Binding of Trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early *Drosophila* embryogenesis

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In *Drosophila*, the maintenance of developmentally important transcription patterns is controlled at the level of chromatin structure. The *Polycomb* group (PcG) and *trithorax* group (trxG) genes encode proteins involved in chromatin remodelling. PcG genes have been proposed to act by packaging transcriptional repressed chromosomal domains into condensed heterochromatin-like structures. Some of the trxG proteins characterized so far are members of chromatin opening complexes (e.g. SWI/SNF and GAGA/NURF) which facilitate binding of transcription factors and components of the basal transcriptional machinery. Genetic and biochemical data suggest that these two groups of regulatory factors may act through a common set of DNA elements. In the present study, we have investigated the binding of Trithorax (TRX) and Polycomb (PC) protein in the bithorax complex (BX-C) during embryogenesis. In addition, we have identified the minimal fragments from the Ultrabithorax (Ubx) regulatory region that are capable of recruiting TRX to chromosomal sites containing them. Comparative analysis of the binding of the two proteins shows that TRX and PC bind target sequences (PcG-regulated elements, PREs) by cellular blastoderm, when BX-C transcription begins. At the same stage, TRX but not PC is strongly associated with core promoters. Later, at germ band extension, the time of derepression in Polycomb mutants, PC binding is also detected outside core PREs and additionally binds to the fragments containing promoters.

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Introduction

In *Drosophila*, the basic body plan is laid down in the early embryo through the action of transiently expressed transcription factors that establish the spatially restricted patterns of homeotic gene expression. These patterns and the determined cell fates they implement are faithfully maintained throughout the rest of development by the action of another set of factors encoded by the Polycomb group (PcG) and trithorax group (trxG) genes. The PcG

proteins are required to maintain the transcriptionally inactive state, whereas the trxG proteins are necessary to counteract silencing and maintain the transcriptionally active state. Both PcG and trxG proteins are thought to function by establishing closed or open chromatin configurations at their target genes (for reviews see Paro and Harte, 1996; Pirrotta, 1997).

Mutations in the trithorax (*trx*) gene result in transformations of body structures reminiscent of loss-of-function mutations in homeotic genes (Ingham, 1985). After disappearance of the transiently acting patterning factors like those encoded by the segmentation genes, maintenance of the initial transcriptional patterns of homeotic genes requires the trithorax (TRX) protein. *trx* is expressed both maternally and zygotically, and TRX protein is detectable in very early embryos (E.P.Jane and P.J.Harte, unpublished data). Genetic analyses indicate that *trx* expression is required continuously throughout development, consistent with its maintenance function, but there also appears to be a critical early requirement, which if compromised cannot be compensated by subsequent continuous expression (Ingham and Whittle, 1980). Comparatively little is known about the molecular environment in which the TRX protein is integrated. However, an elucidation of this issue could be particularly rewarding as chromosomal aberrations involving the human homologue of TRX (MLL, ALL-1, HRX) is one of the most frequent genetic changes in infant leukemias of myeloid and lymphoid lineage and in treatment-induced secondary leukemias (for review see Look, 1997).

Additional trxG genes have also been identified as suppressors of the derepression phenotypes caused by the breakdown of silencing in PcG mutants (Kennison and Tamkun, 1988). However, this does not necessarily mean that all trxG proteins play a specific role in countering or modulating PcG repression. While some trxG proteins may function as specific 'anti-repressors', directly modulating PcG-mediated repression, others are likely to be involved in more general aspects of transcriptional activation. Indeed, several of the trxG proteins appear to be likely to influence PcG repression indirectly through a more general role in transcription. For example, Moira encodes the SWI2-related TAFII170 protein, which appears to be an integral component of the basal transcription machinery (van der Knaap *et al.*, 1997). Brahma and ISWI (SWI2 homologues; Tamkun *et al.*, 1992), and also the trithorax-like GAGA factor (Farkas *et al.*, 1994) are associated with chromatin remodelling complexes possessing ATP-dependent nucleosome displacement activities, which facilitate the binding of other transcription factors and clearly have more global roles in transcriptional activation (reviewed by Kingston *et al.*, 1996).

All PcG proteins analysed so far have been found to co-localize at many specific chromosomal sites (De Camillis

et al., 1992; Rastelli *et al.*, 1993; Lonie *et al.*, 1994; Carrington *et al.*, 1996), indicating that they interact directly with their target genes to silence them. Some PcG proteins have been further shown to be associated in high molecular weight complexes *in vivo* (Franke *et al.*, 1992; Jones *et al.*, 1998; Kyba and Brock, 1998), suggesting that silencing requires the assembly of large multiprotein complexes at target genes. So far, none of the characterized PcG proteins has been shown to bind DNA in a sequence-specific manner. However, genetic and biochemical studies clearly identified specific DNA elements responsive to PcG proteins (PcG response elements, PREs) (Castelli-Gair and Garcia-Bellido, 1990; Gyurkovics *et al.*, 1990; Zink *et al.*, 1991; Simon *et al.*, 1993; Chan *et al.*, 1994; Chang *et al.*, 1995; Gindhart and Kaufmann, 1995). A general feature of PREs when analysed in transgenic flies is that a large PRE works better than a minimal PRE. In addition, the latter performs best in combination with regulatory elements taken from its natural gene (Müller and Bienz, 1991; Chan *et al.*, 1994; Müller, 1995).

Co-localization of TRX and PcG proteins on chromosomes suggests that PREs may be closely associated with *trxG* response elements (TREs). Transgene studies have shown that DNA fragments containing the PRE identified in the 5' regulatory region of the Ultrathorax (*Ubx*) gene also contain a TRE (Chan *et al.*, 1994; Chang *et al.*, 1995; Chinwalla *et al.*, 1995). PREs and TREs have been identified within the same 11 kb fragment of the homeotic gene, *Scr* (Gindhart and Kaufman, 1995). GAGA factor is intimately associated with PREs and despite its general function in transcriptional activation, also appears to be required for PcG repression, probably by facilitating binding of PcG proteins (Hagstrom *et al.*, 1997; Strutt *et al.*, 1997). So far, a large proportion of members of the *trxG* and PcG that have been analysed constitutively co-localize at common chromosomal elements, independent of the current transcriptional activity that the element is sustaining. This finding eliminates the simple view that mutually competing activating and repressing complexes maintain the expression status as binding alone appears not to be a hallmark of function. As such it will be important to define the molecular interplay between the various members of the *trxG* and PcG at the common target sites, in order to assess their respective roles in establishing and maintaining closed versus open chromatin conformations.

We have utilized the formaldehyde crosslinking chromatin-IP methodology (X-CHIP) to map TRX- and PC-binding sites within the BX-C of embryonic chromatin at high resolution. This allowed us to compare the temporal dynamics of TRX- and PC-binding at PREs/TREs during subsequent stages of embryogenesis. Additionally, both proteins were found to interact with promoters of homeotic genes. We find that PC and TRX first bind at their primary sites around cellular blastoderm. Interestingly, at that stage PC is not present on chromosomal regions flanking PREs as is seen later when implementation of PcG silencing is completed. In contrast, by germ band extension high levels of PC are found on fragments encompassing core PREs as well as on flanking regions.

Fig. 1. Mapping of TRX binding sites within the large 5' regulatory region of the *Ubx* gene on polytene chromosomes. A schematic view of the *Ubx* 5' regulatory region is shown at the top. The co-ordinates are in relation to the numbering of the BX-C sequence (DDBJ/EMBL/GenBank accession No. U31961; Martin *et al.*, 1995). The arrow indicates the start of transcription. The thick line indicates the beginning of the coding sequence. The major PRE (Chan *et al.*, 1994; Strutt *et al.*, 1997) is indicated below the line as a black bar and selected enhancers are indicated as white blocks (Chan *et al.*, 1994). The PRE region is expanded above, corresponding to the clone p3384 used in this study. The fragments of the *Ubx* regulatory region contained in P element constructs analysed are shown below, with the restriction sites defining their ends indicated (S, *StuI*; K, *KpnI*; Sa, *Sau3AI*; E, *EcoRI*; P, *PstI*; N, *NdeI*; St, *StyI*). Whether each construct creates new TRX binding sites at their insertion sites is indicated (+/-) to the right of each construct. The constructs used have been previously described (see Materials and methods). Note that only constructs containing the principle PRE bind TRX in this assay. 19UZ has an internal 2 kb deletion generated by removal of two *Sau3AI* fragments that span the PRE.

Results

Mapping TRX binding sites on *Ubx* regulatory sequences in larval polytene chromosomes

The TRX protein binds to at least 75 discrete sites on the polytene chromosomes of the larval salivary glands, including sites of known target genes; the BX-C, the Antennapedia complex (ANT-C; Kuzin *et al.*, 1994; Chinwalla *et al.*, 1995) and the *engrailed* gene (Breen *et al.*, 1995). PcG proteins co-localize with TRX at more than half of these sites. For comparison with sites identified by the X-CHIP method (see below), we have also used this chromosome binding assay to map TRX binding sites within the *Ubx* gene, which is transcriptionally inactive in the salivary gland and has no prior history of transcriptional activity in the progenitors of this tissue. We previously showed that a P-element construct containing 14 kb of the 5' regulatory region of *Ubx* produces a novel TRX binding site at its chromosomal insertion site, indicating that a TRX binding site lies within this 14 kb (Chinwalla *et al.*, 1995).

Figure 1 summarizes results of further analyses of constructs covering the 36 kb of 5' sequence flanking the *Ubx* coding sequence. The only detectable TRX binding in this entire region occurs within a single small 670 bp fragment corresponding to the core of the major *Ubx* PRE

Fig. 2. TRX binding to transgenes containing *Ubx* regulatory regions. On polytene chromosomes, TRX binds to a single region within the large 5' regulatory region of the *Ubx* gene, corresponding to the principle *Ubx* PRE. (A) Chromosome arm 2L from a wild-type larva. (B) The same arm from a transformant containing the 670 bp *PstI*–*NdeI* fragment of the PRE inserted at 65B. Note the new TRX binding site at 65B. The same result was obtained using HRP-linked secondaries.

identified previously (Chan *et al.*, 1994; Figures 1 and 2). We detect no TRX binding to other fragments that do not contain the core PRE. To allow for the possibility that there might be some weak TRX binding sites, whose occupancy might depend on the presence of other neighbouring or even distant sites, we tested two large fragments flanking the PRE that together cover the entire region analysed minus the PRE: the 22 kb *KpnI*–*StuI* fragment extending from the PRE to within the *Ubx* coding sequence (including the promoter, transcription start site and 1 kb of 5' untranslated sequence) and the adjacent 14 kb *KpnI*–*XbaI* fragment extending further 5' towards the *abd-A* gene and containing an internal deletion of the PRE. The binding of several PcG proteins to these constructs was also monitored (data not shown) and parallels the results seen with TRX: no TRX- or PcG-binding is detected on fragments that do not contain the PRE (Figure 1; Chan *et al.*, 1994). The failure to detect TRX binding to these large fragments in salivary gland nuclei suggests that if additional TRX binding sites do exist outside the PRE, binding to them either does not occur in this tissue, or is dependent on the presence of the PRE on the same fragment for their stable binding, or is not detectable in this assay. As we suggested previously (Chinwalla *et al.*, 1995), given the transcriptional inactivity of *Ubx* in the salivary gland, binding of TRX to the PRE is likely to be constitutive or depend on other factors that bind constitutively in this region.

Co-localization of trithorax and Polycomb proteins in the BX-C analysed by chromatin immunoprecipitation

We have adjusted the X-CHIP methodology to be suitable for the analysis of material derived from embryos. The choice of the DNA targets of the BX-C to be analysed

Developmental profile of PC and TRX binding

Fig. 3. Restriction sites of the BX-C PRE and promoter containing subclones used in this study. The nucleotide position as referred to the BX-C sequence is indicated at the edge of the fragment. PREs and transcription start sites are indicated. For each fragment proximal is to the right. Restriction enzymes: E, *EcoRI*; K, *KpnI*; P, *PstI*; A, *AccI*; C, *ClaI*; B, *BamHI*; H, *HindIII*; E47, *Eco47III*; X, *XhoI*; Xb, *XbaI*; S, *SalI*.

(Figure 3) is based on our previous detection of PC, GAGA factor and TRX distributions in SL2 tissue culture cells (Strutt *et al.*, 1997; V.Orlando and R.Paro, unpublished data).

To determine when TRX binds to the BX-C and whether its binding exhibits temporal changes during early development when PcG silencing is established, *in vivo* fixed chromatin was prepared from embryos of three different stages; 0–2, 2–5 and 5–8 h after egg laying. It should be noted that chromatin collected from 0–2 h embryos will largely represent the late embryonic stages of this window as the very early zygotes will only have a few nuclei. The hybridization signals obtained with the TRX-IP-enriched DNAs are shown in Figure 4. The corresponding signal intensities were quantitated and plotted according to their position on the tested fragments (Figures 5 and 6). At pre-blastoderm stages (0–2 h) TRX is not associated with any BX-C sequences. Between 2 and 5 h (early blastoderm to extended germ band) TRX becomes associated with the *Fab7*-PRE (p4344; Mihaly *et al.*, 1997), the PRE in *Mcp* (p5989; Busturia *et al.*, 1997), and the *Ubx* core PREs in the *bxd* and *bx* regions (p3384 and p2571) (Figure 5). In addition, TRX is also found at the transcription start site of the *Abd-B* γ -promoter (p8106; see also note in legend to Figure 6), the *abd-A*

Fig. 4. Temporal binding profiles of TRX and PC in the BX-C during *Drosophila* embryogenesis. Equimolar amounts of insert-DNA of the BX-C genomic sub-clones shown in Figure 3 were analysed. After digestion with restriction enzymes, individual restriction fragments were resolved on a 1.2% agarose gel and transferred onto nitro-cellulose filters. The loading order of the lanes is indicated in the box. Top panels, TRX-enriched immunoprecipitated chromatin DNA obtained from formaldehyde-fixed staged embryos (0–2, 2–5 and 5–8 h) was hybridized to the filters. Signals were analysed with a phosphorimager. Middle panels, the same filters used for TRX analysis were stripped and re-hybridized with PC-enriched chromatin DNA obtained from staged embryos. Bottom panels, the corresponding control hybridizations using DNA obtained from immunoprecipitations from the same embryo chromatin preparations are shown.

promoter (p1894) and the *Ubx* promoter (p3901). This early binding of TRX to the BX-C sequences coincides with the previously described early temperature-sensitive period of *trxl* (Ingham and Whittle, 1980). Interestingly, within these sites substantial changes in the TRX binding patterns occur between 2 and 5, and 5 and 8 h and coincide with changes in PC binding at these same sites during this period (Figures 5 and 6; see below).

Binding profile of PC during embryo development

In PcG mutants, the breakdown of silencing and consequent ectopic expression of the ANT-C and BX-C homeotic genes is first detectable at the beginning of germ band extension. This indicates that PC is not required for establishing the initial boundaries of homeotic gene expression and suggests that it might not associate with the BX-C and ANT-C genes until just before derepression is detected in PcG mutants. However, PC protein is present during oogenesis and a substantial amount of maternally synthesized PC is present in the early embryo, and may also have a role at earlier stages of embryogenesis (Denell, 1982; Haynie, 1983; Sato and Denell, 1985; Paro and Zink, 1993). In addition, early roles of certain PcG proteins in repression of segmentation genes have been documented (McKeon *et al.*, 1994; Pelegri and Lehmann, 1994).

To determine when PC first associates with the BX-C and whether its pattern of binding changes with time, we have studied PC binding to the BX-C during the same stages of early embryogenesis examined for TRX binding. The same blots used for studying TRX distribution were stripped and reprobed with DNA obtained from PC-IPs (Figure 4 and corresponding plots in Figures 5 and 6). At pre-blastoderm stage (0–2 h) PC is present in significant amounts on sites in the *iab-2* (p3870) and *iab-4* (p7652) regions, on the *abd-A* promoter (p1894) and to a minor extent in the *bxd* region (p3384). This PC must be of maternal origin since zygotic transcription has not yet begun at this stage. Interestingly, a number of early noncoding transcripts, whose functions, if any, remain elusive, originate from the *bxd* and *iab-4* regulatory regions where PC is bound at this stage (Lipshitz *et al.*, 1987; Zavortink and Sakonju, 1989). After 2–5 h, PC is found on most core PREs.

In comparison with previously reported PC binding in SL2 cells, several features of PC binding in embryos at pre-blastoderm stage are worth noting. The first is the general absence or low-level binding to regions adjacent to PREs. This suggests that PC binding to target sites in the BX-C is restricted to small chromosomal elements at this stage. We do not believe that this is due to unequal

Fig. 5. Binding of PC and TRX at PREs during embryogenesis. Signals shown in Figure 4 were analysed by ImageQuantTM and values plotted by Microsoft ExcelTM software. Each panel is a schematic view of PC (upper) and TRX (lower) binding profiles to particular restriction fragments of BX-C PRE-containing clones. Intensity values of each band were normalized on an arbitrary 1 kb fragment and subtracted from the corresponding background value obtained from the control hybridizations. The x-axis represents the restriction fragments (the clone structure is featured below each panel); the y-axis shows the time window of staged embryos collection (0–2, 2–5 and 5–8 h); the z-axis represents TRX and PC enrichments over background, expressed in arbitrary units. Note that the units expressed for TRX and those for PC should not be directly compared. In fact, the products of immunoprecipitations from diverse antigens may not be quantitatively comparable by the X-CHIP technique. Fragments around 250 bp and smaller were not quantifiable in the gel system used.

PCR amplification, since the input DNA in the immunoprecipitation and the quantity of PCR products were the same in all samples.

The second feature is a series of temporal changes in the PC-binding pattern that occur concomitantly with changes in the TRX binding pattern. For example, at 2–5 h the *Fab7*-PRE is poorly enriched for PC, whereas TRX is present prominently present. PC is present on a distal *EcoRI*–*XhoI* 1482 bp fragment (p4344). After 5–8 h the situation has changed: PC is strongly present on the 1482 bp fragment, adjacent fragments (*XhoI*–*XbaI* 884 and 778 bp in p4344) and the core PRE, while remarkably, TRX is removed from the core PRE. We also analysed later stages of embryogenesis and did not observe any further changes in the distribution of PC and TRX

(not shown), suggesting that the pattern of binding of both proteins becomes mature and stable during germ band extension (5–8 h).

The PC binding seen in early embryogenesis (0–2 h) in the *iab-2*, *iab-4*, *bxd* and *abd-A* promoter regions is probably not related to the inactive state of the BX-C genes at this time. It is more likely to represent constitutive binding to certain ‘core’ sites, some of which probably play important roles in organizing the mature pattern that emerges after 5–8 h. However, the strong and dynamic association of TRX and PC with transcription start sites is intriguing and may represent their ultimate sites of action. Promoter association also seems very likely to be dependent on the transcriptional status of each gene in a particular cell and may involve the recruitment of PC

Fig. 6. Binding of PC and TRX to *Ubx*, *abd-A* and *Abd-B* promoters during different embryonic stages. Each panel is a schematic view of PC (upper)- and TRX (lower)-binding to particular restriction fragments of BX-C promoter-containing clones (see Figure 3). Transcription start sites are indicated by an arrow. Note that in the case of the *Abd-B*- γ promoter the TRX peak is in the *AccI*-*AccI* fragment 5' to the published site of the promoter located 153 bp downstream. However, the beginning of the *Abd-B* γ transcript was deduced from cDNA analysis and not from 5' extension analysis (Kuziora and McGinnis, 1988). As such, the real 5' end of the transcript might be located within the adjacent *AccI* fragment encompassing the TRX peak. Images were processed as in Figure 5.

and/or TRX from PREs by specific activators or components of the general transcriptional machinery assembled at the promoter.

Discussion

This study investigates the binding of TRX and PC proteins to the BX-C and their dynamic changes during embryogenesis. By using the X-CHIP method we show that binding of PC and TRX occurs much earlier than previously thought, and that the binding profile changes significantly during the first several hours of embryogenesis involving PREs, flanking regions and core promoters.

However, the interpretation of this set of data has to take into account the intrinsic heterogeneity of the material analysed. Unlike the previously analysed material from SL2 tissue culture cells, embryonic material contains a mixture of cells expressing various patterns of BX-C genes. In fact, at cellular blastoderm the *Ubx* and *abd-A* genes show rather broad transcription domains that only later become spatially restricted (Beachy, 1990; Karch *et al.*, 1990). The *Abd-B* gene shows a fairly restricted pattern already at cellular blastoderm (Kuziora and McGinnis, 1988). At the same stages another large untranslated transcript located upstream the *Ubx* promoter (*bxd*; Lipshitz *et al.*, 1987) and including the *bxd*-PRE is produced in PS 6–13 (Irish *et al.*, 1989). Therefore, concerning BX-C gene transcriptional activity, a certain degree of heterogeneity is also intrinsic in some cases at the single-cell level. Nevertheless, we appear to observe defined binding profiles at the different PREs and promoters, despite the different ratios of cells in the mixture expressing specific subsets of BX-C genes. As such, the

X-CHIP results certainly forbid an extrapolation of the found PC–TRX interactions to the single-cell level. However, the observed developmental profiles still appear to allow an assessment of how the two proteins bind to the majority of their target sites.

Binding of PC and TRX to the BX-C regulatory regions becomes evident at cellular blastoderm. PC is already associated with all the BX-C PREs at this stage, but not significantly with flanking regions and promoters as is evident at later stages. TRX is also associated with all the BX-C PREs at this stage, before reductions in BX-C transcription become evident in *trx* mutants (Breen and Harte, 1991; Sedkov *et al.*, 1994). This suggests that PREs/TREs are pre-loaded with both *trxG* and *PcG* components poised for maintenance of both active and repressed transcriptional states before either are apparently required. In addition to PREs/TREs, both TRX and PC show strong association with transcription start sites in the BX-C. In gastrulating stages TRX is strongly associated with the *Ubx*, *abd-A* and *Abd-B*- γ core promoters (Figure 6), whereas PC is virtually absent at this stage. At later stages, as the expression domains of the homeotic genes become fixed, TRX is much reduced at promoters and at most PREs/TREs, though this reduction might also reflect the increasing complexity of the material used. These dynamic changes in the binding of PC and TRX to PREs/TREs and promoters of target genes may point to important transitions necessary for the establishment of the cellular memory mechanism supported by the *PcG* and *trxG*.

Extension of PC-binding profile over developmental time

Our results show an increase in binding sites of PC during embryonic development. While in early embryos,

fragments containing core PREs are co-precipitated with PC, in material from 5–8 h embryos PC is also bound in adjacent fragments, extending in some cases over several thousand base pairs. This late pattern, which according to the genetic data should represent the fully functional state, resembles the results we obtained with SL2 tissue culture cells (Orlando and Paro, 1993; Strutt *et al.*, 1997). However, such an extended binding pattern differs substantially from what we observe with a sequence-specific protein like the GAGA factor, where only single fragments encompassing the consensus binding sites are immunoprecipitated (Strutt *et al.*, 1997). Also, TRX depicts a much more restricted binding profile in late stages. Unfortunately, the underlying structures connected to the individual binding profiles remain elusive. PcG proteins were proposed to keep their target genes inactive by using heterochromatin-like structures (Paro, 1990). However, our current knowledge of the structure of heterochromatin is limited to yeast telomeres, where a spreading by an interaction of the regulating SIR protein complexes with the nucleosomal backbone has been demonstrated, utilizing different means including a X-CHIP analysis (Hecht *et al.*, 1995; Strahl-Bolsinger *et al.*, 1997; Boscheron *et al.*, 1996). In the case of *Drosophila* it remains undetermined whether the extended binding profile seen for PC uncovers a similar path. Alternative explanations that suggest an interaction of PcG proteins with weak PREs scattered along the BX-C (Pirrotta, 1997) are also supported by our data as there is insufficient resolution to distinguish between the two possibilities. On similar lines, the binding of PC and TRX to promoter regions found here might indicate a direct interaction of PREs/TREs with promoters (Bienz and Müller, 1995). However, whether this is achieved by looping, as has been suggested for enhancer–promoter interactions, is an issue we cannot resolve on the basis of our X-CHIP results presented here. Clearly, additional methodology and analyses are needed to increase the resolution and uncover the molecular interactions involved in generating the higher order chromatin structures regulated by the PcG and *trxG*.

PRE and TRE: common elements involved in repression and anti-repression

It appears that the early transcriptional state of a promoter at the time PcG silencing is implemented is decisive in conferring subsequent repressive or activating function to PREs/TREs in a particular cell (Poux *et al.*, 1996). In this light, the communication of PREs/TREs with specific subsets of enhancers is likely to be important for their function (Sigrist *et al.*, 1997; Zhou *et al.*, 1997). This suggests a direct interference with some aspects of enhancer-mediated activation in chromatin. In mammalian cells enhancers have been shown to directly counteract repressive chromatin (Walters *et al.*, 1996). In yeast, UAS-regulated transcription requires SWI/SNF gene products. Interestingly, promoters with a deleted UAS are capable of directing stable high levels of transcription when combined with mutations in repressing chromatin components (Prelich and Winston, 1993). Therefore, at least UAS-dependent activated transcription may be by-passed if chromatin-mediated repression is defective. This suggests an intriguing interdependence between a stable dominant

effect of repressing chromatin and specific activation pathways that involve remodelling of repressive chromatin.

By definition, PREs are responsible for the maintenance of the inactive state of homeotic genes in the absence of the early *trans*-acting repressors. PcG defects are suppressed by *trxG* mutations by preventing the ectopic overexpression of homeotic genes. One possibility is that PcG loss of function could be suppressed by loss of enhancer function involving general transcription activators. Another possibility is that the dominant-negative action of the PRE on the enhancer could be counteracted specifically by *trxG* proteins at TREs, when these have to act to maintain the active state. This would imply the action of more specialized activators, like TRX, at TREs. Genetic analyses of deletions of the BX-C Fab-7 boundary/PRE element shows that *trxG* are required for preventing the PRE-PcG repression. When boundary and PRE elements are both deleted *trxG* alleles show no effect (Mihaly *et al.*, 1997). Therefore, the TRE-function in the PRE could be specifically responsible for counteracting PcG repression.

An anti-repression model would explain why the dose of *trxG* and PcG proteins at a certain time and place in the embryo plays an important role in stabilizing early determined states in chromatin. It was previously shown that increasing amounts of a transcriptional activator can displace proportional amounts of PC at an ectopic PC binding site (PRE) on polytene chromosomes created by insertion of an inducible reporter-flanked PRE (Zink and Paro, 1995). TRX may exert or mediate an analogous competitive effect on PcG silencing. Consistent with this possibility, the dominant homeotic derepression phenotypes seen in Pc mutants are enhanced by extra copies of *trx*⁺ and suppressed in flies carrying only one copy of the *trx* gene (Capdevila and Garcia-Bellido, 1981; Capdevila *et al.*, 1986; Kennison and Russell, 1987). Elimination of the maternally provided component of PC has been shown to increase the Pc phenotype (Denell, 1982). Finally, co-transfection experiments with the *Ubx* PRE/TRE carrying reporter constructs have shown dose-dependent competitive interactions between PC and TRX in the activation and repression of the reporter (Chang *et al.*, 1995). This suggests that the relative amounts of PC and TRX proteins at early stages of embryogenesis may be crucial. While TRX is required continuously throughout development (Ingham, 1985), the early temperature-sensitive period (~0–4 h) determined using the *trx*^l allele (Ingham and Whittle, 1981) very probably reflects a critical early requirement for TRX, which cannot be compensated by providing it later (Ingham, 1981). We start to see a co-localization of PC and TRX during this time, suggesting that its role involves preventing implementation of the beginning of PcG silencing.

Interaction of PC and TRX with core promoters

The interaction of TRX and PC with both PREs/TREs and promoters seems to be an important issue of PcG function. In transgenic flies, it has been shown that the major *Ubx* PRE (bxd) works more efficiently when assayed together with the *Ubx* promoter (Chan *et al.*, 1994; Müller, 1995), which is also found in co-transfection experiments using tissue culture cells (Chang *et al.*, 1995). Also, the transcriptional activity of a gene at early stages

of embryogenesis interferes with establishment of PcG silencing (Poux *et al.*, 1996). Our finding that in early embryogenesis PC and TRX are dynamically associated with core promoters suggests that these proteins somehow interfere with enhancer–promoter interactions.

The mechanism of enhancer action is not fully understood. In particular, typical enhancer binding proteins like the segmentation gene products have been shown to act by direct interference with distinct elements of the basal transcription apparatus (Sauer *et al.*, 1995). In addition, tissue-specific TAFs have been described to support the action of developmentally regulated genes (Sauer *et al.*, 1996). It could be that in the initial transition phase PC stabilizes a repressed conformation of enhancer–promoter by contacting some repressive components of the basal transcriptional machinery. In a subsequent step the inactive expression state might become stabilized by extending PcG complexes over the respective *cis*-regulatory elements. Such structures might be accompanied by specific chromatin modifications inducing changes in the acetylation patterns of histones and other associated chromosomal proteins (reviewed by Pazin and Kadonaga, 1997). In such a scenario maintenance of the repressed state would imply stable association of histone de-acetylase at PREs. Histone acetylases and de-acetylases are relatively abundant. Thus the memory system, in the absence of specific *trans*-acting effectors, would rely on general factors specifically targeted by the PcG/trxG proteins to enhancers and promoters.

Materials and methods

Mapping TRX binding sites on polytene chromosomes

TRX binding sites within the large 5' *Ubx* regulatory region were mapped on polytene chromosomes from the larval salivary gland by immunostaining chromosomes from P element transformants containing constructs with various portions of the 5' *Ubx* regulatory region and determining whether the site of insertion creates a new TRX binding site. The constructs analysed have been previously described and characterized, and include: 35UZ and 22UZ (Irvine *et al.*, 1991), bxd 14.5 (Simon *et al.*, 1990) and D19UZ. The other constructs (2.2 kb *KpnI*–*EcoRI*, 670 bp *PstI*–*NdeI* and 0.5 kb *StyI*–*PstI*) were very kindly provided by V.Pirrotta and are described in Chan *et al.* (1994). Chromosomes were stained with anti-TRX antibodies (Chinwalla *et al.*, 1994), which were visualized with FITC-labelled secondary antibodies and separately with HRP-linked secondaries as previously described (Chinwalla *et al.*, 1994). Chromosomes were counterstained with propidium iodide when fluorescent secondary antibodies were used to allow visualization of chromosome morphology. Cytology was confirmed with HRP stained samples. Images were obtained with a confocal microscope (Zeiss/Bio-Rad) and optical sectioning was performed to ensure that TRX binding signals were distributed throughout the new TRX binding sites associated with the constructs to avoid artefacts.

In vivo formaldehyde fixation and chromatin IP (X-CHIP) from embryos

Approximately 1–3 g of staged embryos were collected and dechorionated utilizing standard methods (Ashburner, 1989). Embryos were transferred in 50 ml phosphate-buffered saline (PBS) (Falcon), supplemented with 0.01% Triton X-100 and allowed to settle to the bottom. Washing buffer was removed by aspiration and the wash repeated once. Embryos were fixed in 10 ml fixing solution [50 mM HEPES pH 7.6, 100 mM NaCl, 0.1 mM Na-EDTA, 0.5 mM Na-EGTA, 2% formaldehyde (from a 37% formaldehyde/10% methanol stock)] and 30 ml heptane by vigorous shaking for 15 min at room temperature. Embryos were centrifuged at 4000 g for 1 min. The heptane and the fixing solution were removed by aspiration and the fixed embryos washed twice in PBS, 0.01% Triton X-100 and 0.125 M glycine.

Fixed embryos were transferred in 15 ml tubes (Falcon) and washed

sequentially for 10 min each in 15 ml of 0.25% Triton X-100, 10 mM Na-EDTA, 0.5 mM Na-EGTA, 50 mM HEPES pH 8, and then 0.01% Triton X-100, 0.2M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES pH 8. Sedimented fixed embryos were resuspended in 6 ml of ice-cold 10 mM HEPES pH 8, 1 mM Na-EDTA, 0.5 mM Na-EGTA. Sonication was performed using a sonifier Branson 250 equipped with a 0.5 cm tip in the presence of 0.5 mm glass beads (~1/4 v/v) with three bursts of 30 s each at maximum power. Subsequent chromatin purification was performed as described (Orlando *et al.*, 1997). Equal amounts of chromatin (~50 mg DNA) were used for each immunoprecipitation. The anti-TRX antibodies used are described in Chinwalla *et al.* (1995). Subsequent steps, and in particular the improved PCR-amplification of immunoprecipitated chromatin fraction DNA were done as previously described (Orlando *et al.*, 1997; Strutt *et al.*, 1997). Quantitative analyses of Southern experiments were performed using a phosphorimager (Orlando *et al.*, 1997; Strutt *et al.*, 1997).

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References

- Ashburner, M. (1989) *Drosophila: A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.
- Beachy, P.A. (1990) A molecular view of the Ultrabithorax homeotic gene of *Drosophila*. *Trends Genet.*, **6**, 46–51.
- Bienz, M. and Müller, J. (1995) Transcriptional silencing of homeotic genes in *Drosophila*. *BioEssays*, **17**, 775–784.
- Boscheron, C., Maillet, L., Marcand, S., Tsai-Pflugfelder, M., Gasser, S. and Gilson, E. (1996) Co-operation at a distance between silencers and proto-silencers at the yeast HML locus. *EMBO J.*, **15**, 2184–2195.
- Breen, T.R. and Harte, P.J. (1991) *Trithorax* regulates multiple homeotic genes in the bithorax and Antennapedia complexes and exerts different tissue-specific effects on each. *Development*, **117**, 119–134.
- Breen, T.R., Chinwalla, V. and Harte, P.J. (1995) *Trithorax* is required to maintain *engrailed* expression in a subset of engrailed-expressing genes. *Mech. Dev.*, **52**, 1–10.
- Busturia, A., Wightman, C.D. and Sakonju, S. (1997) A silencer is required for maintenance of transcriptional repression throughout *Drosophila* development. *Development*, **124**, 4343–4350.
- Capdevila, M.P. and Garcia-Bellido, A. (1981) Genes involved in the activation of the bithorax complex of *Drosophila*. *Wilhelm Roux's Arch. Dev. Biol.*, **190**, 339–350.
- Capdevila, M.P., Botas, J. and Garcia-Bellido, A. (1986) Genetic interactions between the *Polycomb* locus and the Antennapedia and bithorax complexes of *Drosophila*. *Wilhelm Roux's Arch. Dev. Biol.*, **195**, 417–432.
- Carrington, E.A. and Jones, R.S. (1996) The *Drosophila* enhancer of *zeste* gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. *Development*, **122**, 4073–4083.
- Castelli-Gair, J.E. and Garcia-Bellido, A. (1990) Interactions of *Polycomb* and *trithorax* with *cis* regulatory regions of *Ultrabithorax* during the development of *Drosophila melanogaster*. *EMBO J.*, **9**, 4267–4275.
- Chan, C.S., Rastelli, L. and Pirrotta, V. (1994) A *Polycomb* response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *EMBO J.*, **13**, 2553–2564.
- Chang, Y., King, B.O., O'Connor, M., Mazo, A. and Huang, D. (1995) Functional reconstruction of trans regulation of the *Ultrabithorax* promoter by the products of two antagonistic genes. *Mol. Cell. Biol.*, **15**, 6601–6612.
- Chinwalla, V., Jane, P.E. and Harte, P.J. (1995) The *Drosophila* trithorax protein binds to specific chromosomal sites and is co-localized with *Polycomb* at many sites. *EMBO J.*, **14**, 2056–2065.
- De Camillis, M., Cheng, N.S., Pierre, D. and Brock, H. (1992) The *Polyhomeotic* gene of *Drosophila* encodes a chromatin protein that shares polytene chromosome binding sites with *Polycomb*. *Genes Dev.*, **6**, 223–232.
- Denell, D.E. (1982) Homoeosis in *Drosophila*: evidence for a maternal effect of the *Polycomb* locus. *Dev. Genet.*, **3**, 103–113.

- Farkas,G., Gausz,J., Galloni,M., Reuter,G., Gyurkovics,H. and Karch,F. (1994) The *Trithorax*-like gene encodes the *Drosophila* GAGA-factor. *Nature*, **371**, 806–808.
- Franke,A., DeCamillis,M., Zink,D., Cheng,N., Brock,H. and Paro,R. (1992) Polycomb and Polyhomeotic are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*. *EMBO J.*, **11**, 2941–2950.
- Gindhart,J.G. and Kaufman,T.C. (1995) Identification of Polycomb and trithorax group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. *Genetics*, **139**, 797–814.
- Gyurkovics,H., Gausz,J., Kummer,J. and Karch,F. (1990) A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. *EMBO J.*, **9**, 2579–2585.
- Hagstrom,K., Muller,M. and Schedl,P. (1997) Fab-7 functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila* bithorax complex. *Genes Dev.*, **10**, 3202–3215.
- Haynie,J. (1983) The maternal and zygotic roles of the gene *Polycomb* in embryonic determination in *Drosophila melanogaster*. *Dev. Biol.*, **100**, 399–411.
- Hecht,A., Strahl-Bolsinger,S. and Gruenstein,M. (1995) Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature*, **383**, 92–96.
- Ingham,P.W. (1981) trithorax: A new homeotic mutation of *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.*, **190**, 365–369.
- Ingham,P.W. (1985) A clonal analysis of the requirement for the trithorax gene in the diversification of segments in *Drosophila*. *J. Embryol. Exp. Morphol.*, **89**, 349–365.
- Ingham,P.W. and Whittle,R. (1981) trithorax: a new homeotic mutation of *Drosophila melanogaster* causing transformations of abdominal and thoracic segments. I. Putative role during embryogenesis. *Mol. Gen. Genet.*, **179**, 607–614.
- Irish,V.F., Martinez-Arias,A. and Akam,M. (1989) Spatial regulation of the *Antennapedia* and *Ultrabithorax* homeotic genes during *Drosophila* early development. *EMBO J.*, **8**, 1527–1537.
- Irvine,K.D., Helfand,S.L. and Hogness,D.S. (1991) The large upstream control region of the *Drosophila* homeotic gene *Ultrabithorax*. *Development*, **111**, 407–424.
- Jones,C.A., Ng,J., Peterson,A.J., Morgan,K., Simon,J. and Jones,R. (1998) The *Drosophila* esc and E1 (z) proteins are direct partners in Polycomb group-mediated repression. *Mol. Cell. Biol.*, **18**, 2825–2834.
- Karch,F., Bender,W. and Weiffenbach,B. (1990) *abdA* expression in *Drosophila* embryos. *Genes Dev.*, **4**, 1573–1587.
- Kennison,J.A. and Russell,M.A. (1987) Dosage-dependent modifiers of homeotic mutations in *Drosophila melanogaster*. *Genetics*, **116**, 75–86.
- Kennison,J.A. and Tamkun,J.M. (1988) Dosage dependent modifiers of *Polycomb* and *Antennapedia* mutations in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **85**, 8136–8140.
- Kingston,R.E., Bunker,C.A. and Imbalzano,A. (1996) Repression and activation by multiprotein complexes that alter chromatin structure. *Genes Dev.*, **10**, 905–920.
- Kuzin,B., Tillib,S., Sedkov,Y., Mizrokhi,L. and Mazo,A. (1994) The *Drosophila* trithorax gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene *fork head*. *Genes Dev.*, **8**, 2478–2490.
- Kuziora,M.A. and McGinnis,W. (1988) Different transcripts of the *Drosophila* Abd-B gene correlate with distinct genetic sub-functions. *EMBO J.*, **7**, 3233–3244.
- Kyba,M. and Brock,H.W. (1998) The *Drosophila* Polycomb group protein Psc contacts Ph and Pc through specific conserved domains. *Mol. Cell. Biol.*, **18**, 2712–2720.
- Lipshitz,H.D., Peattie,D.A. and Hogness,D.S. (1987) Novel transcripts from the *Ultrabithorax* domain of the bithorax complex. *Genes Dev.*, **1**, 307–322.
- Lonie,A., D'Andrea,R., Paro,R. and Saint,R. (1994) Molecular characterization of the Polycomb like gene of *Drosophila melanogaster*, a *trans*-acting negative regulator of homeotic gene expression. *Development*, **120**, 2629–2636.
- Look,T.A. (1997) Oncogenic transcription factors in the human acute leukemias. *Science*, **278**, 1059–1064.
- Martin,C.H., Mayeda,C.A., Davis,C.A., Ericsson,C.L., Knafels,J.D., Mathog,D.R., Celniker,S.E., Lewis,E.B. and Palazzolo,M.J. (1995) Complete sequence of the bithorax complex of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **92**, 8398–8402.
- McKeon,J., Slade,E., Sinclair,D.A., Cheng,N., Couling,M. and Brock,H. (1994) Mutations in some group genes of *Drosophila* interfere with regulation of segmentation genes. *Mol. Gen. Genet.*, **244**, 474–483.
- Mihaly,J., Hogga,I., Gausz,J., Gyurkovics,H. and Karch,F. (1997) *In situ* dissection of the *Fab7* region of the bithorax complex into a chromatin domain boundary and a Polycomb-response element. *Development*, **124**, 1809–1820.
- Müller,J. (1995) Transcriptional silencing by the Polycomb protein in *Drosophila* embryos. *EMBO J.*, **14**, 1209–1220.
- Muller,J. and Bienz,M. (1991) Long range repression conferring boundaries at *Ultrabithorax* expression in the *Drosophila* embryo. *EMBO J.*, **10**, 3147–3155.
- Orlando,V. and Paro,R. (1993) Mapping Polycomb-repressed odmins in the bithorax complex using *in vivo* formaldehyde cross-linked chromatin. *Cell*, **75**, 1187–1198.
- Orlando,V., Strutt,H. and Paro,R. (1997) Analysis of chromatin structure by *in vivo* formaldehyde cross-linking. *Methods*, **11**, 205–214.
- Paro,R. (1990) Imprinting a determined state into the chromatin of *Drosophila*. *Trends Genet.*, **6**, 416–421.
- Paro,R. and Zink,B. (1993) The Polycomb gene is differentially regulated during oogenesis and embryogenesis of *Drosophila melanogaster*. *Mech. Dev.*, **40**, 37–46.
- Paro,R. and Harte,P.J. (1996) The role of *Polycomb* group and trithorax group chromatin complexes in the maintenance of determined cell states. In Russo,E. *et al.* (eds), *Epigenetic Mechanisms of Gene Regulation*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 507–528.
- Pazin,M.J. and Kadonaga,J.T. (1997) What's up and down with histone deacetylation and transcription? *Cell*, **89**, 325–328.
- Pelegri,F. and Lehmann,R. (1994) A role for Polycomb group genes in the regulation of *gap* gene expression in *Drosophila*. *Genetics*, **136**, 1341–1425.
- Pirrotta,V. (1997) PcG complexes and chromatin silencing. *Curr. Opin. Genet. Dev.*, **7**, 249–258.
- Poux,S., Kostic,C. and Pirrotta,V. (1996) Hunchback-independent silencing of late *Ubx* enhancers by a Polycomb group response element. *EMBO J.*, **15**, 4713–4722.
- Prelich,G. and Winston,F. (1993) Mutations that suppress the deletion of an upstream activating sequence in yeast: involvement of a protein kinase and histone H3 in repressing transcription *in vivo*. *Genetics*, **135**, 665–676.
- Rastelli,L., Chan,C.S. and Pirrotta,V. (1993) Related chromosome binding sites for zeste, suppressor of zeste and Polycomb group proteins in *Drosophila* and their dependence on Enhancer of zeste function. *EMBO J.*, **12**, 1513–1522.
- Sauer,F., Fondell,J.D., Ohkuma,Y. and Roeder,R. (1995) Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta. *Nature*, **375**, 162–164.
- Sauer,F., Wassarman,D.A., Rubin,G.M. and Tjian,R. (1996) TAFIIIs mediate activation of transcription in the *Drosophila* embryos. *Cell*, **87**, 1271–1284.
- Sato,T. and Denell,R.E. (1985) Homeosis in *Drosophila*: anterior and posterior transformations in Polycomb lethal embryos. *Dev. Biol.*, **110**, 53–64.
- Sedkov,Y., Tillib,S., Mizrokhi,L. and Mazo,A. (1994) The bithorax complex is regulated by *trithorax* earlier during *Drosophila* embryogenesis than is the Antennapedia complex, correlating with a bithorax-like expression pattern of distinct early trithorax transcripts. *Development*, **120**, 1907–1917.
- Sigrist,C.J.A. and Pirrotta,V. (1997) Chromatin insulator elements block the silencing of a target gene by the *Drosophila* Polycomb response element (PRE) but allows *trans* interactions between PREs on different chromosomes. *Genetics*, **147**, 209–221.
- Simon,J., Peifer,M., Bender,W. and O'Connor,M. (1990) Regulatory elements of the bithorax-complex that control expression along the anterior-posterior axis. *EMBO J.*, **9**, 3945–3956.
- Simon,J., Chaing,A., Bender,W., Shimell,M. and O'Connor,M. (1993) Elements of the *Drosophila* bithorax complex that control repression by Polycomb-group products. *Dev. Biol.*, **158**, 131–144.
- Strahl-Bolsinger,S., Hecht,A., Luo,K. and Gruenstein,M. (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.*, **11**, 83–93.
- Strutt,H., Cavalli,G. and Paro,R. (1997) Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic genes expression. *EMBO J.*, **16**, 3621–3632.
- Tamkun,J.W., Deuring,R., Scott,M.P., Kissinger,M., Pattatucci,A.M., Kaufman,T.C. and Kennison,J.A. (1992) *brahma*: a regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*, **68**, 561–572.

- van der Knaap,J.A., Borst,J.W., van der Vliet,P.C., Gentz,R. and Timmers,H.T. (1997) Cloning of the cDNA for the TATA-binding protein-associated factor II170 subunit of transcription factor B-TFIID reveals homology to global transcription regulators in yeast and *Drosophila*. *Proc. Natl Acad. Sci. USA*, **94**, 11827–11832.
- Walters,M.C., Magis,W., Fiering,S., Eidemiller,J., Scalzo,D., Groudine,M. and Martin,D.I.K. (1996) Transcriptional enhancers act *in cis* to suppress position-effect variegation. *Genes Dev.*, **10**, 185–195.
- Zavortink,M. and Sakonju,S. (1989) The morphogenetic and regulatory functions of the *Drosophila Abdominal-B* gene are encoded in overlapping RNAs transcribed from separate promoters. *Genes Dev.*, **3**, 1969–1981.
- Zhou,J., Barolo,S., Szymanski,P. and Levine,M. (1996) The Fab-7 element of the bithorax complex attenuates enhancer–promoter interactions in the *Drosophila* embryo. *Genes Dev.*, **10**, 3195–3201.
- Zink,D. and Paro,R. (1995) *Drosophila* Polycomb-group regulated chromatin inhibits the accessibility of a *trans*-activator to its target DNA. *EMBO J.*, **14**, 5660–5671.
- Zink,B., Egstrom,Y., Gehring,W.J. and Paro,R. (1991) Direct interaction of the Polycomb protein with Antennapedia regulatory sequences in polytene chromosomes of *Drosophila melanogaster*. *EMBO J.*, **10**, 153–162.

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