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Transcription through the *iab-7 cis*-regulatory domain of the bithorax complex interferes with maintenance of *Polycomb*-mediated silencing

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SUMMARY

The *Fab-7* chromatin domain boundary insures functional autonomy of the *iab-6* and *iab-7 cis*-regulatory domains in the bithorax complex (BX-C). We have previously shown that chromatin insulators such as *gypsy* or scs^{min} are potent insulators that cannot substitute for *Fab-7* function within the BX-C. During the early stages of these swapping experiments, we initially used a fragment of scs that was slightly larger than a minimal scs element (scs^{min}). We report that this scs fragment, unlike scs^{min}, interferes in an orientation-dependent manner with the output of a regulatory region covering 80 kb of DNA (from *iab-4* to *iab-8*). At the core of this orientation-dependent phenotype is a promoter located immediately adjacent to the scs insulator. In one orientation, the promoter traps the activity of the *iab-3* through *iab-5 cis*-regulatory domains, diverting them from the *abd-A* gene. In the opposite orientation, the promoter is transcribing the *iab-7 cis*-regulatory domain, resulting in ectopic activation of the latter. Our data suggest that transcription through a *Polycomb*-Response Element (PRE) interferes with the maintenance of a *Polycomb* repression complex.

Key words: Boundary, Insulator, Scs, *Fab-7*, Bithorax complex, *Polycomb*, Transcription, Silencing, *Drosophila*

INTRODUCTION

The bithorax complex (BX-C) contains three homeotic genes, *Ubx*, *abd-A* and *Abd-B*, that are responsible for determining the identity of parasegments 5 to 14 (PS5-14). These parasegments will form the posterior thorax (T2 and T3) and all eight abdominal segments of the adult fly (A1 to A8) (Lewis, 1978; Sanchez-Herrero et al., 1985). The PS-specific expression pattern of *Ubx*, *abd-A* and *Abd-B* is controlled by a large *cis*-regulatory region that covers 300 kb of DNA and that is subdivided into nine functionally autonomous *cis*-regulatory domains (*abx/bx*, *bxd/pbx*, *iab-2* to *iab-8*) (for reviews, see Duncan, 1987; Peifer et al., 1987). For example, the *iab-5 cis*-regulatory domain regulates *Abd-B* expression in a pattern that confers PS10/A5 identity to the cells of that PS. Similarly, the *iab-6*, *iab-7* and *iab-8 cis*-regulatory domains activate *Abd-B* expression in patterns appropriate for PS11/A6, PS12/A7 and PS13/A8 identity, respectively (Celniker et al., 1990; Sanchez-Herrero, 1991). When one of the *cis*-regulatory domains is inactivated, the parasegment specified by the affected regulatory domain is transformed into a copy of the PS immediately anterior. Thus, in a deletion of *iab-7*, PS12/A7 is transformed into PS11/A6. In this case, *Abd-B* expression in both PS11 and PS12 is driven by the *iab-6 cis*-regulatory domain alone (Galloni et al., 1993).

The regulation of the BX-C homeotic genes during embryogenesis is subdivided into two phases: initiation and maintenance. In the initiation phase, the products of the gap and pair-rule segmentation genes are responsible for initiating the parasegment specific expression of the BX-C homeotic genes. These proteins interact with target sequences (called initiation elements) in the nine *cis*-regulatory domains (Simon et al., 1990; Qian et al., 1991; Muller and Bienz, 1992; Shimell et al., 1994). However, the products of the segmentation genes are present only transiently in the early embryo. Maintenance of the initial pattern requires the *trithorax-Group* (*trx-G*) and *Polycomb-Group* (*Pc-G*) genes. The *trx-G* genes function to keep the homeotic genes on, while the *Pc-G* genes function to maintain the inactive state of the homeotic genes (reviewed by Paro, 1990; Simon and Tamkun, 2002). Experiments with reporter constructs have identified elements, called Polycomb Response elements or PREs, in several of the BX-C *cis*-regulatory domains, that appear to be targets for *Pc-G* action. When these PREs are combined with a parasegment-specific initiation element, they maintain the parasegmentally restricted expression pattern conferred on the reporter by the initiation element (Muller and Bienz, 1991; Busturia and Bienz, 1993; Simon et al., 1993; Chan et al., 1994; Chiang et al., 1995; Poux et al., 1996). In addition to this maintenance activity, PREs are also able to repress the activity of the mini-white reporter gene used to establish transgenic lines. Usually, transgenic lines carrying the mini-white gene harbor darker eye color when the inserts are homozygous. When they are included in a *mini-white* transgene, the PREs repress or even eliminate *mini-white* expression when the animals are homozygous (a phenomenon referred to as the pairing-sensitive repression assay) (Kassis et al., 1991; Chan et al., 1994; Gindhart and Kaufman, 1995;

Hagstrom et al., 1997; Muller et al., 1999) (for a review, see Pirrotta and Rastelli, 1994).

Genetic and molecular analysis has identified chromatin domain boundaries that demarcate the *cis*-regulatory domains, insuring the functional autonomy of each regulatory domain (Gyurkovics et al., 1990; Galloni et al., 1993; Mihaly et al., 1997; Zhou et al., 1999; Barges et al., 2000) (for a review, see Mihaly et al., 1998). For example, in PS11, the *Fab-7* boundary protects the active *iab-6* cis-regulatory domain from the inactive *iab-7* domain, preventing inappropriate regulatory interactions between the two domains. Immediately adjacent to the *Fab-7* boundary, lies the *iab-7*PRE, which is involved in maintaining inactivity of *iab-7* in parasegments anterior to PS12 (Hagstrom et al., 1997; Mihaly et al., 1997).

Two classes of mutations affect the *Fab-7* region. Class II mutations, such as *Fab-7*^{2}, delete the boundary alone and leave the nearby *iab-7* PRE intact. They lead to a mixed gain- and loss-of-function phenotype in PS11/A6; there are groups of cells acquiring PS10/A5 identity, because in these cells both *iab-6* and *iab-7* are inactive. The remaining cells of PS11 adopt a PS12/A7 fate, because both *iab-6* and *iab-7* are active in these cells. This mixed gain- and loss-of-function phenotype arises because there is a competition in the fused cis-regulatory domain between positive elements in *iab-6* that ectopically activate *iab-7* and negative elements in *iab-7* that ectopically silence *iab-6*. Class I mutations, such as the original *Fab-7*^{1} allele, are larger deletions that remove not only the boundary but also the nearby *iab-7* PRE. In this class of mutation, the balance between gain- and loss-of-function phenotype is shifted towards gain-of-function, and A6 is completely transformed into A7 (see Fig. 2B) (Mihaly et al., 1997).

Most chromatin domain boundaries in higher eukaryotes have been identified by their ability to block enhancer-promoter interactions when intercalated between them (enhancer-blocking assay) (for reviews, see Gerasimova and Corces, 1996; Geyer, 1997; Sun and Elgin, 1999; Bell et al., 2001). In our terminology, we call elements defined in the enhancer-blocking assay chromatin insulator. In *Drosophila* two insulators, scs/scs' (Kellum and Schedl, 1991; Kellum and Schedl, 1992) and *gypsy* (Geyer and Corces, 1992; Roseman et al., 1993) have been extensively studied in the enhancer-blocking assay. We have previously described that *gypsy* or a minimal scs fragment (scs^{min}) cannot substitute for *Fab-7*; their enhancer-blocking activity prevents the *iab-5* and *iab-6 cis*-regulatory domains from interacting with the *Abd-B* target promoter (Hogga et al., 2001). We describe the results of experiments in which we replace *Fab-7* by a slightly larger scs fragment that was used in enhancer-blocker experiments by different laboratories (Kellum and Schedl, 1991; Kellum and Schedl, 1992; Vazquez and Schedl, 1994; Dunaway et al., 1997; Krebs and Dunaway, 1998; Parnell and Geyer, 2000). Surprisingly, this scs fragment behaves differently than scs^{min} and leads to opposite gain- and loss-of-function phenotype depending on its orientation within the context of the *Fab-7* region. The orientation-dependent effect is due to the presence of a promoter immediately adjacent to the scs insulator. Our results suggest that transcription through the *iab-7* PRE interferes with the maintenance of a *Polycomb* repression complex on the *iab-7* domain.

MATERIALS AND METHODS

Gene conversion

Fab-7 was replaced by scs$_{prom}$ (or $_{prom}$scs) using the gene conversion strategy described by Hogga et al. (Hogga et al., 2001).

DNA techniques, fly work, antibody staining and in situ hybridization

DNA techniques, fly work, antibody staining and in situ hybridization have been described previously (Mihaly et al., 1997; Hogga et al., 2001; Zhou et al., 1999). The antibody against ABD-B was kindly provided by Sue Celniker (Celniker, 1990).

Abdominal cuticles

Adult abdominal cuticles were mounted as described elsewhere (Mihaly et al., 1997), examined and photographed on an Axioplan microscope with a 5× lens. Only half cuticles are shown in Fig. 2. The dorsal surface of each abdominal segment has a rectangular plate of hard cuticle called the tergite (only half of the tergites are visible on the left of each panel, as well as the genitalia at the bottom). The ventral surface of abdominal segments is composed of soft cuticle called the pleura. On the ventral midline of the second (A2) and more posterior segments, there are small plates of harder cuticle called sternites. In males only six abdominal segments are visible. The tergites on A5 and A6 are pigmented and can be therefore distinguished from more anterior tergites. On the ventral side, the sixth sternites can be distinguished from the more anterior sternites by its different shape and by the absence of bristles. Homeotic transformations associated with *Fab-7* are best visible in males where most (*Fab-7*^{2}) or all of A6 (*Fab-7*^{1}) is missing (see Fig. 2B). As A7 and A8 do not contribute to any visible cuticle after metamorphosis in males, homeotic transformations associated with *Fab-8* are detected in females where A7 develops as a smaller segments than the anterior segments (see Fig. 2C).

RESULTS AND DISCUSSION

Orientation dependent effects of scs when integrated in the BX-C

The slightly larger scs fragment used to replace *Fab-7* in the present studies is depicted in Fig. 1. The extra-DNA (relative to scs^{min}) consists in the 282bp *Mlu*I-*Pst*I fragment at one edge of scs^{min}. In the $_{prom}$scs convertant line, the 282 bp extra DNA fragment faces *iab-6*. In scs$_{prom}$, this extra DNA is juxtaposed to *iab-7*.

Fig. 2A shows the phenotype observed in homozygous males in which the $_{prom}$scs construct replaces *Fab-7*. In these flies, A3 to A6 were transformed into a mixture of A2-A3 identity, indicating that *iab-3* through *iab-6* are affected by the $_{prom}$scs element. In prior experiments, we have shown that replacement of *Fab-7* by the minimal scs insulator (scs^{min}) in both orientations results in a consistent phenotype in which *iab-5* and *iab-6* are prevented from interacting with *Abd-B* by the intervening insulator (Hogga et al., 2001). Thus, the extra 282 bp DNA element appeared to interfere at a distance with *iab-3* and *iab-4*. Interference with *iab-3* and *iab-4* functions in $_{prom}$scs is surprising, because these *cis*-regulatory domains regulate *abd-A* (see Fig. 5A) and are distant from $_{prom}$scs. This result implies that, $_{prom}$scs exerts a negative polar effect that can spread 40 kb away into *iab-3*. In addition, we have previously provided evidence that, upon insulation from *Abd-B* by the intervening scs^{min} insulator, *iab-5* is targeted instead to the *abd-A* gene, which it activates in a pattern appropriate

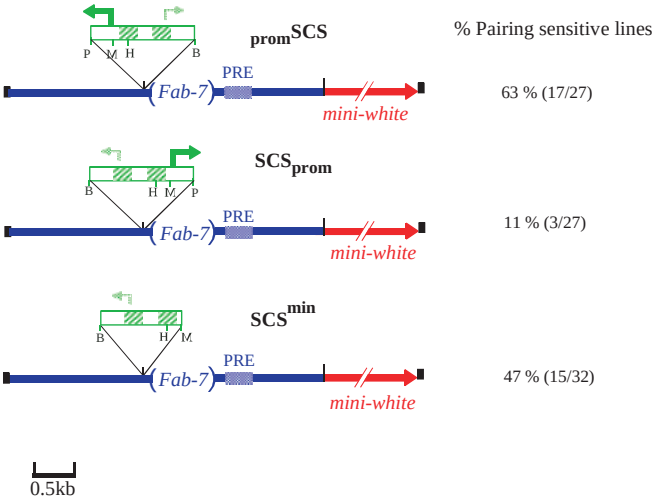

Fig. 1. Structure of the different P elements used to induce the swapping of *Fab-7* by scs. The 3.1 kb fragment (containing the *Fab-7*^{2} deletion) that serves as ectopic donor in the gene conversion experiment is drawn in dark blue at the scale indicated at the bottom of the figure. The *iab-7*PRE abutting the *Fab-7* boundary is indicated. This *Fab-7* fragment is inserted in front of a *miniwhite* gene within a P-element (the feet of the P element are indicated by black rectangles; the miniwhite gene in red is not drawn at scale). The structure of the SCS element that was inserted in the *Nsi*I site just upstream from the *Fab7*^{2} deletion endpoint is shown in green above the *Fab-7* DNA line with a few relevant restriction sites (drawn at the same scale; B, *Bam*H1; H, *Hpa*I: M, *Mlu*I; P, *Pst*I). The promoter driving transcription under the control of the *iab cis*-regulatory domains from within the BX-C is indicated in dark green. The promoter described by Avramova and Thikonov (Avramova and Thikonov, 1999) is shown in light green. The percentage of pairing sensitive lines from each construct is indicated with the numbers of lines scored (only homozygous viable lines are reported).

for specifying a A5-like identity (see Hogga et al., 2001). In promscs, A5 is transformed into A2-A3, indicating that the negative polar effect exerted by promscs also affects *iab-5/abd-A* interaction. This phenotype was seen in four independent conversion lines and a whole genome Southern analysis has verified that there are no large rearrangements affecting the *iab-3* through *iab-5* regions of the promscs chromosome. Finally, as heterozygous flies are wild type, the negative polar effect of promscs on *iab-3* through *iab-5* is only acting in *cis*.

Fig. 2B shows the phenotype observed in homozygous males in which the same scs fragment replaces *Fab-7* in the opposite orientation (scsprom). Instead of observing the loss-of-function phenotype described above, where A3, A4, A5 and A6 are transformed to a more anterior segment, we found a gain-of-function phenotype in which A6 took the identity of a more posterior segment, A7. This is similar to removal of *Fab-7* entirely. Because in these conversions we

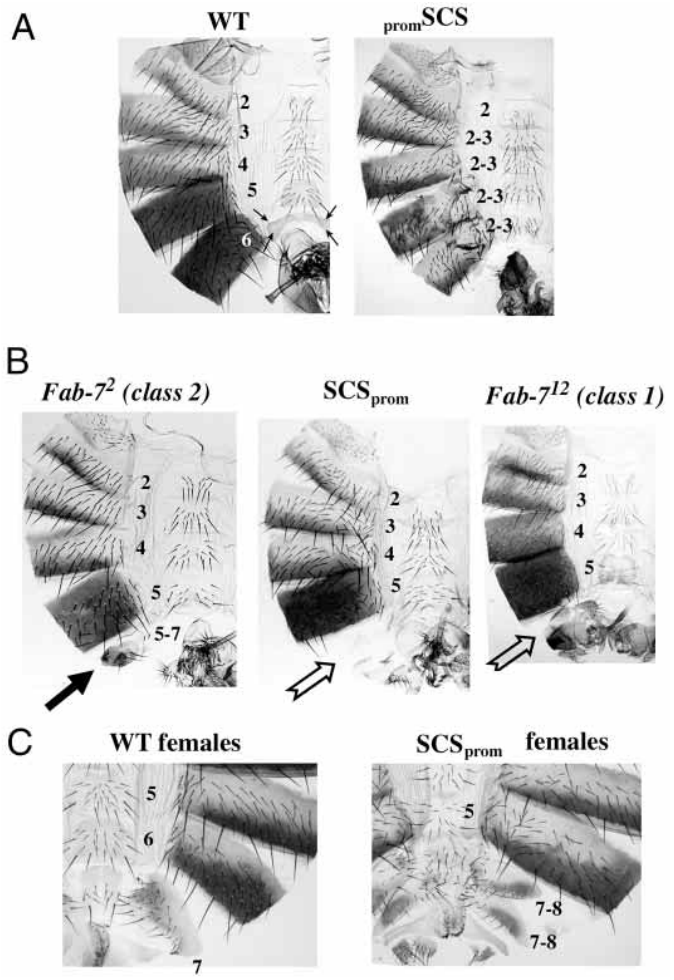

Fig. 2. Homeotic transformations in promscs and scsprom. Wholemounts of abdominal cuticles (see Materials and Methods for description of the abdominal segments). (A) In homozygous promscs males, the presence of bristles on the sixth sternite (shown by arrows in wild type, left) indicates a homeotic transformation of A6 towards a more anterior segment. On the dorsal side, A5 and A6 tergites have a patchy pigmentation, indicating a transformation into a more anterior abdominal segment. This could reflect a transformation into A4. However, dissection of whole abdomen revealed that they contain only rudimentary gonads. As the somatic part of the gonads is derived from A3 (Bender and Hudson, 2000), rudimentary gonads reflect a transformation towards a more anterior segment. Taken altogether, these homeotic transformations indicate that A3, A4, A5 and A6 are transformed into a more anterior abdominal segment [a mixture of A2 and A3 (2-3)]. (B) A wild-type male has six abdominal segments. The seventh abdominal segment (A7), which is present in larvae, is suppressed during metamorphosis. In *Fab-7*^{2}, *iab-7* (left) is ectopically activated in most cells of A6. As a consequence, A6 assumes A7 identity and most of the sixth tergite and sternite are absent. There are, however, cells of A6 in which ectopic activation of *iab-7* does not take place. These cells, which are visible as a small tergite (shown by an arrow), adopt A5 identity, indicating that not only *iab-7* is inactive, but also *iab-6*. In *Fab-7*^{12} homozygotes (right), *iab-7* is ectopically expressed in all cells of A6, giving rise to a fly with no apparent tissue in A6 (open arrow). In scsprom homozygotes, A6 is completely transformed into A7, as revealed by the complete absence of tergite or sternite tissue in A6 (open arrow). (C) The derepression of *iab-8* in A6 and A7 (*Fab-7* and *Fab-8* phenotype). The A7 into A8 transformation is detectable in females where A7 develops. scsprom homozygous females show a phenotype reminiscent of an *Fab-8* boundary deletion, as revealed by the strong reduction of the seventh tergite. Because in scsprom, the *Fab-7* boundary function is also affected, the sixth tergite is also reduced in size.

removed *Fab-7* by introducing the *Fab-7*^{2} deletion, we confirmed by sequencing that scs$_{prom}$ is intact. Moreover, the same phenotype is observed in the five other independent conversion events that we recovered. The dominant gain-of-function phenotype associated with scs$_{prom}$ was confirmed by the observation that heterozygotes (scs$_{prom}$/+) displayed the same phenotype, although not as severe. Because the dominant gain of function associated with scs$_{prom}$ was absent from the flies in which *Fab-7* was replaced by scs^{min}, we conclude that the same extra 282 bp fragment (*Mlu*I-*Pst*I) is responsible for the loss-of-function phenotype in $_{prom}$scs and the dominant gain-of-function phenotype in scs$_{prom}$.

An anti-PRE at the edge of scs$_{prom}$

Closer examination of the males shown in Fig. 2B revealed that the phenotype of scs$_{prom}$ flies was slightly different from the phenotype generated by the *Fab-7*^{2} deletion alone. As mentioned in the Introduction, class 2 mutations such as *Fab-7*^{2}, which remove only the boundary and leave an intact *iab-7*PRE, caused a mixed gain- and loss-of-function phenotype: most cells of A6 adopted A7 identity while the remaining adopted A5 identity (see Fig. 2B). If the scs$_{prom}$ construct had no effect on the region, we should have observed a *Fab-7*^{2} phenotype simply because of the removal of the *Fab-7* element. Fig. 2B shows that this was not the case: A6 was completely transformed into A7 in homozygous scs$_{prom}$ flies. This phenotype is identical to the phenotype of the class I *Fab-7*^{12} allele where the boundary and nearby *iab-7*PRE are deleted (Fig. 2B). Thus, introduction of the scs$_{prom}$ element converts a class II allele into a class I allele, as if the extra 282 bp fragment (*Mlu*I-*Pst*I) interfered with the activity of the nearby *iab-7* PRE. To test this hypothesis, we decided to verify how scs$_{prom}$ affected PRE-mediated pairing-sensitive repression of a miniwhite reporter construct (see Introduction). Although transformants with the scs element in the $_{prom}$scs orientation are pairing sensitive in 63% of the lines, when the scs element is in the opposite orientation (scs$_{prom}$) the pairing-sensitive frequency decreases to 11% (Fig. 1). Thus, our hypothesis that scs$_{prom}$ within the BX-C contains an anti-PRE activity is supported by these ectopic constructs. To localize this anti-PRE activity, we analyzed the pairing-sensitivity of scs$_{prom}$ derivatives in which the scs$_{prom}$ fragment was progressively shaved from one end (see Fig. 1). A deletion removing 282 bp from the end (*Mlu*I-*Pst*I deletion; scs^{min}) restored pairing-sensitivity to a frequency of 47%. Because a deletion that extended further towards the *Hpa*I site did not significantly increase the proportion of pairing-sensitive lines (50%), we conclude that most of the anti-PRE element of scs is located within the *Mlu*I-*Pst*I fragment.

The anti-PRE associated with scs$_{prom}$ interferes with *iab-8* silencing

Examination of homozygous scs$_{prom}$ females indicated that *iab-8* was also partially activated in the sixth and seventh abdominal segments, both of which show an identity intermediate between A7 and A8, a phenotype that is reminiscent of *Fab-8* boundary deletions (Fig. 2C) (Barges et al., 2001). These observations suggest that the anti-PRE activity contained in scs$_{prom}$ is not only interfering with the functioning of the nearby *iab-7* PRE, but spreads across the whole *iab-7* domain and reaches *iab-8*.

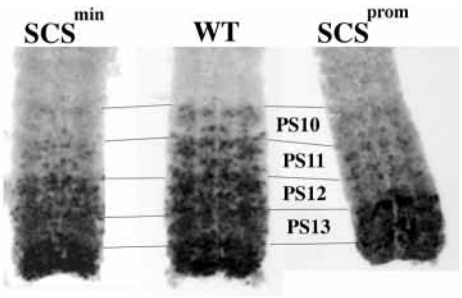

Fig. 3. *Abd-B* expression patterns in the central nervous system of homozygous scs^{min}, wild type and scs$_{prom}$ embryos. Central nervous systems (CNS) were dissected out from embryos stained with an antibody directed against ABDB as described elsewhere (Hogga et al., 2001). In wild type, the typical graded ABDB expression pattern from PS10 to PS14 is visible. In scs^{min} CNS, a partial block between *iab-5/iab-6* and *Abd-B* is visible by the weaker signal in PS10 and PS11. In scs$_{prom}$, the same impediment appears in PS10. Note however, that in PS11 and PS12, the ABDB expression patterns are similar (unlike in scs^{min}). The PS11 and PS12 levels and patterns of ABDB expression are not a reiteration of the PS12-specific pattern in wild type (as it would be expected in a *Fab-7* embryo). They reach a level intermediate between the PS10 and PS11 from wild type. The regulatory output of the fused *iab-6-iab-7* domain may be shared between the scs$_{prom}$ and *Abd-B* promoters (see text).

The gain-of-function effect associated with scs$_{prom}$ is post-embryonic

We also examined the *Abd-B* expression pattern in embryos (Fig. 3). In scs^{min}, the partial block between *iab-5/iab-6* and the *Abd-B* promoter was visualized by the great reduction of *Abd-B* expression in PS10 and PS11 (Hogga et al., 2001). In scs$_{prom}$, because of the posterior transformation of A6 into A7, we were expecting to monitor a reiteration of the PS12/A7 *Abd-B* expression pattern in PS11/A6 (Galloni et al., 1993). This is not what we found. In PS10 and PS11, the expression patterns were similar to the patterns detected in scs^{min}. In PS12, the expression pattern was much lower than in wild type and was similar to the pattern detected in PS11. In fact, this expression pattern resembled that of an *iab-7* deletion mutant (Galloni et al., 1993). This should lead to a transformation of PS12 into PS11, exactly the opposite of the phenotype that we observe in adults. Unfortunately, there are no distinguishing morphological landmarks in embryos and larvae that definitively identify these two parasegments earlier in development. It is not clear why, in the adult, the readout of scs$_{prom}$ results in a gain-of-function phenotype, whereas in the embryo, *Abd-B* expression resembles loss of function. We believe these findings indicate that *iab-7* misexpression is more pronounced in the pupa, when the adult structures are forming, strongly supporting the idea that scs$_{prom}$ affects primarily the maintenance phase. Bender and Fitzgerald have described similar mutations that affect the maintenance phase of *iab-2* silencing in PS6/A1 (Bender and Fitzgerald, 2002). In their case, the identity of the affected abdominal segment PS6/A1 could be easily recognized in embryos and larvae from PS7/A2. Intriguingly, the dominant gain-of-function phenotype associated with their mutations was also only detectable in adult.

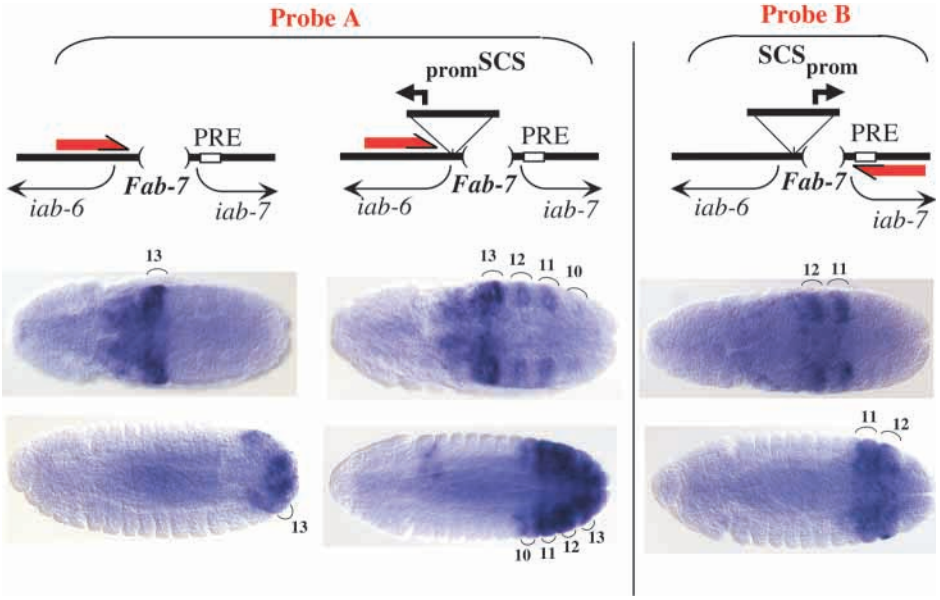

Fig. 4. Transcripts arising from the scs promoter in the BX-C. Organization of the genomic DNA around *Fab-7* in *Fab-7*^{2} (left), $_{prom}$scs (middle) and scs$_{prom}$ (right) flies. The promoter active in $_{prom}$scs and scs$_{prom}$ is drawn. The A and B strand-specific probes are shown in red: probe A, bases 84,732 to 86,947 in the BX-C sequence (Martin et al., 1995); probe B, 82,554-84,732. The expression pattern of the transcripts in wild type (or *Fab-7*^{2}), $_{prom}$scs and scs$_{prom}$ embryos at 4 and 12 hours of development are shown below. Parasegments are indicated. In scs$_{prom}$, we detected a similar expression pattern with a probe spanning the *iab-8*PRE (not shown; from 59,446 to 62,117). It should be noticed that the intensity of rightwards transcription is higher from scs$_{prom}$ than leftwards transcription originating from $_{prom}$scs. Although the signal usually appears after 1 hour of incubation with the alkaline substrates, with probe B, we had to wait more than twice as long as we did with probe A.

The region responsible for the orientation-dependent effect contains a promoter

We have previously shown that scs contains a chromatin insulator element (scs^{min}) that is able to interfere with long-range enhancer-promoter interactions (Hogga et al., 2001). In addition to the insulator, the 1.2 kb scs fragment contains at one extremity an element that, in conjunction with scs^{min}, is able to induce a gain-of or loss-of-function phenotype when replacing *Fab-7*. When facing *iab-7*, this element destabilizes *iab-7* and *iab-8* silencing in A6. However, when facing *iab-6*, this element exerts a negative polar effect on *iab-5*, *iab-4* and *iab-3*. These two gain- and loss-of-function effects are difficult to reconcile. Previously, Avramova and Tikhonov (Avramova and Tikhonov, 1999) discovered that scs contained a promoter and challenged the idea that scs was a neutral chromatin domain boundary. Their finding supported the promoter decoy hypothesis of Geyer (Geyer, 1997), in which insulation is achieved by a promoter-trapping mechanism. Although the promoter described by Avramova and Thikonov (Avramova and Thikonov, 1999) maps to the other side of scs (see Fig. 1), promoter trapping could account for the polar effect on *iab-5*, *iab-4* and *iab-3*. In this scheme, insertion of a promoter at the *iab-6* edge of *Fab-7* would attract the nearby *cis*-regulatory elements and divert them from their normal *abd-A* and/or *Abd-B* promoter (see Fig. 5A). In this case, transcription of sequences near the promoter should be detected in parasegments affected by $_{prom}$scs (and scs$_{prom}$). In order to test this hypothesis, we performed whole-mount in situ hybridization on embryos of the $_{prom}$scs and scs$_{prom}$ lines. We synthesized strand-specific probes from the *iab-6* (probe A; Fig. 4) or *iab-7* DNA (probe B; Fig. 4) flanking the scs element. Using both probes on $_{prom}$scs, scs$_{prom}$ and scs^{min} lines, we found that the promoter described by Avramova and Thikonov (Avramova and Thikonov, 1999) remained silent in the context of the BX-C. There was, however, a promoter that became active in the context of the BX-C at the other side of the scs fragment. Fig. 4 shows the results obtained with a probe from the *iab-6* side of $_{prom}$scs (probe A). In wild type (or *Fab-7*^{2}), transcription was detected from a very early stage throughout embryogenesis in PS13 and PS14. The expression profile of this transcript was reminiscent of a transcript that originates from a promoter localized just downstream from the *Abd-B* transcription unit (Zhou et al., 1999). In $_{prom}$scs embryos, however, we detected additional transcripts in PS10/A5, PS11/A6 and PS12/A7. Interestingly, we failed to detect these transcripts when the truncated version of $_{prom}$scs (scs^{min}) replaced *Fab*-7, indicating that the *Mlu*I-*Pst*I fragment contains the promoter (or sequences necessary for its activity). Appearance of PS10-specific transcripts may explain the polar effect of $_{prom}$scs on *iab-5* if we assume that *iab-5* activity is trapped by the scs promoter and thus diverted from the *abd-A* promoter [in the $_{prom}$scs context, *iab-5* is insulated from *Abd-B* by the intervening scs^{min} insulator, see Hogga et al. (Hogga et al., 2001)]. A similar mechanism would explain the polar effect on *iab-4* and *iab-3* (Fig. 5A). However, we failed to detect transcription in PS9 and PS8, where both regulatory domains are active (Fig. 4). The discrepancy between the observed pattern of embryonic transcription and the adult phenotype may reflect a higher affinity of the promoter to *iab-3,4* in adults.

It is not entirely clear which regulatory domain activates transcription in PS12. In the $_{prom}$scs context, the scs insulator is located between the promoter and the *iab-7 cis*-regulatory domain. As reported by Hogga et al. (Hogga et al., 2001), the insulator alone does not completely impair interactions between the distal *iab-5*/*iab-6* cis-regulatory domains and their *Abd-B* target promoter (see also Fig. 3). Thus, in $_{prom}$SCS, *iab-7* may not be completely insulated from the promoter and activates transcription in PS12. As an alternative explanation we believe that transcription in PS12 in $_{prom}$scs embryos results from activation by the more anterior *cis*-regulatory domains (*iab*-3, *iab-4*, *iab-5* or *iab-6*), which once activated in a given parasegment, remain active in the more posterior parasegments, as first proposed in Ed Lewis' model (Lewis,

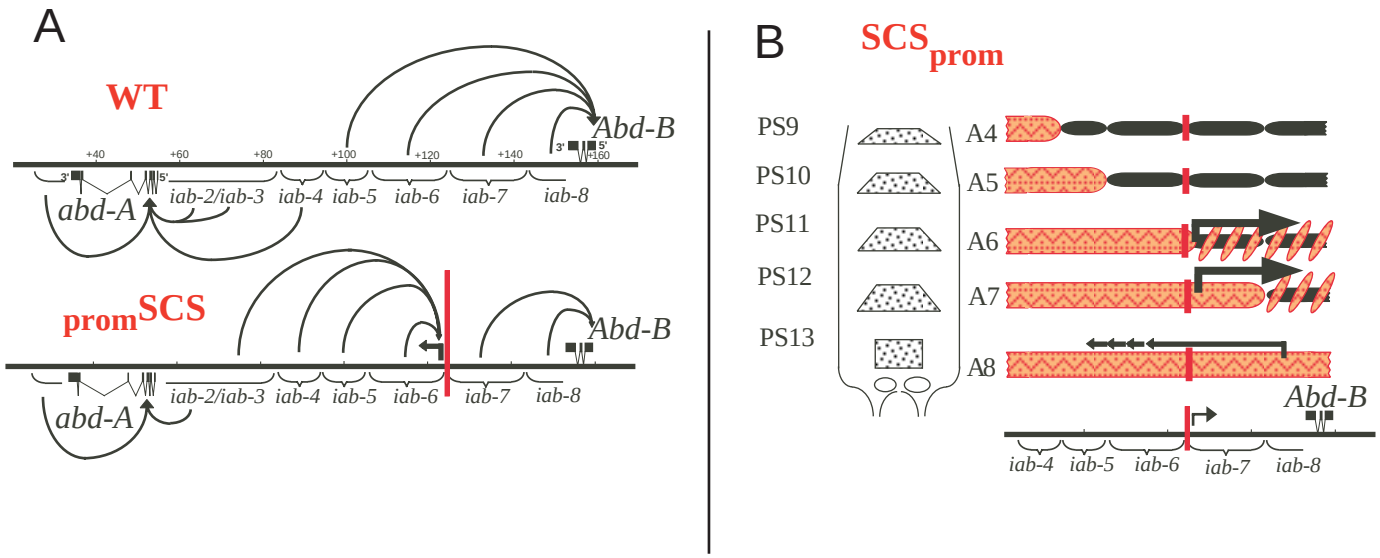

Fig. 5. Summaries of the effects caused by the replacement of *Fab-7* by $_{prom}$scs or scs$_{prom}$. (A) The thin horizontal line represents the genomic DNA of the abdominal region of the BX-C marked off in kb according to Karch et al. (Karch et al., 1985). The structures of the *abd-A* and *Abd-B* transcription units are shown. The *cis*-regulatory interaction between the *iab* domains and their respective target promoters are shown by loops. While *iab-2*, *iab-3* and *iab-4* regulate *abd-A* in PS7, PS8 and PS9 respectively, *iab-5*, *iab-6*, *iab-7* and *iab-8* regulate *Abd-B* expression in PS10, PS11, PS12 and PS13. The red vertical bar represents the scs^{min} insulator that prevents *Abd-B* activation by *iab-5* and *iab-6*. Upon isolation from *Abd-B*, *iab-5* is able to interact with *abd-A* (Hogga et al., 2001). In $_{prom}$scs, the promoter on the left of the insulator would be activated by *iab-3*, *iab-4* and *iab-5*, diverting these regulatory domains from the *abd-A* promoter. This results in the loss-of-function phenotype of *iab-3*, *iab-4* and *iab-5* (loss-of-function of *iab-6* is due to the inability of *iab-6* to regulate *Abd-B* properly because of the intervening scs^{min} insulator) (Hogga et al., 2001). (B) The posterior abdomen of a larva from A4/PS9 to A8/PS13 is shown on the left. On the right, the activity states of *iab-4* to *iab-8 cis*-regulatory domains are shown in their respective segments/parasegments. For example, in A4/PS9, *iab-4* is active (red), whereas the remaining *iab-5,6,7* and *iab-8* are kept inactive by the Pc-G-repressing complex (shown in black). In A5/PS10 the next adjacent *cis*-regulatory domain *iab-5* is activated. In A6/PS11, *iab-6* becomes active. As the scs^{min} insulator (red bar) does not insulate *iab-6* fully from *Abd-B*, we envision that *iab-6* activates the scs promoter across the insulator in A6/PS11, leading to transcription through *iab-7* and *iab-8* (shown by the thick arrow). As transcription would interfere with Pc-G complex silencing, ectopic activation of *iab-7* and *iab-8* occurs in A6/PS11, giving rise to the *Fab-7/8* homeotic transformations.

1978). It should be noted that although promoter-trapping is an attractive explanation accounting for the polar effect of $_{prom}$scs on the *iab*-3, *iab-4*, *iab-5* and *iab-6*, we cannot exclude the possibility that transcription through these cis-regulatory domains is the cause of their inactivation.

Transcription through the *iab-7*PRE may interfere with *iab-7* silencing

In scs$_{prom}$, the edge of scs containing the promoter is facing the *iab-7 cis*-regulatory domain. Not surprisingly, we detected intense transcription in PS12 with a probe from the *iab-7* edge (probe B, Fig. 4). The same probe failed to detect any transcript in wild type or in embryos in which *Fab-7* is replaced by scs^{min}. These results indicate that the PS12-specific transcript very likely originates from the same promoter that is firing in PS10-12 in $_{prom}$scs. We also detected equally intense transcription in PS11. Thus, the fragment harboring anti-PRE activity when associated with scs$_{prom}$, contains a promoter that fires transcription across the *iab-7*PRE with which it interferes. As mentioned above, the anti-PRE activity contained in scs$_{prom}$ not only interferes with the functioning of the nearby *iab-7*PRE, but spreads across the whole *iab-7* domain and reaches *iab-8*. Notably, the same transcription pattern in PS11 and PS12 is observed with a strand-specific probe originating from *iab-8* (data not shown, see Fig. 4).

Fig. 5B suggests how the transcription from scs$_{prom}$ might cause the posterior transformation in the sixth and seventh abdominal segments (PS11 and PS12). The promoter in scs$_{prom}$ is activated in PS11 and PS12 (Fig. 4), sending transcripts across the *iab-7* and *iab-8* regulatory regions. In PS11, both of these regulatory regions are normally silent, but the act of transcription apparently reverses the silencing, causing the cells in PS11 to differentiate in the same way as those of PS12 or PS13. Likewise, in PS12, transcription across the *iab-8* region activates it, transforming PS12 cells towards PS13 character. We do not observe transcription from scs$_{prom}$ in PS13. In this parasegement, however, the *iab-8* promoter is activated, giving rise to leftwards transcription (Zhou et al., 1999). It is possible that this leftwards transcription interferes with rightwards transcription from scs$_{prom}$. It is perhaps surprising that the transcription from scs$_{prom}$ begins in PS11, as the PS12-specific regulatory region (*iab-6*) is separated from the promoter of scs$_{prom}$ by the scs insulator. However, the insulator in an scs^{min} conversion at the same site does not completely insulate *iab-6* from the *Abd-B* target promoter (Hogga et al., 2001) (see also Fig. 3), making it likely that *iab-6* can activate the scs$_{prom}$ across the insulator. It is also possible that the function of an insulator depends on neighboring sequences. If, for example, insulator function is enhanced by a nearby PRE, then partial loss of the *iab-7*PRE function might weaken the scs$_{prom}$ insulator.

There are precedents where transcription has been suggested

to play a role in chromatin remodeling. For example, the human β-globin locus is subdivided into three chromatin domains, each of which become more accessible to nuclease digestions upon gene activation (Ashe et al., 1997; Gribnau et al., 2000; Plant et al., 2001). Interestingly, large intergenic transcripts delineate each of these domains and chromatin remodeling of each domain is preceded by its transcription. Another example has been reported by Rank et al. (Rank et al., 2002). Using a transgenic context, they found that transcription across a PRE could interfere with silencing. Finally, in the accompanying paper, Fitzgerald and Bender provide evidence that transcription across the *iab-2 cis*-regulatory domains in PS6/A1 interferes with *iab-2* silencing, resulting in the posterior transformation of PS6/A1 into PS7/A2 (Bender and Fitzgerald, 2002). In this case, the identity of the affected abdominal segment can easily be recognized in embryos and larvae. Despite the existence of intense transcription of *iab-2* in embryos, the dominant gain-of-function phenotype associated with *iab-2* misexpression is only detectable in the adult. Thus, as in our case, transcription across the *iab* regulatory regions appear to interfere with silencing during the late maintenance phase, when the adult structures are forming.

If transcription can interfere with Pc-G silencing, what are the mechanisms responsible for this activity? Factors that affect RNA polymerase II (RNAPII) transcript elongation have been shown to have an effect on chromatin. For example, it has been suggested that histone acetyl transferases (HAT) such as PCAF (Cho, 1998) or *ELP3* (Wittschieben, 1999) assist RNAPII in relieving inhibition caused by nucleosome arrays. Although active chromatin requires acetylation of specific lysine residues in the H3 and/or H4 histone tails (Moazed, 2001), the recent purification of Pc complexes suggests that histone deacetylation is required for establishing a stable long-term Pc-G silencing complex (Saurin et al., 2001; Tie et al., 2001). In the case of scs_{prom}, perhaps the frequent passage of RNAPII and its associated histone acetylation activities though the PREs interferes with the assembly of the Pc-G silencing. Involvement of acetylated histones in antagonizing PcG-dependent silencing is supported by the findings of Cavalli and Paro (Cavalli and Paro, 1999) showing that high levels of acetylated histone H4 are associated with non-repressive PRE sequences. Alternatively, it has been recently found that variant histone H3.3 was deposited on active chromatin during transcription, providing a mechanism for the immediate activation of genes that are silenced by histone modification (Ahmad and Henikoff, 2002). It may be possible that transcription across *iab-7* (and also *iab-8*) results in deposition of new nucleosome marked by H3.3, interfering thereby with the maintenance of silencing by the Pc-G complex.

Concluding remarks

It has been known for a long time that the large *cis*-regulatory regions of the bithorax complex are transcribed (Lipshitz et al., 1987; Sanchez-Herrero and Akam, 1989; Cumberledge et al., 1990). In blastoderm stage embryos, the *iab-2* though *iab-8* regions can be divided into three domains, each transcribed in a region that extends from a specific anterior limit to the posterior limit of the segmented part of the embryo. These domains are only broadly defined but their order on the chromosome reflects the anterior limit of expression for each of them. In the light of our data, it is tempting to speculate that transcription of the *iab* domains convey a regulatory signal, preventing assembly of the *Polycomb*-repressing complex on the *iab* domains that need to remain active. If this is true, transcripts should appear in the anteriormost parasegments/segments where each *cis*-regulatory domain is activated. However, so far, we have not seen transcripts in every regulatory region, which would account for the sequential activation of each regulatory domain. Moreover, this model predicts that the *iab-7* PRE and *iab-7* domains should be transcribed from PS12, where *iab-7* is first active. So far transcripts across the *iab-7* domain have only been detected in PS13 and 14 (this study) (Zhou et al., 1999). Thus, it remains unclear whether intergenic transcription plays a role in wild-type animals to create and/or maintain open chromatin, or whether the existence of intergenic transcripts is the consequence of an open structure. However, our experiments strongly suggest that forced transcription through an inactive *cis*-regulatory domain interferes with the maintenance of silencing, highlighting an incompatibility between transcription and Pc-G mediated silencing. This activity probably reflects a fundamental mechanism to protect an actively transcribed gene from being inactivated by the Pc-G proteins that are present in all cells.

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