig. 7. GAL-GCN5 at blastoderm causes loss of silencing memory in larvae. (A) BHL4G4 expression in early embryos (left) and after germ band retraction (right) with no induction of the GAL-GCN5 protein (no hs). GAL-GCN5 induction at 2-3 hours does not affect the expression of BHL4G4. (B) When both GAL-GCN5 and hs-LexA-PC (hs-LexA-PC) proteins are produced at 2-3 hours, repression induced by hs-LexA-PC is prevented by GAL-GCN5, resulting in a strong ectopic expression in late embryos. In contrast, when α1-tubulin-LexA-PC (α1T-LexA-PC) is provided maternally, GAL-GCN5 does not interfere with PcG silencing. (C) When targeted to BHL4G4PRE in the 2-3 hours embryo, GAL-GCN5 does not interfere with repression in late embryos but results in loss of repression in larval imaginal discs. When the TRX level is reduced (*trx*), targeting GAL-GCN5 to BHL4G4PRE leads only to a very weak and partial derepression in larvae.

in larvae but the derepressed domains were much reduced, relative to the wild-type control. Instead of a general derepression in all larval segments, we observed only patches of ectopic expression (compare Fig. 6A with Fig. 6D), showing that the GAL-fusion proteins in the early embryo can still counteract PRE repression in a mitotically heritable way. However, in the absence of normal levels of endogenous TRX, repression is restored in some cells.

Targeting histone acetylation causes loss of repressive memory

Histone acetylation is generally associated with transcriptional activation as well as with actively transcribing promoters. It is possible therefore that histone acetylation is part of the mechanism that prevents a PRE from establishing repression of an active gene. To test this hypothesis, we targeted the yeast histone acetyltranferase GCN5, fused to the GAL4 DNA binding domain, to the BHL4G4 reporter either alone or together with LexA-PC. Alone, GAL-GCN5 had no detectable effect on the expression of BHL4G4 when induced before blastoderm or during later development (Fig. 7A). When targeted together with hs-LexA-PC in the pre-blastoderm embryo, hs-GAL-GCN5 prevents the establishment of repression by LexA-PC and results in the appearance of completely derepressed expression after germ band extension. If, however the LexA-PC is driven by the constitutive α1-tubulin promoter and inherited maternally, hs-GAL-GCN5 does not prevent its normal silencing activity (Fig. 7B). This strongly suggests that GCN5 interferes with the early stages of establishment of silencing. If these have already occurred in the pre-blastoderm embryo, GCN5 activity does not prevent silencing. As described by Poux et al. (Poux et al., 2001b), early events at the PRE that might be sensitive to acetylation involve the assembly of a transient complex that includes both PC and ESC components, as well as the RPD3 histone deacetylase.

We then tested the effect of acetylation in the presence of the PRE by targeting GAL-GCN5 to BHL4G4PRE. Induction of hs-GAL-GCN5 in the 2- to 3-hour embryo had no visible effect on the embryonic repression instituted by the PRE but, when these embryos were allowed to develop to larvae, the pre-blastoderm induction of GAL-GCN5 resulted in partial loss of silencing in imaginal discs. Strong ectopic patches of expression appeared in eye, wing and all leg discs, as well as in the anterior compartment of the haltere, showing that the silenced state that prevailed during embryonic development could not be maintained in the larva (Fig. 7C). Induction of GAL-GCN5 after blastoderm or later embryonic or larval stages had no detectable effect.

These results are consistent with the idea that PcG complex formation at the PRE initiates well before blastoderm with maternally supplied components (Poux et al., 2001b). GAL-GCN5 expressed at the blastoderm stage does not interfere with the establishment of a silencing complex at the PRE or with its silencing activity in the embryo. Most interestingly, however, these results suggest that GCN5-induced acetylation still has an effect at this time, not to prevent PRE silencing as such but to interfere with a step during early embryonic development that is necessary for the establishment of epigenetically stable silencing.

DISCUSSION

TRX stimulates expression

Our results with the YGfPfMG construct show that, depending on the insertion site, the *bxd* PRE acts mainly as a PRE or mainly as a TRE. This variability even when the PRE-*miniwhite* is flanked by *su(Hw)* insulators has been reported previously and attributed to the presence of the PRE (Sigrist and Pirrotta, 1997), however, the variability persists when the PRE is removed. Lines with predominant TRE activity still have a relatively low eye pigmentation after excision of the *bxd* PRE, suggesting that the transposon inserted in sites that do not promote strong expression. In contrast, lines with predominant TRE activity continue to have higher levels of eye pigmentation even after excision of the *bxd* PRE, suggesting that the genomic location of the transposon favors transcriptional activity. Taken together, these results suggest that the nuclear environment favors expression or repression, calling into play the TRE or PRE activities, respectively. Our results suggest that the choice may depend on the level of histone acetylation of the transgene in the early embryo.

Simultaneous targeting of LexA-PC and GAL-TRX to a reporter gene supports the idea that either silencing or stimulation will occur, depending on the initial state of activity. Thus LexA-PC maintains repression in the thorax, where the gene was inactive, while GAL-TRX stimulates the expression in the abdomen, where the gene was already active. The balance between the two seems to be dynamic and may be swayed by their relative abundance, as suggested by the genetic evidence (Capdevila et al., 1986). We suppose that reducing PC weakens repression and favors ectopic expression that is enhanced by high levels of TRX. When the level of both PC and TRX proteins is reduced, neither activity can overwhelm the other, and the effects partially cancel each other out (Sato and Denell, 1985).

Our results show that TRX is not an autonomous activator and does not maintain continuous expression initiated by early-acting enhancers but stimulates the expression of an otherwise active gene. In the absence of the appropriate enhancer factors, transcription does not occur despite the presence of TRX. Furthermore, when BHL4G4PRE is derepressed by either GAL4 or GAL-TRX, no *lacZ* expression is induced in tissues where it is not activated by the 2212H1 enhancer. We do not know how TRX stimulates expression. A possible explanation is that it recruits the CREB binding protein (CBP), a well-known coactivator with histone acetylase activity that interacts genetically with ASH1, a direct partner of TRX (Bantignies et al., 2000; Rozovskaia et al., 1999). Another, not incompatible hypothesis would be that TRX stimulates expression by facilitating the access of activators or RNA polymerase. Rozenblatt-Rosen et al. (Rosen et al., 1998) found that TRX interacts with SNR1, a component of the *Drosophila* SWI/SNF complex, suggesting that it could recruit this remodelling activity.

PcG, TRX complexes and GAGA binding sites

The immunoprecipitation experiments show that both TRX and PC complexes bind in vitro to most PRE subfragments. However, in vivo results indicate that only part of PRE fragment AB, part of BP and part of HS are involved in the *trx* response (Tillib et al., 1999; Orlando et al., 1998). It is important to note that the binding detected in vitro is probably the result of complexes preassembled in vivo and does not necessarily reflect the in vivo specificity (Horard et al., 2000). From this point of view, the presence of GAGA factor in a TRX complex would account for the binding of TRX to any DNA fragment that contains GAGA consensuses, whether or not it can recruit a TRX complex in vivo. In the case of the BP fragment, however, the genetic analysis confirms that it has a TRE activity.

Our results indicate that GAGA factor is associated with both PcG and TRX complexes. Tillib et al. (Tillib et al., 1999) showed that small PRE deletions affect the response to either *Pc* mutations or *trx* mutations, implying that PcG and TRX complexes bind neighboring but separable sequences. Their results do not contradict our finding that mutating GAGA sites abolishes both PC and TRX in vitro binding and in vivo response to *Pc* and *trx* mutations. The two complexes could require different sets of recruiting sequences while sharing a dependence on GAGA binding sites. GAGA factor was classified as a trxG protein because its mutations (*Trl*) cause homeotic loss-of-function phenotypes (Farkas et al., 1994). However, a connection with repressive functions is suggested by the presence of GAGA protein in some PcG complexes (Horard et al., 2000; Hodgson et al., 2001), and by its interaction with SAP18, a component of the Sin3-HDAC co-repressor complex (Espinas et al., 2000). The effects of *Trl* mutations on PcG silencing are contradictory. Reducing GAGA dosage decreases silencing in some cases (Hagstrom et al., 1997; Horard et al., 2000) but has the opposite effect in others (Cavalli and Paro, 1999; Strutt and Paro, 1997). This might be explained if GAGA sites contribute to the formation of both PcG and TRX complexes. Depending on the nature of the PRE, the insertion site or the reporter gene, GAGA mutations might have either a positive or negative overall effect on expression.

If PC complexes and TRX complexes both utilize GAGA binding sites, the binding of one complex might exclude the binding of the other. However, on polytene chromosomes, TRX is found at loci that are repressed by a PRE (Chinwalla et al., 1995), suggesting that the presence of PcG complexes is not incompatible with the binding of TRX. The fact that other subfragments of the *bxd* PRE bind TRX in vitro but lack GAGAG sequences (Tillib et al., 1999), suggests that there exist alternative TRX recruitment modes and explains why *Trl* mutations have weaker effects than *trx* mutations on homeotic gene expression.

TRX is required to prevent late repression

Reduction of TRX dosage in our BHL4G4PRE reporter lines leads to loss of expression in patches of cells in the posterior haltere, a segmental domain in which expression had been activated at blastoderm. This is consistent with the idea that PRE and TRE functions are antagonistic and normally kept in balance. In absence of sufficient TRX, PcG silencing can apparently be established at later stages, leading to complete repression in the third leg disc, where expression is normally weak, or stochastic repression in patches of cells of the haltere disc, where expression is stronger. The fact that this is also observed in homozygous trx^1 larvae that had been transferred to a restrictive temperature after hatching indicates that repression can be established de novo during larval

development. We suppose that the level of expression of the reporter gene varies during development, as the activators that bind to the two different enhancers rise and fall. The presence of TRX might then prevent PcG silencing from being re-established in the intervals between the end of one enhancer activity and the onset of the other. The same mechanism might account for the epigenetic memory function associated with PREs. In fact, the ectopic repression observed in *trx* larvae might be thought of as a loss of memory of the derepressed state.

TRX and epigenetic memory

Reducing the TRX level has two effects on our BHL4G4PRE reporter: expression is lower and the derepressed state is more subject to re-repression. It is difficult to determine whether the *trx* mutation affects the activation of the reporter gene, resulting in weak or incomplete derepression, or the memory of the derepressed state. The two alternatives merge into one if, for example, TRX induces a chromatin structure that both enhances transcription and interferes with the re-establishment of PcG silencing.

Our results support this dual role. For reasons already mentioned, it is unlikely that the derepressed state implies continuous and permanent transcriptional activity. Although Cavalli and Paro found that their stably derepressed reporter maintained some degree of expression even when the activator was no longer present, this may correspond to a heightened basal level of the minimal TATA box promoter they used. The basal level of such a promoter might be increased simply by histone acetylation, as has been observed in yeast (Candau et al., 1997). In our case, the activators were targeted to sites adjacent to the PRE but more than 3 kb distant from the nearest promoter. Our results show that although TRX stimulates an already active gene, it does not maintain active transcription throughout development. It does antagonize the establishment of PcG repression and favor the action of appropriate activators. We suppose that these two functions might be carried out by the same biochemical activity that sets a molecular mark on active chromatin, which is both heritable and stimulatory when appropriate enhancer factors are present.

Although we have not proved that they depende on the catalytic domain, the experiments with GAL-GCN5 strongly suggest that histone acetylation can selectively interfere with the memory of the silenced state without affecting the silencing activity of the PRE. The preferred target of GCN5 acetylation in vitro and in vivo is histone H3 (Kuo et al., 1998; Suka et al., 2001), suggesting that this core histone is important for establishing a mark of the repressed state but not necessarily for the repressive activity as such. GAL-GCN5 behaves differently, however, from GAL-TRX, which prevents PRE silencing completely. One interpretation of these results is that, while GAL-GCN5 prevents the memory of the silenced state from being established, GAL-TRX goes a step further and establishes the memory of a derepressed state. Experiments are in course to verify this interpretation.

Epigenetically stable chromatin states imply the emplacement of chromatin modifications that are regenerated every round of replication. Either the derepressed state or the repressed state, or both, could be specifically marked to convey an epigenetic memory of that state. Histone acetylation is frequently associated with active genes. Recent reports suggest that it may have another significance in controlling histone modifications such as methylation that may be even more important for the maintenance of chromatin states. Acetylation at histone H3 lysine9 prevents the methylation of this lysine by SUV39 that is important for heterochromatic silencing (Rea et al., 2000; Bannister et al., 2001). The state of acetylation might therefore determine whether chromatin can be marked with a histone methylation imprint. It may be significant that the methyltransferase activity of SUV39 is due to its SET domain, a structural motif shared by TRX and by the PcG protein EZ. Recently, methylation has also been found associated with transcriptional activation: the PMRT1 protein directs the specific methylation of histone H4 arginine3, which facilitates a subsequent acetylation (Wang et al., 2001). This mechanism is particularly interesting in light of the specific acetylation of histone H4 that has been found associated with the epigenetically maintained derepressed state (Cavalli and Paro, 1999).

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