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

Jozsef Mihaly¹, Ilham Hogga¹, Janos Gausz², Henrik Gyurkovics²,* and François Karch¹,*

¹Department of Zoology and Animal Biology, University of Geneva, 30 quai E. Ansermet, 1211 Geneva 4, Switzerland
²Institut of Genetics, Biological Research Center, Hungarian Academy, H-6701 Szeged, Hungary

*Authors for correspondence (e-mail: Karch@sc2a.unige.ch: sipi@everx.szbk.u-szeged.hu)

**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). Deletions 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-7S*; 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 segment 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.,...
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-71 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-71 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-71 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 blueset 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 blueset transposon. This prediction was confirmed by mobilizing the blueset 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 blueset 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 blueset 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 blueset 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 btl transposon

Several schemes were used to recover excisions of the blueset transposon. In some instances, the source of transposase was provided by a TM3 balancer chromosome carrying P(ry)2Δ2-31 (Reuter et al., 1993).

In the set of experiments in which we recovered the iab-6.7Ps1, iab-
Fab-7 contains a boundary and a PRE

6,7P14.1 and iab-6,7P18.1 derivatives, we had crossed dysgenic males iab-7B/+;TM3,P[(ry)+]D2-3 to iab-4,5D0/TM3 females. The iab-4,5D0 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-4,5D0 males associated with visible homeotic transformations were crossed again to iab-4,5D0/TM3 females to establish stocks. Since both the iab-4,5D0 and TM3 chromosomes carry the recessive marker p3, 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.

![Diagram of the BX-C region showing the homeoboxes and regulatory elements.](image)

**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 \textit{blt} transposon from the \textit{iab-6}, \textit{iab-7}, \textit{iab-8}, \textit{iab-9}, and \textit{iab-10} lines, dysgenic males were generated over the usual \textit{Sh/Y} chromosome of (Robertson and Engels, 1989) and crossed to \textit{Cy+;TM2,ry;MKRS} virgins. Altogether, 156 \textit{ry} individuals were kept and analyzed further by PCR and/or Southern analysis.

In order to increase the frequency of deletions in \textit{Fab-7}, we have also mobilized the \textit{blt} transposon over a \textit{Sh}, \textit{\Delta2-3} chromosome carrying the \textit{Fab-7} deletion (Hoggan and Karch, 1995). Such jump-starter males were crossed to \textit{Cy+;TM2,ry;MKRS} virgins. Among the \textit{ry} progeny, we found 26 independent lines with dominant \textit{Fab-7} phenotype. Finally, we have used the same scheme to recover deletions in the region, regardless of their phenotype. Of 334 \textit{Fab-7} minus progeny, we have found 26 independent lines with dominant carriers of the \textit{Fab-7} deletion (Hoggan and Karch, 1995). Such jump-starter males were crossed to \textit{Cy+;TM2,ry;MKRS} virgins. Among the \textit{ry} progeny, we have found 26 independent lines with dominant \textit{Fab-7} phenotype. Finally, we have used the same scheme to recover deletions in the region, regardless of their phenotype. Of 334 \textit{Fab-7} minus progeny, we have found 26 independent lines with dominant carriers of the \textit{Fab-7} deletion (Hoggan and Karch, 1995). Such jump-starter males were crossed to \textit{Cy+;TM2,ry;MKRS} virgins. Among the \textit{ry} progeny, we have found 26 independent lines with dominant \textit{Fab-7} phenotype. Finally, we have used the same scheme to recover deletions in the region, regardless of their phenotype. Of 334 \textit{Fab-7} minus progeny, we have found 26 independent lines with dominant carriers of the \textit{Fab-7} deletion (Hoggan and Karch, 1995). Such jump-starter males were crossed to \textit{Cy+;TM2,ry;MKRS} virgins. Among the \textit{ry} progeny, we have found 26 independent lines with dominant \textit{Fab-7} phenotype. Finally, we have used the same scheme to recover deletions in the region, regardless of their phenotype.
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*<sup>-2</sup>, *Fab-7*<sup>-3</sup>, *Fab-7*<sup>-14</sup>, *Fab-7*<sup>-28</sup>, *Fab-7*<sup>-32</sup>, *Fab-7*<sup>-19</sup>, *Fab-7*<sup>-10</sup>, *Fab-7*<sup>-12</sup>, *Fab-7*<sup>-21</sup>, *Fab-7*<sup>-14</sup>, *Fab-7*<sup>-P18.1</sup>, *Fab-7*<sup>-P18.19</sup> and *Fab-7*<sup>-P18.18</sup>) is strongly enhanced by Pc-g mutations like *Pc-c*, *Pc-F1*, *Scm*<sup>1d1</sup> and an Asx deficiency, *Df(2R)kto<sup>2</sup>*. 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*<sup>-7</sup>, *Fab-7*<sup>-7</sup>, *Fab-7*<sup>-7</sup>, *Fab-7*<sup>-P18.1</sup>, *Fab-7*<sup>-P18.19</sup> and *Fab-7*<sup>-P18.18</sup>) 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*<sup>F2</sup> or *brm*<sup>20</sup> point mutations or the *Df(2R)kto<sup>2</sup>* 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 I deletions, *Fab-7*<sup>P18.18</sup>, 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*<sup>-7</sup> 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-719, 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-71 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-7PRE56 increases to about 1/10 (data not shown) when heterozygous with iab-7P2, a deletion that remove DNA from the insertion site of the blt transposon to position +143 kb (Galloni et al., 1993; Fig. 3).

![Fig. 3. Interactions of class I and III Fab-7 deletions with mutations in Pcl and trx-g.](image)
The presence of two bristles on the 6th sternite is due to visible by a partial loss of pigments on the 5th tergite. Moreover, the presence of two bristles on the 6th sternite is due to haplo-insufficiency. For example, the bithorax complex causes few partial loss-of-function phenotypes due to haplo-insufficiency. For example, the reduced size of the 6th tergite.

The expression pattern is greatly enhanced when the absence of the homologous iab-7PRE 56 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-6,7p14.1, iab-6,7p18.1 and iab-6,7p14.1, 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-6,7p14.1), 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-6,7p18.1 and iab-6,7p18.1) 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-6,7p6.1, iab-6,7p18.1 and iab-6,7p14.1 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-72 or other class II alleles (see above) was observed. Representative of the new lines were selected and named Fab-7p6.1, Fab-7p18.1 and Fab-7p14.1, 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-6,7p6.1, iab-6,7p18.1 and iab-6,7p14.1 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-6,7p6.1, iab-6,7p18.1 and iab-6,7p14.1 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,
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.

**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-
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., 1993). 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 a ‘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., 1993; 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 expression pattern, we proposed that the Parasegments. Based on this 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-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1, 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-6,7P14.1 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-6,7P6.1 and iab-6,7P18.1, 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-6,7P6.1 and iab-6,7P18.1 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-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1 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-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1 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 transgenic 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., 1996). 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 experiments 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-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1, 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-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1 lines produces the expected Fab-7 class II phenotype. From our analysis of imprecise excision derivatives of iab-6,7P6.1, iab-6,7P18.1 and iab-6,7P14.1, 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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