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In situ dissection of the *Fab-7* region of the bithorax complex into a chromatin domain boundary and a *Polycomb*-response element

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SUMMARY

Parasegmental (PS)-specific expression of the homeotic genes of the bithorax-complex (BX-C) appears to depend upon the subdivision of the complex into a series of functionally independent *cis*-regulatory domains. *Fab-7* is a regulatory element that lies between *iab-6* and *iab-7* (the PS11- and PS12-specific *cis*-regulatory domains, respectively). Deletion of *Fab-7* causes ectopic expression of *iab-7* in PS11 (where normally only *iab-6* is active). Two models have been proposed to account for the dominant *Fab-7* phenotype. The first considers that *Fab-7* functions as a boundary element that insulates *iab-6* and *iab-7*. The second model envisages that *Fab-7* contains a silencer element that keeps *iab-7* repressed in parasegments anterior to PS12. Using a P-element inserted in the middle of the *Fab-7* region (the *blt* transposon), we have generated an extensive collection of new *Fab-7* mutations that allow us to subdivide *Fab-7* into a boundary element and a *Polycomb*-respond element (PRE). The boundary lies within 1 kb of DNA on the proximal side of the *blt* transposon (towards *iab-6*). Deletions removing this element alone cause a complex gain- and loss-of-function phenotype in PS11; in some groups of cells, both *iab-6* and *iab-7* are active, while in others both *iab-6* and *iab-7* are inactive. Thus, deletion of the boundary allows activating as well as repressing activities to travel between *iab-6* and *iab-7*. We also provide evidences that the boundary region contains an enhancer blocker element. The *Polycomb*-response element lies within 0.5 kb of DNA immediately distal to the boundary (towards *iab-7*). Deletions removing the PRE alone do not typically cause any visible phenotype as homozygotes. Interestingly, weak ectopic activation of *iab-7* is observed in hemizygous PRE deletions, suggesting that the mechanisms that keep *iab-7* repressed in the absence of this element may depend upon chromosome pairing. These results help to reconcile the previously contradictory models on *Fab-7* function and to shed light on how a chromatin domain boundary and a nearby PRE concur in the setting up of the appropriate PS-specific expression of the *Abd-B* gene of the BX-C.

Key words: DNA domain boundary, *Polycomb*-response element, bithorax complex, *Drosophila*, *Fab-7*, *iab-6*

INTRODUCTION

The bithorax complex (BX-C) of *Drosophila* contains three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), which are responsible for specifying the identities of the parasegments (PS) that form the posterior half of the thorax and the abdomen of the fly (PS5-14; Lewis, 1978; Sanchez-Herrero et al., 1985; Casanova et al., 1987). A very large *cis*-regulatory region of 300 kb is responsible for generating the PS-specific expression patterns of the three BX-C homeotic genes. Genetic and molecular studies indicate that this large regulatory region is subdivided into nine PS-specific *cis*-regulatory units (*abx/bx*, *bxd/pbx*, *iab-2*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7* and *iab-8,9*; Bender et al., 1983; Karch et al., 1985; Duncan, 1987; Peifer et al., 1987). The *abx/bx* and *bxd/pbx cis*-regulatory units are responsible for directing the appropriate pattern of *Ubx* expression in PS5 and PS6, respectively (White and Wilcox, 1984; Beachy et al., 1985). Similarly, the *iab-2*, *iab-3* and *iab-4 cis*-regulatory units direct *abd-A* expression in PS8, PS9 and PS10 (Karch et al., 1990; Macias et al., 1990; Sanchez-Herrero, 1991). Finally, the *iab-5* through *iab-8,9 cis*-regulatory units direct *Abd-B* expression in PS10-14. (Celniker et al., 1990; Sanchez-Herrero, 1991). Loss-of-function mutations in any one of these nine PS-specific *cis*-regulatory units transforms the corresponding parasegment into a copy of the parasegment immediately anterior. For example, the *iab-7 cis*-regulatory unit is responsible for controlling the expression of the short *Abd-B* transcription unit in PS12 (see Fig. 1A). A mutation that deletes much of *iab-7* (*iab-7*^{Sz}; Fig. 1A) results in the transformation of PS12 into a copy of PS11. Consistent with this phenotypic transformation, the normal *Abd-B* expression in PS12 is replaced by a PS11-like pattern that is generated by the *iab-6 cis*-regulatory unit (Galloni et al., 1993).

During early embryogenesis, when segmental identity is initially selected, the PS-specific *cis*-regulatory units are the targets of the gap and pair-rules gene products (Simon et al., 1990; Qian et al., 1991; Muller and Bienz, 1992; Shimell et al.,

1994). When the segmentation gene products decay, BX-C regulation switches from the initiation to the maintenance phase. The expression of the homeotic genes is maintained during the remainder of the life cycle by the *Polycomb* and *trithorax* group genes (*Pc-g* and *trx-g*). Though their precise mode of action is unknown, the products of the *Pc-g* and *trx-g* are thought to stabilize the expression patterns in each parasegment by imprinting inactive or active chromatin conformation of the PS-specific *cis*-regulatory subregions (Shearn, 1989; Paro, 1990; Pirrotta and Rastelli, 1994; Simon, 1995).

Molecular studies have revealed the existence of elements within the PS-*cis*-regulatory units that seem to be responsible for the initiation and maintenance phases of BX-C regulation. Some DNA fragments are able to initiate expression of a *Ubx-lacZ* reporter gene in the proper parasegments during early embryonic development (Qian et al., 1991; Shimell et al., 1994; Simon et al., 1990; Muller and Bienz, 1991). In most cases, however, these patterns are not maintained and expression expands into more anterior parasegments around the time when BX-C regulation would switch to the maintenance mode. Other BX-C DNA fragments are capable of retaining the appropriate parasegmental restrictions in *lacZ* expression after the gap and pair-rule gene products disappear. These fragments contain 'maintenance elements' also known as *Pc-g* response elements (PRE) because their activity depends on *Pc-g* gene products (Simon et al., 1990, 1993; Muller and Bienz, 1991; Zhang and Bienz, 1992; Busturia and Bienz, 1993; Chan et al., 1994; Christen and Bienz, 1994; Chiang et al., 1995; Poux et al., 1996). Finally, a third type of regulatory element that has been identified in experiments with *Ubx-lacZ* reporter constructs is tissue- or cell-type-specific enhancers. They induce *lacZ* expression in specific tissue or cell type with no restriction along the anteroposterior axis.

Many observations suggest that the PS-specific *cis*-regulatory units are organized into functionally independent domains. This is best illustrated by the expression patterns of 'enhancer trap' transposons integrated in different domains of the complex (McGall et al., 1994; Galloni et al., 1993). These enhancer traps are subject to regulatory elements located within the same domain, but are insensitive to regulatory elements in adjacent domains. The autonomy of each domain is ensured by elements that are believed to function as boundaries. Two such regulatory elements, *Mcp* and *Fab-7*, have been identified genetically (Gyurkovics et al., 1990; Karch et al., 1994). *Mcp* is located between the *iab-4* and *iab-5 cis*-regulatory units or domains, while *Fab-7* is located between *iab-6* and *iab-7* (see Fig. 1A). Unlike loss-of-function mutations in the *cis*-regulatory domains, which show a transformation of the affected parasegment into the parasegment immediately anterior, deletions that remove either of the boundary elements have an opposite gain-of-function phenotype; they transform the affected parasegment into a copy of the parasegment immediately posterior. For example, in the adult fly, the $Fab\text{-}7^{1}$ deletion results in the development of the 6th abdominal segment (A6 which corresponds to PS11) into a copy of A7 (PS12). This transformation appears to be due to the inappropriate activation of the *iab-7 cis*-regulatory domain in PS11 where *Abd-B* is normally controlled by *iab-6* (see Fig. 1A). Consistent with this hypothesis, in $Fab\text{-}7^{1}$ mutant embryos, the pattern of *Abd-B* protein expression in PS11 is identical to that found in PS12 (Galloni et al., 1993). Since the dominant gain-of-function phenotype of the $Fab\text{-}7^{1}$ deletion can be reverted by second site mutations that eliminate either *iab-6* or *iab-7* function, the ectopic activation of *iab-7* in PS11 depends upon inappropriate interactions between elements in the *iab-6* and *iab-7 cis*-regulatory domains. A similar interaction between *iab-4* and *iab-5* is required for the dominant gain-of-function phenotype associated with *Mcp* mutations. These findings have led to the suggestion that *Mcp* and *Fab-7* may correspond to the boundaries of the PS-specific *cis*-regulatory domains. They are responsible for ensuring that the adjacent *cis*-regulatory domains in the BX-C are functionally autonomous. Consequently when one of these insulating element is deleted, adventitious interactions between adjacent *cis*-regulatory domains are possible.

Additional evidence that *Fab-7* functions as a boundary was provided by the insertion of a P-element transposon that carries a *Ubx-lacZ* reporter gene close to *Fab-7*, at the proximal edge of the *iab-7 cis*-regulatory region (the *bluetail* transposon shown in Fig. 1A; Galloni et al., 1993). Since the *lacZ* reporter gene is equidistant from both *iab-6* and *iab-7*, it should be in principle regulated by both *cis*-regulatory subregions. However, *lacZ* expression is detected only in PS12 indicating that the *Ubx-lacZ* reporter gene is subject to regulation by *iab-7* but not by *iab-6* (Fig. 5). A prediction from this finding is that sequences required for *Fab-7* boundary function reside to the left of the insertion site for the *bluetail* transposon. This prediction was confirmed by mobilizing the *bluetail* transposon and isolating imprecise excisions that have the *Fab-7* gain-of-function phenotype. These *Fab-7* alleles have deletions that remove DNA to the left of the *bluetail* insertions site (Galloni et al., 1993; see also below).

As has been observed for other elements that function as domain boundaries (Udvardy et al., 1985; Kellum and Schedl, 1991; Chung et al., 1993), the *Fab-7* region is organized into a chromatin structure that contains four discrete nuclease hypersensitive sites (see Fig. 1B). Three of these hypersensitive sites (two strong, HS1 and HS2 and one weak, ∗) are located to the left of the *bluetail* insertion site towards *iab-6*, while the fourth hypersensitive site (HS3) is to the right of the insertion site towards *iab-7*. In the studies reported here, we have analyzed the functional organization of the *Fab-7* region by inducing an extensive collection of new deletions in the BX-C complex. This *in situ* analysis of *Fab-7* demonstrates that the boundary function in the context of the BX-C is dependent upon DNA sequences that span nuclease hypersensitive sites I and II. Deletion of this region alone allows both positive and negative signals to travel between *iab-6* and *iab-7*. Hypersensitive site 3, to the right of the *bluetail* transposon, is not required for *Fab-7* boundary function; instead it functions as an *iab-7* PRE.

MATERIALS AND METHODS

Drosophila culture

Fly stocks were maintained on standard yeast-cornmeal medium.

Mobilization of the *blt* transposon

Several schemes were used to recover excisions of the *bluetail* transposon. In some instances, the source of transposase was provided by a TM3 balancer chromosome carring P[(ry^{+})Δ2-3] (Reuter et al., 1993).

In the set of experiments in which we recovered the $iab\text{-}6,7^{P6.1}$, *iab-*

6,7$^{P14.1}$ and *iab-6,7$^{P18.1}$* derivatives, we had crossed dysgenic males *iab-7^{blt}/TM3,P[(ry^{+})Δ2-3]* to *iab-4,5^{DB}/TM3* females. The *iab-4,5^{DB}* chromosome deletes the *iab-4* through *iab-6 cis*-regulatory regions (Karch et al., 1985; Duncan, 1987). Among the progeny of this cross, individual *Blt*/iab-4,5^{DB}* males associated with visible homeotic transformations were crossed again to *iab-4,5^{DB}/TM3* females to establish stocks. Since both the *iab-4,5^{DB}* and *TM3* chromosomes carry the recessive marker *p^{p}*, it was easy to recognize the *blt*/TM3* progeny and construct the stock. 25 *Blt*/TM3* stocks were generated and characterized by whole genome Southern analysis.

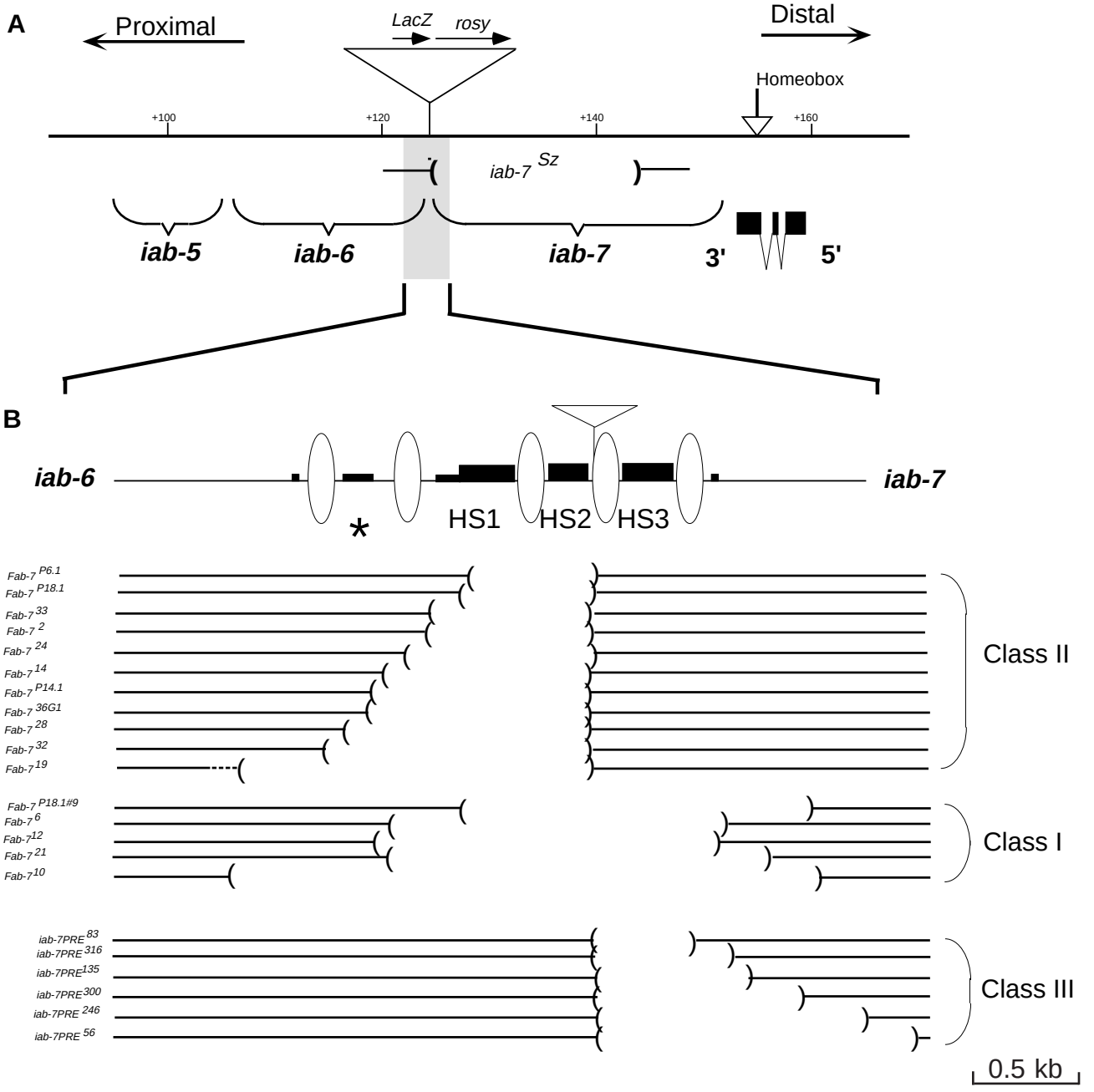

Fig. 1. (A) Distal part of the BX-C. The thin horizontal line represents the genomic DNA of the distal part of the BX-C marked off in kilobases. The *Abd-B* homeobox is indicated by a vertical arrow. For simplicity, only the class A *Abd-B* transcript, which is required for morphogenic functions of PS10 to 13 is shown below the DNA line (Celniker et al., 1989, 1990; Zavortink and Sakonju, 1989; Boulet et al., 1991). The horizontal brackets below the genomic DNA indicate the extent of the *iab-5*, *iab-6* and *iab-7 cis*-regulatory subregions, which regulate the class A *Abd-B* transcript in PS10, PS11 and PS12, respectively. The proximal and distal deficiency endpoints of the *iab-7^{Sz}* deletion are also indicated below the DNA line. The position of the P[lacZ] transposon in the *blt* line (drawn at the same scale) is indicated above the genomic DNA. While proximal points towards centromere, distal indicates the direction of the telomere. (B) Molecular map of the *Fab-7* deletions. The thin line represents the DNA of the *Fab-7* region drawn at the scale indicated below the figure. The nuclease hypersensitive regions (HS) are shown by black rectangles (while thick black rectangles indicate strong hypersensitive sites, weak sites are shown by thin black rectangles). HS nomenclature is according to Karch et al. (1994). The ovals indicate putative nucleosome core particles. The triangle at the distal edge of HS2 indicate the position of the *blt* transposon. The extent of the class I, class II and class III deletions are indicated below the DNA line.

In order to excise the *blt* transposon from the *iab-6,7$^{P6.1}$*, *iab-6,7$^{P14.1}$* and *iab-6,7$^{P18.1}$* lines, dysgenic males were generated over the usual *Sb[, Δ2-3]* chromosome of (Robertson and Engels, 1989) and crossed to *Cy/+;TM2,ry,/MKRS* virgins. Altogether, 156 *ry* individuals were kept and analyzed further by PCR and/or Southern analysis.

In order to increase the frequency of deletions in *Fab-7*, we have also mobilzed the *blt* transposon over a *Sb, [Δ2-3]* chromosome carrying the *Fab-7^{1}* deletion (Hogga and Karch, 1995). Such jump-starter males were crossed to *Cy/+; TM2,ry/MKRS* virgin. Among the *ry* minus progeny, we have found 26 independent lines with dominant *Fab-7* phenotype. Finally, we have used the same scheme to recover deletions in the region, regardless of their phenotype. Of 334 *ry* individuals analyzed by PCR, we have isolated 15 independent deletions on the right of the *blt* transposon insertional site.

Genetic interactions between class I, II *Fab-7* deletions and mutations in the *trx-g* and *Pc-g*

In a pilot experiment, we have crossed *Fab-7^{1}* and *Fab-7^{2}* to the following alleles of the *Pc*- and *trx*-group of genes: *Pc^{3},Pc^{1}, ph^{410}, Pcl^{T1}, Df (2R) trix* as *Asx* deficiency, *esc^{10}, Scm^{D1}, Su(z)3^{02}, Psc^{1}, Su(z) 2^{5}* and *Df* (3R) *red trx, trx^{E2}, brm^{20}, kto^{1}, kto^{2}* (see Lindsley and Zimm, 1992) for a description of these mutations). The stronger interactions with *Fab-7^{2}* were found with *Pc^{3}*, *Pcl^{T1}* and *Scm^{D1}* as well as with *Df(3R)red,trx, brm^{20}* and *kto$^{2.}* These latter were then tested with all of the *Fab-7* mutations listed in Fig. 1B.

DNA techniques

Whole genome Southern analysis were performed as described in Gyurkovics et al. (1990). The DNA lesions of all the mutations listed in Fig. 1B as well as *iab-6,7$^{P6.1}$*, *iab-6,7$^{P14.1}$* and *iab-6,7$^{P18.1}$* were recovered by PCR using Appligene Taq polymerase. PCR products were then cloned into pBluescript II KS+ (Stratagene) and sequenced using double-strand template with the USB Sequenase 2.0 system. PCR reactions were carried out on genomic DNA templates prepared according to Bender et al. (1983) or on DNA isolated from a single fly according to a protocol of Bill Engels (Gloor et al., 1993).

Mounting abdominal cuticle

Adult abdominal cuticles were mounted as described by Duncan (1982). Flies were kept for at least one night in a solution of 1 part glycerol in 3 parts ethanol. Abdomens were separated from thorax, split mid-dorsally with a razor blade and transfered in 10% KOH for 15 minutes. The cuticules were then spread out on a slide and gently flattened under a coverslip. After 3 hours on a slide warmer at 50°C, the cuticules were washed, dehydrated in n-propanol and mounted in Euparal 'Vert' (BDH Chemicals).

Antibody staining

Embryos were stained as described in Karch et al. (1990) using mouse monoclonal antibodies against β-galactosidase from Promega. Secondary antibodies were goat anti-mouse coupled to horseradish peroxidase from BioRad. The ventral nerve cord were dissected out of the embryos with thin tungsten needles and mounted in Immu-Mount media from Shandon.

RESULTS

Deletions of the Fab-7 boundary fall into two phenotypic classes

In previous studies, we isolated and characterized three deletions in the *Fab-7* region, *Fab-7^{1}*, a 4.3 kb deletion, and two smaller deletions *Fab-7^{2}* and *Fab-7^{3}*, which disrupt boundary function (Gyurkovics et al., 1990; Galloni et al., 1993). Although all three cause a transformation of PS11 into PS12, there are subtle but clear cut phenotypic differences between the larger *Fab-7^{1}* allele and the two smaller *Fab-7^{2}* and *Fab-7^{3}* alleles. Since these phenotypic differences suggested that the *Fab-7* region may be functionally complex, we decided to further dissect the properties of this region by generating an extensive collection of deletions. For this purpose, we took advantage of the *bluetail* P transposon (*blt*), fortuitously inserted between the nuclease hypersensitive sites HS2 and HS3 (Fig. 1; Galloni et al., 1993). We mobilized the *blt* transposon and isolated excision derivatives based on several different criteria (see Materials and Methods for details).

The first excision derivatives that we examined had lost the *ry^{+}* marker of the transposon and had a dominant *Fab-7* gain-of-function phenotype. Altogether, we isolated 26 *ry$^{−}$ blt* derivatives that had a *Fab-7*-like phenotype. These new *Fab-7* alleles could be grouped into two phenotypic classes, I and II, based on how they affect the development of cuticle structures in abdominal segment A6 of adult males. Six of the new *Fab-7* mutants fell into class I, and the phenotype of these (with one possible exception, *Fab-7$^{P18.1\#9}$*) is indistinguishable from that of the original *Fab-7^{1}* deletion (Gyurkovics et al., 1990). As illustrated for one of these new alleles, *Fab-7^{12}*, in Fig. 2B, A6 is completely transformed into A7 in homozygous animals. This complete transformation of A6 into A7 would suggest that the *iab-7 cis*-regulatory domain is ectopically activated in all PS11 cells in the class I mutants as has been observed previously for *Fab-7 ^{1}* (Galloni et al., 1993).

The 20 remaining derivatives were class II, and the phenotype of these mutants closely resembles that observed in *Fab-7^{2}* (Galloni et al., 1993). As illustrated in Fig. 2C for one of the class II alleles, *Fab-7$^{P18.1}$*, homozygous animals show an incomplete transformation of A6 into A7. This is evident on the dorsal side of adult males by the formation of structures of varying shapes that resemble rudimentary 6th tergite. These rudimentary tergites appear to be of clonal origin and are composed of small, discontinuous patches of darkly pigmented cuticle. This incomplete transformation can also be seen on the ventral side where clones of sternite tissue are observed in A6 (arrows in Fig. 2C). Since tergites and sternites are formed in A6 (or more anterior segments) but not in A7, it would appear that, in contrast to the class I alleles, the *iab-7 cis*-regulatory domain is not ectopically activated in all A6 cells in the class II mutants. Interestingly, the morphology of the cuticular structures produced by these groups of cells is different from that expected for A6 or PS11 identity. Usually the tergite patches on the dorsal side are covered by trichomes, and on the ventral side by bristles. Since such structures are not characteristic of A6, but rather of more anterior segments (i.e. A5), this observation would suggest that *iab-6* must also be inactivated in these clones. Thus, unlike the class I mutants where all cells in A6 assume an A7 identity, the A6 cells in the class II mutants can apparently assume either an A7 or an A5 identity.

A boundary and a PRE in the *Fab-7* region

The results described in the previous section suggest that class II mutants have both a gain-of-function and a loss-of-function phenotype in A6. The gain-of-function phenotype is presumably attributable to an ectopic activation of the *iab-7 cis*-regulatory domain in PS11 (A6) cells as is the case for the original *Fab-7^{1}* allele (see Galloni et al., 1993) and for class I alleles

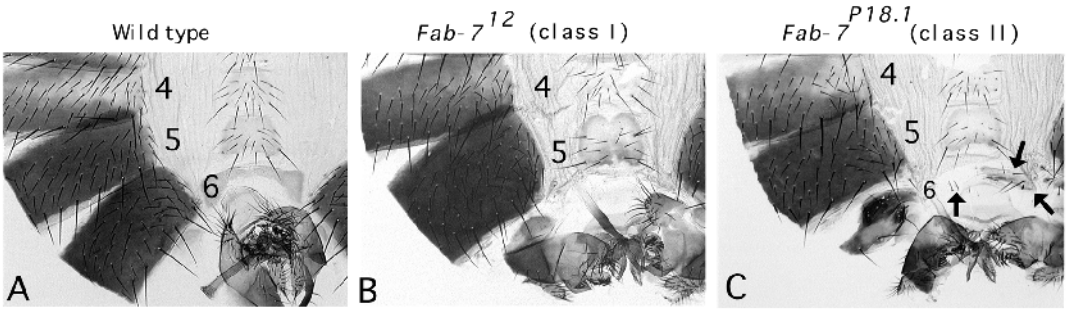

Fig. 2. Adult male phenotypes of homozygous class I and II *Fab-7* mutations. Whole mounts of abdominal male cuticles of wild type (A), *Fab-7*^{12} (B) and *Fab-7*$^{P18.1}$ (C). Male abdomens were cut along the dorsal midline and flattened on a slide. The dorsal surface of each abdominal segment has a rectangular plate of hard cuticle called the tergite. Only half of the tergites of the 4th, 5th and 6th abdominal segments (numbered) are visible on the left of each panel, as well as the genitalia at the bottom. Note that the 5th and 6th tergites are pigmented. The ventral surface of abdominal segments is composed of soft cuticle called the pleura. On the ventral midline of the pleura, there are small plates of harder cuticle called sternites. In wild type, the 6th sternite can be easily distinguished from the more anterior sternites by its different shape and by the absence of bristles. A wild-type male (A) has six abdominal segments. The 7th abdominal segment (A7), present in larvae, is suppressed during metamorphosis. In *Fab-7*^{12} (B), *iab-7* is ectopically expressed in A6. As a consequence, A6 assumes A7 identity, giving rise to a male with only five abdominal segments. In *Fab-7*$^{P18.1}$ (C), ectopic activation of *iab-7* does not occur in all cells, resulting into the differentiation of a 6th tergite of reduced size. Observation of this reduced 6th tergite in pseudo dark field (not shown) reveals that it is covered by trichomes, a characteristic of more anterior tergites. This indicates that *iab-6* is inactive in these cells (since the tergite is darkly pigmented, *iab-5* is active). On the ventral side, clones of sternites also appear in A6 (shown by black arrows), indicating that ectopic activation of *iab-7* did not take place in these cells. Since these sternite clones are covered by bristles, *iab-6* appears to be inactive in these cells.

described here. In contrast, the loss-of-function phenotype is probably due to some type of inactivation or silencing of the *iab-6 cis*-regulatory domain in PS11 (A6) cells. Silencing in the bithorax complex is usually mediated by PREs which provide sites for the assembly of a repressive complex of Polycomb group proteins (for reviews, see Paro, 1990; Pirrotta and Rastelli, 1994; Simon, 1995). Hence, one plausible explanation for the difference between class I and class II alleles is that both the *Fab-7* boundary and an adjacent PRE element are deleted in the former, while only the boundary is missing in the latter, leaving the PRE intact. In class II alleles, the deletion of the boundary may allow the PRE from *iab-7* to inactivate *iab-6*, giving rise to the phenotype observed.

Two predictions can be drawn from this hypothesis. First, we should be able to enhance ectopic activation of *iab-7* and suppress *iab-6* silencing in PS11 of the class II alleles by introducing mutations in the genes encoding *Polycomb* group proteins. By contrast, mutations in *Polycomb* group genes may be expected to have little or no effect on the phenotype of the class I alleles. Second, there should be a discernible pattern in the sequences that are missing in the class I and class II alleles. Both should lack sequences essential for boundary function, while only the class I alleles should delete sequences essential for PRE function. These predictions are tested below.

(1) Class I and Class II mutants respond differently to mutations in the *Polycomb* and *trithorax* group genes

We first asked whether the class I and class II mutants respond differently to mutations in *Polycomb* group genes. Consistent with the first prediction, we find that the gain-of-function transformation of A6 into A7 in animals heterozygous for class II alleles (we tested *Fab-7*^{2}, *Fab-7*^{3}, *Fab-7*^{33}, *Fab-7*^{24}, *Fab-7*^{14}, *Fab-7*^{28}, *Fab-7*^{32}, *Fab-7*^{19}, *Fab-7*$^{P6.1}$, *Fab-7*$^{P18.1}$, *Fab-7*$^{P14.1}$) is strongly enhanced by *Pc-g* mutations like *Pc*^{3}, *Pcl*^{T1}, *Scm*^{D1} and an *Asx* deficiency, *Df (2R) trix.* As illustrated by the cuticle preparations shown in Fig. 3, the enhancement by *Pc-g* mutations often results in a phenotype close to that of class I deletions (compare the abdomen in Fig. 3F to the abdomen in Fig. 3A). In contrast, the gain-of-function phenotype of class I deletions (we tested *Fab-7*^{1}, *Fab-7*^{6}, *Fab-7*^{10}, *Fab-7*^{12}, *Fab-7*^{21} and *Fab-7*$^{P18.1\#9}$) remains unchanged in *Pc-g* mutant backgrounds (Fig. 3E and not shown).

Mutations in the *trx-g* genes are known to antagonize the effect of *Pc-g* mutations. Hence it was of interest to determine whether *trx-g* mutations alter the phenotype of the class I or class II alleles. For this purpose, we introduced *trx* ^{E2} or *brm*^{20} point mutations or the *Df(2R)kto*^{2} into a background heterozygous for representative class I and class II alleles. As illustrated for *trx* in Fig. 3D), the transformation of A6 into A7 in class II alleles is strongly suppressed by mutations in *trx-g* genes. In addition, it appears that *iab-6* inactivation in PS11 cells is enhanced by the *trx-g* mutation. In contrast, the gain-of-function phenotype of class I alleles is unaffected (Fig. 3C).

(2) The deletions in the class I and class II are different

We used a combination of Southern blotting, PCR amplification and DNA sequencing to characterize the sequence organization of the *Fab-7* region in five of the six class I mutants and in eleven of the twenty class II mutants. As illustrated diagrammatically in Fig. 1B, the results of this analysis are consistent with the second prediction, namely that the deletions in the class I and class II mutants remove overlapping but distinct DNA sequences.

All five class I mutants delete sequences on both sides of the *blt* transposon. The largest of these removes sequences spanning the four nuclease hypersensitive sites (*, HS1, HS2 and HS3), while the three intermediate deletions remove hypersensitive sites HS1, HS2 and HS3. The smallest of the class 1 deletions, *Fab-7*$^{P18.1\#9}$, removes the edge of HS1, and all of HS2 and HS3. This class I allele is somewhat unusual because the transformation of A6 is slightly weaker than in *Fab-7*^{1} or in others members of this class (data not shown).

While the class I mutations remove sequences located to either side of the *blt* insertion site, all eleven of the class II

mutations examined have a common distal end point at the transposon insertion site and only remove sequences proximal to it. The smallest of the class II alleles, *Fab-7* $^{P6.1}$, deletes 510 bp and this includes all of HS2 and part of HS1. The largest, *Fab-7*^{19}, deletes 1262 bp and this includes not only HS1 and HS2, but also the minor proximal hypersensitive site (*; see Fig. 1B).

Taken together with the phenotypes of class I and class II alleles in wild type and *Polycomb* mutant background, this analysis defines two distinct functional elements in the *Fab-7* region. The first is the *Fab-7* boundary. This element is defined by the DNA sequences that are deleted in both the class I and class II alleles, and would correspond to hypersensitive sites HS1 and HS2 (at the minimum). The second is an *iab-7* PRE. This element is only deleted in the class I alleles and would correspond to hypersensitive site HS3 (at the minimum).

Isolation of deletions that remove HS3

In our screen for *blt* derivatives that were *ry*$^{-}$ and had *Fab-7*-like gain-of-function phenotype, we did not recover any deletions that only removed DNA sequences from the region immediately distal to the site of insertion of the *blt* element (i.e., the *iab-7* PRE). Based on the results described in the previous sections, a plausible hypothesis would be that such deletions would not produce a dominant *Fab-7*-like gain-of-function phenotype. To test this hypothesis, we used a PCR-based screen to identify *blt* derivatives that have lost sequences distal to the transposon insertion site. In order to prevent gap repair of imprecise excision by the homologous chromosome, we mobilized the *blt* transposon over the *Fab-7*^{1} deletion (Hogga and Karch, 1995). Individual lines that had lost the *rosy*$^{+}$ marker were established and analyzed by PCR. Out of 334 excision events, we recovered 15 independent class III deletions that remove sequences distal to the *blt* transposon. Fig. 1B shows six class III alleles whose deletion endpoints have been determined by DNA sequence analysis. The smallest of these is 450 bp and it removes most of HS3. The largest is 1500 bp and it removes HS3 plus a large distal flanking region.

In contrast to class I and class II alleles, which cause a dominant transformation in segment identity, the class III mutants have no detectable phenotype as heterozygotes. This is also true for homozygous class III mutant animals; most of the homozygous flies have no visible phenotype (Fig. 4A). However, we do occasionally observe homozygous males in which small patches of the 6th tergite are missing, indicating a very weak *Fab-7* transformation. The penetrance of this phenotype is very low (about 1/40). In addition, in complementation tests, the *Fab-7* phenotype of class I and II mutations is enhanced by the class III deletions. These results indicate that the class III deletions are partially defective in maintaining the inactive state of *iab-7* in A6/PS11. However, since the majority of homozygous flies have no phenotype, *iab-7* usually remains inactive in segments anterior to A7/PS12 of class III deletions, suggesting that there are additional PRE-like elements within the *iab-7 cis*-regulatory domain.

The penetrance of the weak *Fab-7* phenotype of *iab-7PRE*^{56} increases to about 1/10 (data not shown) when heterozygous with *iab-7*^{Sz}, a deletion that remove DNA from the insertion site of the *blt* transposon to position +143 kb (Galloni et al., 1993;

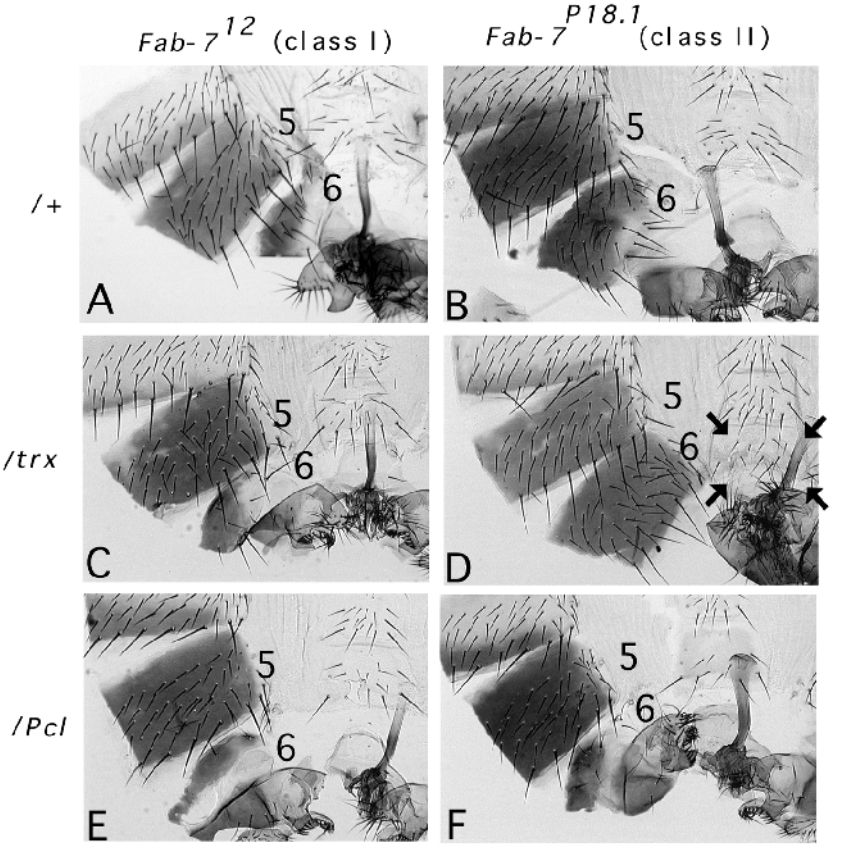

Fig. 3. Interactions of class I and III *Fab-7* deletions with mutations in *Pc-g* and *trx-g*. The cuticles of the respective genotypes were mounted as in Fig. 2. (A,B) As heterozygotes, the *Fab-7*^{12} and *Fab-7*$^{P18.1}$ mutations have weaker *Fab-7* phenotypes than their homozygous counterparts. This is visible by comparing the size of the 6th tergites shown in A and Fig. 2B for *Fab-7*^{12}, and the size of the 6th tergites shown in B and Fig. 2C for *Fab-*$^{P18.1}$. (C,D) The effect of heterozygote *trx* mutation, *Df(3R)red,trx*. (C) The *trx* mutation has no effect on the strength of the *Fab-7*^{12}/+ phenotype. This is visible by the size of the 6th tergite, which remains about the same as in A. In contrast, heterozygote *trx* mutation has a strong suppressor effect on the phenotype of *Fab-7*$^{P18.1}$/+ as can be seen by the size of the 6th tergite, which becomes almost wild type. Moreover, the presence of bristles on the 6th sternite (shown by arrows) indicates a transformation into a copy of the 5th sternite. (E,F) The effect of heterozygote *Pcl* mutation, *Pcl*^{T1}. As for *trx*, mutation in *Pcl* does not appear to have an effect on the class I *Fab-7*^{12} deletion. This is visible by the size of the 6th tergite which remains about the same as in A. However, mutation in *Pcl* seems to enhance the *Fab-7*$^{P18.1}$/+ phenotype as revealed by the size of the 6th tergite, which is reduced compared to B. Note that in a wild-type background, *trx* heterozygotes have partial loss-of-function phenotypes of the BX-C. In the posterior abdomen, there is a weak *iab-5* phenotype which is visible in C and D by a partial loss of pigmentation on the 5th tergite. There is also a weak *iab-6* phenotype revealed by the occasional appearance of one or two bristles on the 6th sternite as can be seen in C.

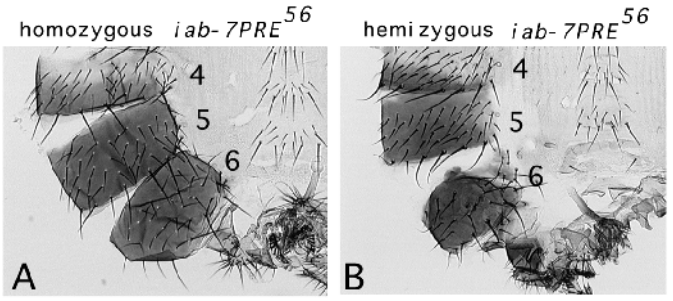

Fig. 4. Phenotypes of males homozygous and hemizygous for *iab-7PRE* deficiencies. The cuticles of the respective genotypes were mounted as in Fig. 2. (A) The cuticle of a homozygous *iab-7PRE*^{56} male. There is no apparent phenotype and this cuticle is similar to the wild-type cuticle shown in Fig. 2A. (B) The cuticle from a hemizygous male, *iab-7PRE*^{56}*/Df(3R)P9*. Hemizygosity for the bithorax complex causes few partial loss-of-function phenotypes due to haplo-insufficiency. For example *iab-5* haplo-insufficiency is visible by a partial loss of pigments on the 5th tergite. Moreover, the presence of two bristles on the 6th sternite is due to *iab-6* haplo-insufficiency. In addition to these loss-of-function phenotypes, *iab-7PRE*^{56} hemizygotes also show an opposite gain-of-function transformation in which *iab-7* appears to be ectopically activated in A6. This is visible by the reduced size of the 6th tergite.

Fig. 1A). This result would indicate that, in class III deletions, the absence of the homologous *iab-7* region in *trans* affects the maintenance of the repressed state of *iab-7* in PS11. Intriguingly, we find that the neighboring *cis*-regulatory regions may also contribute to the stability of inactive state, because the *Fab-7* phenotype is greatly enhanced when the *iab-7PRE*^{56} class III deletion is in *trans* with a deficiency of the entire BX-C (*Df(3R)P9*; Lewis, 1978; Karch et al., 1985). This is illustrated in Fig. 4B, which shows a hemizygous male (*iab-7PRE*^{56}*/Df(3R)P9*) in which the size of the 6th tergite is reduced.

Fab-7 deletions that retain the *bluetail* transposon

In addition to screening for dominant gain-of-function mutations, we also screened for recessive loss-of-function mutations. For this purpose, we crossed the mobilized *blt* chromosomes to a deficiency that removes the *iab-4*, *iab-5* and *iab-6 cis*- regulatory domains (*iab-4,5*^{DB} ; Karch et al., 1985; Duncan, 1987). We recovered a variety of DNA lesions, some of which delete extensive segments of the *iab-6 cis*-regulatory domain (data not shown). However, of most interest here are three derivatives, *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$, that remove DNA sequences to the proximal side of the transposon insertion site (towards *iab-6*, see Fig. 5) but leave the *blt* transposon intact. The size of these deletions are 510, 594 and 1041 bp, respectively. These three *blt* derivatives have two interesting features. The first is their *lacZ* expression pattern, while the second is their phenotype. Each feature is considered below.

(1) *lacZ* expression pattern

The deletion of sequence proximal to the transposon in *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$ alters the expression pattern of the *lacZ* reporter gene carried by the *blt* transposon. In the original *blt* line, only the *iab-7* cis-regulatory region is able to regulate the transgene, presumably because the *Ubx* promoter of the *lacZ* reporter gene is protected from the regulatory influences of *iab-6* (and more proximal *cis*-regulatory regions) by the *Fab-7* boundary element immediately proximal to the transposon (see above and Galloni et al., 1993). As a consequence, *lacZ* expression pattern is restricted to PS12 and more posterior parasegments (Fig. 5). In *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$, however, the anterior border of *lacZ* expression is shifted to PS11 (Fig. 5), indicating that the *lacZ* reporter gene can now be activated by *iab-6*. This observation strengthens the argument that the *Fab-7* element, defined by the hypersensitive sites HS1 and HS2 (and ∗), functions as a chromatin boundary element which can block enhancer-promoter interactions. In the line carrying the largest deletion (*iab-6,7*$^{P14.1}$), the pattern of *lacZ* expression is equivalent in PS11 and PS12. This suggests that all of the sequences required for full enhancer blocking activity have been removed in this mutant, allowing the *Ubx* promoter of the reporter gene to be equally regulated by both *iab-6* and *iab-7* (Fig. 5). In contrast, the two smaller deletions (*iab-6,7*$^{P6.1}$ and *iab-6,7*$^{P18.1}$) appear to retain some residual enhancer blocking activity as *lacZ* expression in PS11 is weaker than in PS12.

(2) Phenotype of the deletions

As described above, deletions that remove all or part of the *Fab-7* boundary element cause the transformation of A6 (PS11) into A7 (PS12). However, *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$ are unusual in that they cause the opposite *iab-6*$^{-}$ and *iab-7*$^{-}$ loss-of-function phenotypes as revealed by the presence of bristles on the 6th sternite and of a small 7th tergite (Fig. 6). An obvious possibility is that these deletion mutants deviate from the expected *Fab-7*-like phenotype because they retain the 14 kb *blt* transposon. To test this possibility, we mobilized the *blt* transposon again and recovered lines in which the P element was precisely excised. In each case, a *Fab-7* phenotype similar to that of *Fab-7*^{2} or other class II alleles (see above) was observed. Representatives of the new lines were selected and named *Fab-7*$^{P6.1}$, *Fab-7*$^{P18.1}$ and *Fab-7*$^{P14.1}$, respectively.

What is the origin of the *iab-6*$^{-}$ and *iab-7*$^{-}$ loss-of-function phenotype of these *blt* derivatives? The original *blt* line has a weak *iab-7*$^{-}$ phenotype, which is revealed in homozygous males by the presence of a rudimentary tergite in A7 (Fig. 6A). This phenotype is most probably due to a competition between the promoter(s) in the transposon and that of the *Abd-B* gene for the enhancers in *iab-7* (Galloni et al., 1993). A similar explanation could account for the *iab-6* (and *iab-7*) loss-of-function phenotype of the *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$ mutants. In these *blt* derivatives, the deletion of the *Fab-7* boundary element exposes the *Ubx*, *rosy* and P promoters in the *blt* transposon to enhancers in the *iab-6 cis*-regulatory region, thereby allowing them to compete with *Abd-B*. This view is consistent with the finding that the *lacZ* reporter gene is activated in PS 11 (Fig. 5; see above).

Sequences in the blt transposon prevent ectopic activation of iab-7 in the absence of the Fab-7 boundary

The presence of the 14 kb long *blt* transposon in *iab-6,7*$^{P6.1}$, *iab-6,7*$^{P18.1}$ and *iab-6,7*$^{P14.1}$ prevents ectopic activation of the *iab-7 cis*-regulatory region in PS11, as revealed by the complete absence of transformation of A6 into A7 in these lines. This indicates that, in the absence of *Fab-7* sequences, the transposon itself is able to substitute for the boundary function. Three mechanisms can be proposed to account for this activity. First,

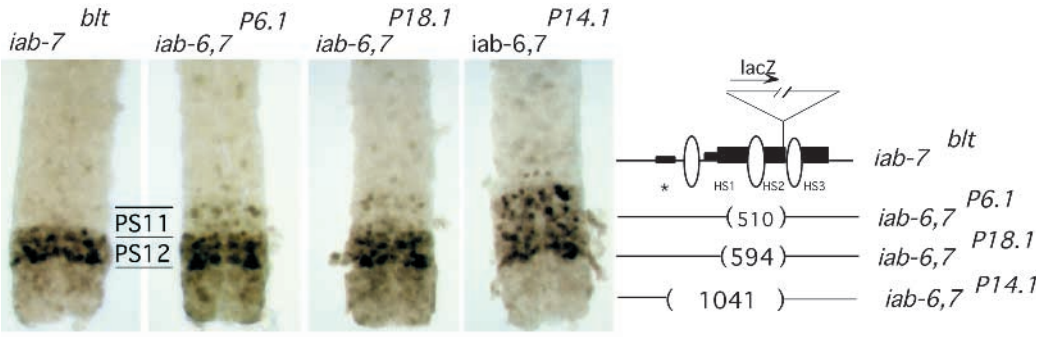

Fig. 5. *lacZ* expression in the central nervous system of *iab-7^{blt}*, *iab-6,7$^{P6.1}$*,*iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$*. Ventral cord dissected out from stage 14 embryos stained with anti-β-galactosidase antibody using peroxidase-coupled secondary antibody. All ventral cords are derived from homozygous embryos of the genotypes indicated. Parasegmental boundaries are shown. The panel on the right shows the molecular lesions in *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$*. The thin horizontal line represents the genomic DNA of the *Fab-7* region as in Fig. 1B. The position of the *blt* transposon is drawn. The sizes of the respective deletions is indicated in nucleotides between the brackets.

it is possible that the interaction between the *iab-6 cis*-regulatory subregion and the *Ubx*, *rosy* and P promoters of the *blt* transposon can somehow interfere with the ectopic activation of *iab-7*. Second, the *blt* transposon itself may contain a boundary element. Of particular concern here is the presence of a 1 kb long DNA fragment derived from the middle of the *iab-7 cis*-regulatory domain (position +143 to +144 of the BX-C walk; see Galloni et al., 1993). This 1kb *iab-7* fragment is inserted just upstream from the *Ubx* promoter and, considering the orientation of the *blt* transposon within the *Fab-7* boundary, it is juxtaposed to the *iab-6 cis*-regulatory domain. Finally, the length of the *blt* transposon may play the role of boundary due to distance effect. To try to distinguish between these three possibilities, we have analyzed derivatives of *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$* in which the *blt* transposon is partially excised.

Altogether, we characterized 70 independent derivatives that had lost different parts of the *blt* transposon. Of these, we recovered 37 lines that had lost the insulation activity of the transposon as judged by their *Fab-7*-like gain-of-function phenotype. Nearly half of these (16) had an insert of less than 1 kb long and did not contain the 1 kb *iab-7* fragment. The other 21 lines had inserts of more than 1 kb. The 1 kb *iab-7* fragment was retained in only 3 of these 21 lines. 33 of the lines exhibited no detectable gain-of-function phenotype even though they had internal deletions of the *blt* transposon. The majority of these lines had inserts of more than 4 kb in length and 29 of them contained the 1 kb *iab-7* fragment. Although this analysis does not allow us to pinpoint which sequences in the transposon are capable of conferring insulating activity, we can draw several tentative conclusions. First, promoter interference is unlikely to be responsible for the insulating activity as we recovered deletion derivatives that had a wild-type phenotype (i.e. did not exhibit a *Fab-7*-like phenotype) which had lost both the *Ubx* and *rosy* promoters. Second, insulating activity may be generated by two different mechanisms. The first is length. DNA fragments larger than about 4-5 kb appear to be capable of preventing the ectopic activation of *iab-7* in A6 (PS11). The second is the presence of the 1 kb *iab-7* fragment. In most case in which insulating activity was retained, the 1 kb *iab-7* fragment was still present.

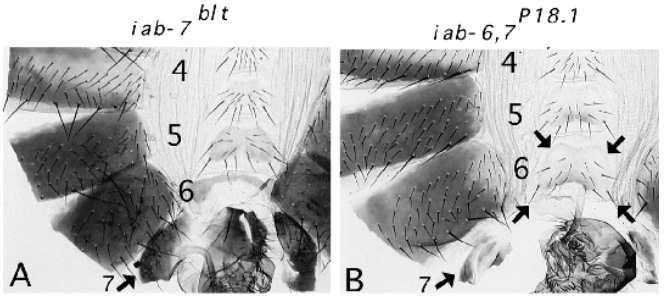

Fig. 6. Homozygous *iab-7^{blt}* and *iab-6,7$^{P18.1}$* males. The cuticles of the respective genotypes were mounted as in Fig.2. (A) Cuticle from a homozygous *iab-7^{blt}* male. As explained in the legend of Fig. 2, the activity of *iab-7^{+}* in males suppresses the development of a 7th abdominal segment. Thus inactivation of *iab-7* leads to the appearance of a 7th abdominal segment, which develops as a copy of A6. In *iab-7^{blt}*, inactivation of *iab-7* is not complete, leading to the appearance of only a small 7th tergite (indicated by an arrow in A). (B) A cuticle from a homozygous *iab-6,7$^{P18.1}$* male. In addition to the presence of a small 7th tergite, this male cuticle also contains a 6th sternite covered by bristles, indicating a transformation of A6 into A5 (*iab-6* phenotype).

DISCUSSION

Parasegment identity in the posterior two thirds of the fruit fly *D. melanogaster* is determined by the precise pattern of expression of the three homeotic genes from the bithorax complex. The expression of the relevant homeotic gene in each parasegment is controlled by a parasegment-specific *cis*-regulatory domain (for review, see Peifer et al., 1987). Early in development, initiation elements within each regulatory domain are responsible for directing the spatial and temporal pattern of homeotic gene expression appropriate for the specific parasegment (Simon et al., 1990; Qian et al., 1991; Muller and Bienz, 1992; Shimell et al., 1994). Once the appropriate expression pattern has been established, maintenance elements within each regulatory domain are responsible for sustaining this pattern during subsequent stages of development (for reviews, see Paro, 1990; Simon et al., 1995). Critical to generating patterns of homeotic gene expression that are specific to each parasegment are mechanisms which ensure the functional autonomy of the individual *cis*-regulatory domains in the bithorax complex. One mechanism that seems to play an important role in shielding *cis*-regulatory domains from the regulatory influences of adjacent domains is the insulating activity of chromatin domain boundaries. Genetic studies have uncovered two unusual elements in the BX-C that are good candidates for chromatin domain bound-

aries, *Mcp* and *Fab-7* (Gyurkovics et al., 1990; Galloni et al., 1993; Karch et al., 1994). In the studies reported here, we have undertaken a functional dissection of the *Fab-7* region of the BX-C. Our results define the sequences required for *Fab-7* boundary function within the context of the BX-C and clarify how this boundary element and a nearby PRE contribute to the parasegment-specific regulation of the *Abd-B* homeotic gene (see Fig. 7).

An 'in situ enhancer blocking' assay

Two different but complementary assays have been used to identify DNA elements that have properties that might be expected for chromatin domain boundaries or insulators. The first assay tests whether the element is able to insulate a reporter gene against chromosomal position effects (Kellum and Schedl, 1991; Roseman et al., l993). The second assay tests whether the putative boundary element can block enhancer-promoter interactions when interposed between the enhancer and the promoter (Kellum and Schedl, 1992; Chung et al., 1993). Both of these assays assess the insulating activity of a putative boundary element in a context that is quite different from the context in which the element must normally function.

In the present studies, we have defined the sequences required for *Fab-7* boundary function using what could be characterized as an 'in situ enhancer blocking' assay. For this purpose, we have taken advantage of a P-element reporter, *blt*, inserted in the BX-C at the edge of the *iab-7 cis*-regulatory domain just distal to the sequences thought to be essential for *Fab-7* boundary function. The *Ubx-lacZ* reporter in this transposon is expressed in PS12 and more posterior parasegments, but not in PS11 or in more anterior parasegments. Based on this *lacZ* expression pattern, we proposed that the *blt* transposon is subject to regulatory elements in the *iab-7 cis*-regulatory domain, but is insulated from the effects of regulatory elements in the proximal *iab-6 cis*-regulatory domain by the intervening *Fab-7* boundary element (Galloni et al., l993; see Figs 1 and 5).

If this hypothesis is correct, that is, if the *Fab-7* boundary is 'blocking' *iab-6* regulatory elements from interacting with the *blt lacZ* reporter, then it should be possible to activate *lacZ*

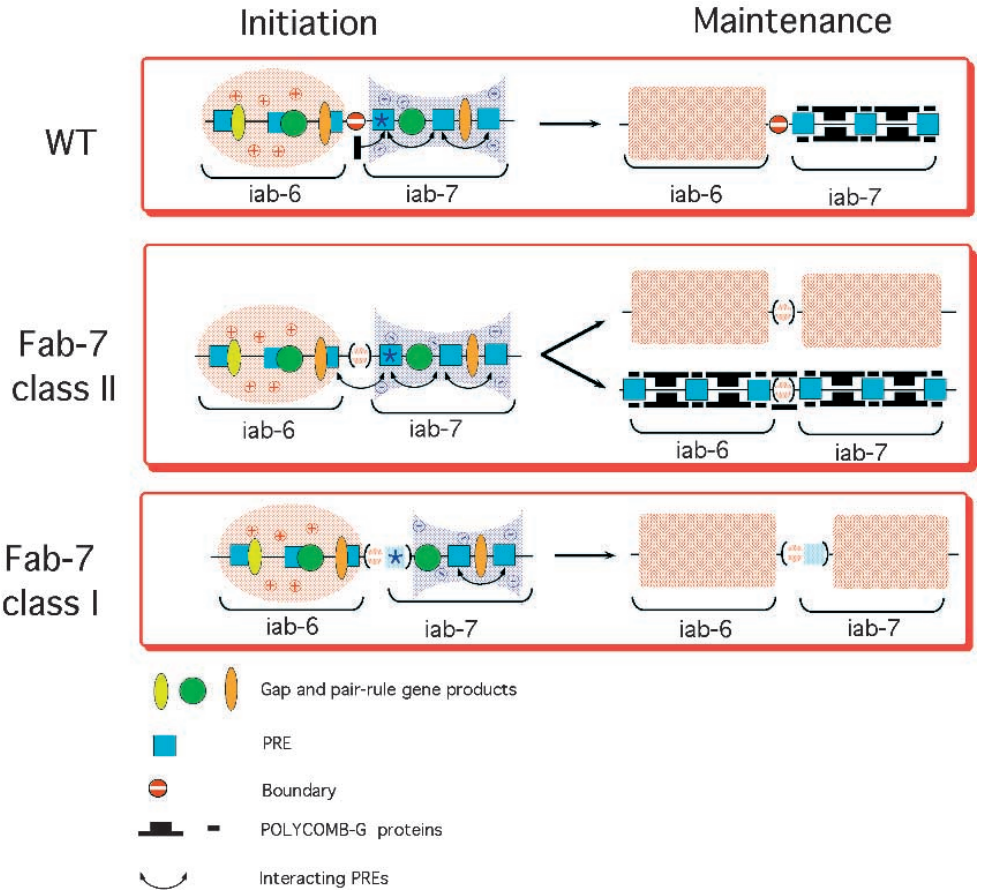

Fig. 7. Schematic representation of the function of the *Fab-7* boundary in insulating *iab-6* and *iab-7*. Activity of the *iab-6* and *iab-7 cis*-regulatory domains in PS11 at initiation and maintenance phases are shown. Early in development, when segmental identity is initially selected, the combination of gap and pair-rule gene products expressed in PS11 (shown in yellow, green and orange) results in the activation of the *iab-6* domain (represented by + in a pink background). The *iab-7* domain, however, remains inactive (represented by – in a bluish background). A first function of the boundary element is to prevent spreading of the active state of *iab-6* into *iab-7*. Because *iab-7* is inactive, the PREs (shown by blue rectangles) are accessible to the *Pc-g* silencing complex, which will then assemble on the *iab-7* domain (shown by black arrows). A second function of the boundary element is to prevent the *iab-7* PRE (shown by a star in a blue rectangle) from interacting with adjacent PREs in *iab-6*. Note that the number and positions of PREs drawn in *iab-6* and *iab-7* are arbitrary except for the *iab-7* PRE immediately distal to the boundary. In the maintenance phase, after gap and pair-rule gene products have decayed, the active state of *iab-6* (shown by a reddish rectangle) is sustained by the activity of the products of the *trx-g.* Meanwhile, the *iab-7* domain is permanently repressed by the *Pc-g* silencing complex that has assembled on the PREs. The *Fab-7* boundary (one-way sign) is responsible for the limit between active and inactive states. In *Fab-7* class II deletions, removal of the boundary alone (shown by brackets) allows activating as well as repressing signals to travel between *iab-6* and *iab-7*. On the one hand, active state of *iab-6* can invade the *iab-7* domain, leading to ectopic activation of *iab-7* in PS11. On the other hand, we envision that the most proximal PRE of *iab-7* (the *iab-7*PRE) can interact with the most distal PRE of *iab-6*, leading to the inactivation of *iab-6* by the *Pc-g* silencing complex. Thus cells in PS11 have either both *iab-6* and *iab-7* active, or both of them repressed. In *Fab-7* class I deletions, the boundary as well as the *iab-7* PRE are deleted (shown by brackets). The fact that *iab-7* is always ectopically activated in class I alleles can be explained by the assumption that the lack of a PRE in *iab-7* weakens the assembly of the *Pc-g* silencing complex on *iab-7*, allowing the active state of *iab-6* to invade *iab-7*. It is also possible that the remaining PREs in *iab-7* are too remote to interact with PREs in *iab-6*.

expression in PS11 by removing the boundary. Our results demonstrate that this is the case. We recovered three derivatives of *blt*, *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$*, in which the transposon is retained after mobilization while sequences immediately proximal to the transposon are deleted. As predicted, *lacZ* expression is activated in PS11 in all three derivatives. Moreover, the level of *lacZ* expression in PS11 is correlated with the size of the deletions. In *iab-6,7$^{P14.1}$* the level of *lacZ* expression in PS11 is close to that in PS12. This is the largest deletion, removing nuclease hypersensitive sites HS2 and HS1 and most of the proximal minor hypersensitive site (see Figs 1 and 5). These deleted sequences would presumably correspond to the 'maximum limits' of the *Fab-7* element required for blocking interactions between the *iab-6* regulatory domain and the *blt* reporter. In the two smaller deletions, *iab-6,7$^{P6.1}$* and *iab-6,7$^{P18.1}$*, the level of *lacZ* expression in PS11 is less than in PS12. These deletions remove HS2 and only a part of HS1. Since boundary function is compromised in these smaller deletions, the deleted sequences must be important for full activity. On the contrary, since the *lacZ* expression in PS11 is less than in PS12, it would appear that some boundary function must be retained in the two deletions. This would argue that sequences proximal to the endpoints of *iab-6,7$^{P6.1}$* and *iab-6,7$^{P18.1}$* also contribute to the blocking activity of the *Fab-7* boundary.

Further evidence that sequences critical for *Fab-7* boundary function are deleted in *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$* comes from the loss-of-function phenotypes associated with these three *blt* derivatives. The original *blt* line has a weak *iab-7* loss-of-function phenotype (Fig. 6A). This *iab-7* phenotype appears to be due to a competition, in PS12 cells, between promoters in the *blt* transposon (the *Ubx*, *rosy* and P-element promoters) and the *Abd-B* promoter for interactions with regulatory elements within the *iab-7 cis*-regulatory domain (Galloni et al., 1993). On the contrary, in PS11 cells, the regulatory elements in *iab-6* are apparently insulated from this promoter competition and are able to interact with the *Abd-B* promoter without interference. If the *Fab-7* boundary is responsible for insulating regulatory elements in the *iab-6* domain from the promoters of the *blt* transposon, then the effects of promoter competition should be evident in PS11 cells when the boundary, but not the *blt* transposon is removed. Consistent with this hypothesis, we find that *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$* all have an *iab-6* loss-of-function phenotype (Fig. 6B).

The conclusions that we have drawn from our in situ analysis of the *Fab-7* boundary are supported by the work of Hagstrom et al. (1996). These authors used conventional 'enhancer blocking' assays (in which putative boundary elements are interposed between an enhancer and a promoter in a transgene reporter construct) to demonstrate that sequences from the *Fab-7* region have boundary function. In these conventional enhancer blocking assays, the sequences required for full blocking activity extend from the edge of the minor proximal nuclease hypersensitive site through hypersensitive sites HS1 and HS2 (Hagstrom et al., l996). This corresponds closely (within 62 bp) to the sequences that appear to be essential for full 'enhancer blocking' as measured in our in situ assays by the pattern of *lacZ* expression in PS11. This is also in agreement with similar exepriments performed by Zhou et al. (1996).

The *blt* transposon can substitute for the *Fab-7* boundary function

Although sequences essential for *Fab-7* boundary function are apparently deleted in the *blt* derivatives *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$*, the (gain- and loss-of function) phenotypes normally observed when the *Fab-7* boundary is removed are not evident in these lines. A plausible explanation for this unexpected finding is that the *blt* transposon somehow substitutes for *Fab-7*, blocking adventitious interactions between regulatory elements in the *iab-6* and *iab-7 cis*-regulatory domains. Consistent with this possibility, a clean excision of the *blt* transposon in the *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$* lines produces the expected *Fab-7* class II phenotype. From our analysis of imprecise excision derivatives of *iab-6,7$^{P6.1}$*, *iab-6,7$^{P18.1}$* and *iab-6,7$^{P14.1}$*, it would appear that at least two factors may be important for the 'artificial' insulating activity of the *blt* transposon. The first is the presence of a foreign DNA fragment of sufficient length. In general, imprecise excision derivatives that retained more than 4 kb of the *blt* transposon exhibited at least some insulating activity. The second was the presence of the 1 kb sequence from the distal end of *iab-7*. Most of the lines that showed 'artificial' insulating activity retained this sequence.

Function of the *Fab-7* boundary in the regulation of BX-C gene activity

While the *Fab-7* boundary can block interactions between regulatory elements in the *iab-6 cis*-regulatory domain and the promoters in the *blt* transposon, this is not its normal function in the regulation of BX-C gene activity. Critical to understanding the contributions of the *Fab-7* boundary to the specification of parasegment identity are the phenotypic effects of deletions that remove the boundary and/or adjacent regulatory elements. Our deletion mutants can be divided into three classes (I-III) based on the location of their end-points and on the nature of their phenotypic effects.

The class II deletions remove all of nuclease hypersensitive site HS2 plus part or all of the HS1 and the minor more proximal hypersensitive site. These sequences are essential for boundary function as defined either by the in situ 'enhancer blocking' assay described here or by conventional transgenic enhancer blocking assays (see above and Hagstrom et al., 1996; Zhou et al., 1996). The class II boundary deletions are dominant and have a complex gain- and loss-of-function phenotype in PS11 (A6). On the one hand, they can cause an ectopic activation of *iab-7* in PS11 cells, transforming these cells from a PS11 to a PS12 identity (A6→A7). On the other hand, less frequently, the boundary deletions cause silencing of *iab-6* in PS11 cells (in this case *iab-7* is off as well), leading to a transformation from a PS11 to a PS10 identity (A6→A5). The relative balance between the gain- and loss-of-function phenotypes in PS11 (A6) depends upon products of the *Polycomb* and *trithorax* groups. Mutations in *Polycomb* group genes (as heterozygotes) shift the balance towards the gain-of-function phenotype, while mutations in the *trithorax* group genes (also as heterozygotes) shift the balance in the opposite direction.

The class I deletions also remove the *Fab-7* boundary; however, they do not have the gain- and loss-of-function phenotypes in PS11 (A6) that are evident in the class II deletions. Instead, there is a complete transformation of PS11 (A6) into a

copy of PS12 (A7). Mapping of the deletion endpoints indicates that the critical difference between the class II and class I alleles is the presence or absence of sequences corresponding to nuclease hypersensitive site HS3. Recent studies by Hagstrom et al. (in press) indicate that HS3 defines an *iab-7* PRE. These authors found that an 800 bp fragment containing HS3 (plus sequences immediately distal to HS3) can mediate the pairing-dependent silencing of *mini-white* and can function to maintain a parasegmentally restricted pattern of expression of a *Ubx-lacZ* reporter when combined with a *bxd* initiation element. Both of these PRE activities are dependent upon *Polycomb* group genes. Our analysis of the effects of *Polycomb* and *trithorax* group mutations on the class I deletions provides further support for the idea that HS3 corresponds to a PRE. We found that in contrast to class II mutations, the deletion of HS3 renders class I mutations insensitive to a reduction in the activity of either the *Polycomb* or *trithorax* group genes.

Finally, the class III alleles only remove the HS3 *iab-7* PRE. Critical for the present discussion is the fact that class III alleles typically have no phenotypic effects. Whereas deletions that remove the *Fab-7* boundary affect the development of PS11 (A6) even as heterozygotes, the class III alleles are essentially wild type not only as heterozygotes but also as homozygotes. This finding resolves a controversy that has arisen regarding the nature of the *Fab-7* mutations. Based on their analysis of transgenes containing DNA fragments from the *Fab-7* region that include HS3 sequences, Busturia and Bienz (1993) and Zink and Paro (1995) have suggested an alternative explanation for the phenotypic effects of the *Fab-7* deletions. They have proposed that *Fab-7* is a silencer or PRE, not a boundary. In this model, the ectopic activation of *iab-7* in PS11 would be explained by the fact that a silencer or PRE, which normally keeps *iab-7* off in this parasegment, is deleted. However, since the silencer/PRE uncovered by these authors in their transgene experiments has been localized to HS3 sequences (Hagstrom et al. in press) and the removal of these sequences has essentially no phenotypic effects, this model can be discounted. The role of the HS3 *iab-7* PRE in BX-C regulation will be discussed further below.

How does the *Fab-7* boundary function in the parasegmental regulation of *Abd-B*? Our results indicate that the *Fab-7* boundary plays a critical role in ensuring the functional autonomy of the *iab-6* and *iab-7 cis*-regulatory domains. During the initiation phase of BX-C regulation, *Fab-7* prevents adventitious interactions between positive and negative regulatory elements in the *iab-6* and *iab-7* domains (see Fig. 7). This enables the gap and pair-rule genes to select the activity state of the *iab-6* and *iab-7 cis*-regulatory domains in PS11 and PS12. As a consequence, *Abd-B* is expressed in the appropriate parasegment-specific pattern in PS11 and PS12. Once the products of the gap and pair-rule genes in each parasegment have selected the activity state of the *cis*-regulatory domains, BX-C regulation switches to the maintenance system. In PS11, where *iab-6* is activated while *iab-7* is not, the maintenance system must keep *iab-7* off. This is presumably accomplished by 'activating' PREs (including the HS3 *iab-7* PRE) in the *iab-7 cis*-regulatory domain, leading to the assembly of a *Polycomb* group protein silencing complex. The *Fab-7* boundary must prevent this *iab-7* silencing complex from nucleating the assembly of a *Polycomb*-group protein complex in *iab-6*. This is presumably accomplished by blocking interactions between the *iab-7* PREs and the *iab-6 cis*-regulatory domain (see Fig. 7). In PS12, where *iab-7* is activated, the on state must be maintained (perhaps through the action of proteins encoded by members of the *trithorax* group).

When the boundary is deleted (as in class II alleles) the *iab-6* and *iab-7 cis*-regulatory domains fuse into a single domain. The fusion of the domain results in the loss of parasegmental specificity. There is a competition in PS11 cells between the ectopic activation of the fused domain by positive regulatory elements in *iab-6* and silencing of the domain by negative regulatory elements in *iab-7*. If the fused domain is activated, then the *Abd-B* expression in PS11 is driven primarily by *iab-7* and is PS12-like. If the fused domain is inactivated, turning off both *iab-6* and *iab-7*, then *Abd-B* expression is driven by *iab-5* and is PS10-like.

Role of the HS3 *iab-7* PRE

To our knowledge, the class III alleles represent the first documented examples of mutations in a PRE from the BX-C. These mutations have a number of interesting features. First, unlike the boundary mutants, the HS3 *iab-7* PRE deletions are not dominant, and typically have no phenotypic effects as homozygotes. This would suggest that the *iab-7 cis*-regulatory domain must contain additional PRE-like sequences that can maintain the inactive state in the absence of the HS3 *iab-7* PRE. This suggestion is in good agreement with the recent model of Poux et al. (1996), which proposed that several PREs of varying strength help to stabilize the repressed state of *Ubx*. Second, although most class III animals are wild type, we infrequently observe a weak transformation in PS11 (A6) of the sort that would be expected for a failure to properly maintain *iab-7* in the off state. This finding provides strong support for our contention that the HS3 PRE is actually one of the PREs in the *iab-7 cis*-regulatory domain. Additionally, it implies that the HS3 *iab-7* PRE does play an important role in maintaining the silenced state of *iab-7*. This suggestion is supported by the finding that the class III deletions can enhance the gain-of-function phenotypes of class I and class II deletions in *trans*. Third, the silenced state of *iab-7* can be stabilized by the presence of a wild-type copy of BX-C in *trans*. This is illustrated by the finding that the frequency of weak transformations in PS11 increases substantially when the class III deletions are in *trans* to deletions that removes part or all of the BX-C. The stabilization of the silenced state by a wild-type copy of BX-C in *trans* would argue that chromosome pairing may help the *Polycomb* -group proteins to maintain a repressed chromatin structure. In this context, it is interesting to note that the silencing of *mini-white* by PREs is greatly enhanced when the *mini-white* transgenes carrying the PREs are paired (Kassis, 1994; Chan et al., 1994; Gindhart and Kaufman, 1995; Kapoun and Kaufman, 1995; Hagstrom et al., 1997). Further studies will be required to understand how this occurs.

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