The *Fab-8* boundary defines the distal limit of the bithorax complex *iab-7* domain and insulates *iab-7* from initiation elements and a PRE in the adjacent *iab-8* domain

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SUMMARY

The *Drosophila* bithorax complex *Abdominal-B* (*Abd-B*) gene specifies parasegmental identity at the posterior end of the fly. The specific pattern of *Abd-B* expression in each parasegment (PS) determines its identity and, in PS10-13, *Abd-B* expression is controlled by four parasegment-specific *cis*-regulatory domains, *iab-5* to *iab-8*, respectively. In order to properly determine parasegmental identity, these four *cis*-regulatory domains must function autonomously during both the initiation and maintenance phases of BX-C regulation. The studies reported here demonstrate that the (centromere) distal end of *iab-7* domain is delimited by the *Fab-8* boundary. Initiators that specify PS12 identity are located on the proximal *iab-7* side of *Fab-8*, while initiators that specify PS13 identity are located on the distal side of *Fab-8*, in *iab-8*. We use transgene assays to demonstrate that *Fab-8* has enhancer blocking activity and that it can insulate reporter constructs from the regulatory action of the *iab-7* and *iab-8* initiators. We also show that the *Fab-8* boundary defines the realm of action of a nearby *iab-8* Polycomb Response Element, preventing this element from ectopically silencing the adjacent domain. Finally, we demonstrate that the insulating activity of the *Fab-8* boundary in BX-C is absolutely essential for the proper specification of parasegmental identity by the *iab-7* and *iab-8* *cis*-regulatory domains. *Fab-8* together with the previously identified *Fab-7* boundary delimit the first genetically defined higher order domain in a multicellular eukaryote.

Key words: Bithorax complex, Chromatin domain boundary, Insulator, Polycomb Response Element, *Drosophila*

INTRODUCTION

The three homeotic genes of the bithorax complex, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) are responsible for specifying the identity of parasegments 5 to 14 (PS5-PS14), which form the posterior half of the thorax and all abdominal segments of the adult fly (Lewis, 1978; Sanchez-Herrero et al., 1985). The PS-specific expression patterns of *Ubx*, *abd-A* and *Abd-B* are generated by a complicated *cis*-regulatory region that spans a DNA segment of 300 kb. This *cis*-regulatory region is organized in a series of nine parasegment-specific domains, *abx/bx*, *bxd/phx*, *iab-2* to *iab-8* (for reviews see Duncan, 1987; Peifer et al., 1987). Each domain directs expression of one of the BX-C homeotic genes in a specific parasegment. For example, *Abd-B* expression in PS10, PS11, PS12 and PS13 is controlled by the *iab-5*, *iab-6*, *iab-7* and *iab-8* *cis*-regulatory domains, respectively (Celniker et al., 1990; Sanchez-Herrero, 1991). These *cis*-regulatory domains are located downstream of the *Abd-B* transcription unit (see Fig. 1) and, as is the case for the other BX-C *cis*-regulatory domains, their proximal-distal order along the chromosome corresponds to the anteroposterior order of the parasegments that they specify.

Regulation of the BX-C homeotic genes can be divided into two phases, initiation and maintenance. The choice of parasegment identity is made during the initiation phase, and depends upon the activity of the gap and pair-rule gene products that are present in the parasegment (Simon et al., 1990; Qian et al., 1991; Muller and Bienz, 1992). These proteins interact with initiation elements in each *cis*-regulatory domain, sequentially activating the domains in progressively more posterior parasegments. For example, the regulatory proteins present in PS12 activate *iab-7*, but not *iab-8*, while the proteins in PS13 activate *iab-8*. Because the products of the...
gap and pair-rule genes are expressed only transiently in the early embryo, the activity state selected during the initiation phase is fixed by the programming of a maintenance system in each cis-regulatory domain. This maintenance system requires the Polycomb-Group (Pc-G) and trithorax-Group (trx-G). The products of the Pc-G genes function as negative regulators, maintaining the inactive state of the homeotic genes, while the products of the trx-G function as positive regulators, maintaining the active state. Pc-G-mediated repression is thought to resemble mating type silencing in yeast and involve the formation of chromatin structures that are inaccessible to trans-acting regulators (McCaff and Bender 1996; Boivin and Dura 1998; for reviews see Paro, 1990; Kennison and Tamkun, 1992; Simon, 1995; Pirrotta, 1997). The repressive Pc-G complexes are first assembled during the transition from the initiation phase to the maintenance phase (Poux et al., 1996) and exert their regulatory effects by interacting with specific elements in each cis-regulatory domain, called Polycomb Response Elements or PREs (Simon et al., 1990, 1993; Müller and Bienz, 1991; Chan et al., 1994; Chiang et al., 1995). The activity state of the PREs in each cis-regulatory domain appears to be programmed by the initiator elements; however, it is not yet clear whether this involves a direct interaction of gap and pair-rule gene products with components of the PRE or requires other intermediary factors.

Implicit in this model for the functional organization of the BX-C cis-regulatory domains is a mechanism(s) to ensure their autonomy. One possible mechanism has been suggested by the discovery of an unusual dominant mutation called Fab-71, which deletes a 4 kb DNA segment between the iab-6 and iab-7 cis-regulatory domains (Gyurkovics et al., 1990). While most mutations in BX-C cis-regulatory domains have a loss-of-function phenotype, Fab-7 is unusual in that it has a gain-of-function phenotype, transforming PS11 into a copy of PS12. This transformation arises from the inappropriate activation of the iab-7 cis-regulatory domain in PS11 where iab-6 normally functions. As a consequence, Abd-B is expressed in a PS12-like pattern in PS11 (Galloni et al., 1993). The novel properties of Fab-71 led to the hypothesis that the mutation deletes a chromatin domain boundary element that insulates the iab-6 cis-regulatory domain from iab-7; in the absence of this boundary, the two domains are no longer autonomous but instead fuse into a single cis-regulatory domain. Consequently, positive elements in iab-6 ectopically activate iab-7 in PS11 (for review, see Mihaly et al., 1998).

This hypothesis has been supported and refined by transgene assays for boundary function and by the analysis of additional deletions in Fab-7. Transgene assays have shown that the DNA segment deleted in the original Fab-7 mutation actually contains two distinct elements. On the proximal side, there is a boundary element that spans two prominent chromatin-specific nucleosome hypersensitive regions. In transgene assays, this element blocks enhancer-promoter interactions (Hagstrom et al., 1996; Zhou et al., 1996). On the distal side, there is a PRE for the iab-7 cis-regulatory domain which spans a single chromatin-specific nucleosome hypersensitive region. In transgene assays, this element functions as a Pc-G-dependent silencer (Hagstrom et al., 1997). The conclusions drawn from transgene assays have been confirmed by the phenotypic properties of a series of BX-C deletions that remove just the boundary element or the PRE (Mihaly et al., 1997). Like the original Fab-71 allele, boundary deletions cause a dominant transformation of PS11 into PS12. However, these deletions differ from Fab-71 in that there are often small clones of cells in PS11 that exhibit a loss-of-function phenotype and assume PS10 identity. In these clones, iab-6 is ectopically silenced in PS11, and, as a consequence, Abd-B expression is controlled by iab-5. The mixed gain- and loss-of-function phenotypes in the deletions that only remove the boundary arise because there is a competition in the fused cis-regulatory domain between positive elements in iab-6 that ectopically activate iab-7 and negative elements in iab-7 that ectopically silence iab-6. It is presumed that the same competition between positive and negative elements occurs in larger deletions like Fab-71; however, because the iab-7 PRE is absent, the silenced state is unstable, leading to the activation of iab-7.

If boundaries ensure the functional autonomy of each BX-C cis-regulatory domain, one would expect to find elements separating each of them. To date only one other candidate boundary element, Mcp, has been identified. Mcp is located between iab-4 and iab-5 and is defined by three small deletions that span a prominent chromatin-specific nucleosome hypersensitive region (Karch et al., 1994). Like Fab-7, the change in segmental identity induced by the Mcp mutations is easily recognized in adult males. Since the other abdominal segments have quite similar morphology, it is possible that mutations in other boundaries in the Abd-B cis-regulatory region would be too subtle to be recognized in genetic screens. In the studies reported here, we have a different strategy to identify and characterize the Fab-8 boundary, which marks the distal end of the iab-7 domain and is responsible for insulating iab-7 from iab-8.

MATERIALS AND METHODS
P element transformation
Germline transformation was performed essentially as described in Mihaly et al. (1997). Plasmid DNA mixes were injected either in ry506 or w1118 host strains.

Generation of Fab-8 mutations
In order to avoid gap repair by the homolog chromosome, the P element in fs(3)5649 was mobilized over a chromosome carrying a deficiency for the distal part of BX-C (Df(3R)S9; Gyurkovics et al., 1990) and the A2-3-producing transposase. Haplosterility of such trans-heterozygotes was covered by a duplication of BX-C on the X chromosome, Dp(t(3)I)xal11. Dysgenic males fs(3)5649/Df(3R)S9, D2-3; Dp(3;1)xal11 were crossed to TM2,ry/MKRS females and putative deletion mutations were recognized on the basis of their ry- and/or dominant gain-of-function Fab-8 phenotype. All putative mutations were analyzed by PCR with a set of oligonucleotide primers that hybridize on both sites of the P element. The endpoints of the deletions were sequenced.

Plasmid constructions
8XS is an 8 kb Xhol-SalI fragment [coordinates 60,241-68,235 of the BX-C sequence from Martin et al. (1995)]. 8XSΔHS-I is a derivative of 8XS in which the central HindIII-PstI fragment (from 62,541 to 64,584) has been deleted. Both inserts were cloned upstream of the Ubx-lacZ reporter gene in vector 1204 (Simon et al., 1990) carrying the ry4 gene selectable marker. All other 8XS constructs were inserted upstream of the Ubx-lacZ reporter gene in the CaSpeR/Ubx- lacZ transposon vector (Qian et al., 1991). In 8XSΔ, the HindIII-PstI
fragment from coordinates 62,541 to 64,584 has been replaced by a 2 kb HindIII-PstI fragment derived from bacteriophage λ. In 8XSahiS1-2, the HindIII-PfM1 fragment from coordinates 63,384 to 64,584 has been deleted from the 8XS fragment. The 3.6XH fragment corresponds to the fragment XhoI-HindIII (64,584-68,235) and the 2.1PP fragment corresponds to a 2.1 kb PstI fragment from 60,451 to 62,380. In the enhancer-blocking assays, an AflII fragment (62,380-65,210) was inserted between the white enhancer and the mini-white gene in the vector of Hagstrom et al. (1996). In subsequent constructs the EcoRI-AflII fragment (62,380-63,788) covering the iab-8 PRE was inserted in the same white enhancer-mini-white vector, while the HindIII-EcoRI fragment spanning Fab-8 (63,788-64,584) was inserted between the UPS-NE elements of the ftz enhancer and the hsp-70-lacZ reporter gene of Hagstrom et al. (1996). All cloning details are available upon request.

Antibody staining
Embryos were stained as described in Mihaly et al. (1997).

Preparation of larval and adult cuticles
Larval cuticles were prepared for microscopy following a procedure of Van der Meer (1977). In order to distinguish homozygous Fab-8 mutant larvae, we used a TM3 balancer containing a duplication of the gene in the vector of Hagstrom et al. (1996). In subsequent constructs the 2.1PP fragment corresponds to a 2.1 kb HindIII-PstI fragment from 60,451 to 62,380. In the enhancer-blocking assays, an AflII fragment (62,380-65,210) was inserted between the white enhancer and the mini-white gene in the vector of Hagstrom et al. (1996). In subsequent constructs the EcoRI-AflII fragment (62,380-63,788) covering the iab-8 PRE was inserted in the same white enhancer-mini-white vector, while the HindIII-EcoRI fragment spanning Fab-8 (63,788-64,584) was inserted between the UPS-NE elements of the ftz enhancer and the hsp-70-lacZ reporter gene of Hagstrom et al. (1996). All cloning details are available upon request.

DNA techniques and chromatin digests
Whole genome Southern analysis were performed as described in Gyurkovics et al. (1990). Chromatin digests were isolated and analyzed by indirect end labeling as described in Galloni et al. (1993) and Karch et al. (1994). The DNA lesions of Fab-8 mutations were recovered by PCR using Appligene Taq polymerase. PCR products were then cloned into pGEM-T vector system I (Promega) and sequenced using double-strand template with the USB Sequenase 2.0 system. PCR reactions were carried out on single flies according to Gyurkovics et al. (1990). Chromatin digests were isolated and analyzed by indirect end labeling as described in Galloni et al. (1993) and Karch et al. (1994). The DNA lesions of Fab-8 mutations were recovered by PCR using Appligene Taq polymerase. PCR products were then cloned into pGEM-T vector system I (Promega) and sequenced using double-strand template with the USB Sequenase 2.0 system. PCR reactions were carried out on single flies according to Gyurkovics et al. (1993).

RESULTS

Chromatin structure of the iab-7:iab-8 junction
Previous studies have shown that the Fab-8 boundary as well as the adjacent iab-7:iab-8 PRE are defined by prominent nuclease hypersensitive regions in chromatin digests (Galloni et al., 1993). We reasoned that if iab-7 and iab-8 are separated by a Fab-8 boundary element, such an element should be marked by one or more nuclease hypersensitive regions. To investigate this possibility we examined the chromatin structure of the DNA segment on the distal side of the iab-7 cis-regulatory domain. Since the precise endpoints of the iab-7 cis-regulatory domain have not been previously defined, we focused our attention on the DNA segment near the most distal DNA lesion known to affect iab-7 function, the R73 deletion (Gyurkovics et al., 1990). As shown in Fig. 1, the R73 deletion maps near the middle of an ~8 kb XhoI-Sall fragment (8XS; coordinates 60,241-68,235 in BX-C in Martin et al., 1995). To analyze the chromatin structure of this DNA segment we used the indirect end labeling technique with probes abutting either the proximal XhoI site (see Fig. 2) or the distal Sall site (not shown). The XhoI probe labels a ~20 kb fragment extending from within the iab-7 cis-regulatory domain distally towards iab-8 and the 3′ end of the Abd-B transcription unit.

In DnaS1 digests of KC cell nuclei (see Fig. 2), there are two major nuclease hypersensitive regions in the DNA segment just beyond the R73 deletion. The first of these, HS1, overlaps the distal edge of the R73 deletion and spans a DNA segment of ~400 bp. HS1 is flanked on both the proximal and distal sides by several weaker DnaS1 cleavage sites. The second DnaS1 hypersensitive region, HS2, is located ~1 kb distal and spans about 150 bp. The same pair of DnaS1 hypersensitive regions is observed in digests of nuclei prepared from embryos. Additionally, these two DNA segments are hypersensitive to digestion by micrococcal nuclease. Equivalent results were obtained using a probe abutting the distal Sall site (not shown).

Boundary and PRE activity
Although a variety of different regulatory elements are known to be marked by nuclease hypersensitive regions, we speculated that HS1 might correspond to the Fab-8 boundary while the more distant hypersensitive region, HS2, might correspond to an iab-8 PRE. Supporting the latter possibility is the finding that HS2 contains a ~12 bp sequence motif, which is present in several PREs in BX-C and elsewhere in the genome and is thought to be a binding site for the Pc-G protein pleiohomeotic (Brown et al., 1998; Mihaly et al., 1998). To test this idea, we inserted a ~2.8 kb AflII restriction fragment from the middle of 8XS that encompasses both HS1 and HS2 (see Fig. 1) into a P-element transgene, white-mini-white, in between the white enhancer and the mini-white reporter (see Fig. 3). In previous studies, we used this same vector to analyze the Fab-7 boundary and the adjacent iab-7 PRE (Hagstrom et al., 1996, 1997). As was found for Fab-7-containing transgenes, nearly half of the white-AflII-mini-white lines showed evidence of enhancer blocking activity. In addition to the boundary activity, the AflII fragment has a silencing activity and mini-white expression was silenced in about a quarter of the lines when the transgene insert was homozygous (Fig. 3C, see Hagstrom et al., 1997).

To further delimit the sequences conferring blocking and silencing activities, we subdivided the 2.8 kb AflII into two smaller restriction fragments. The first is an ~800 bp HindIII-EcoRI fragment spanning HS1 and is expected to contain the Fab-8 boundary (see Fig. 1). To test the HS1 fragment for boundary activity, we used a reporter construct in which the fushi-tarazu enhancer and the hsp70 promoter-lacZ fusion gene (Fig. 3). The UPS enhancer generates a pattern of seven stripes around blastoderm stage, while the NE enhancer activates expression in a subset of cells in the CNS during germ band extension. The ftz vector offers the advantage that it is less sensitive to chromosomal position effects than the white-mini-white assay system, and it is more readily possible to compare the blocking activity of different fragments. We examined the pattern of β-galactosidase expression in eight independent transgenic lines in which the putative Fab-8 boundary element (the 800 bp HindIII-EcoRI fragment) is inserted between the ftz enhancers and the hsp70 promoter. As illustrated for the ftz stripes in Fig. 3, we found that the blocking activity is equivalent to that of Fab-7 boundary; stripe and CNS β-galactosidase expression was reduced to the same extent as that seen with Fab-7. We conclude from these results that the HS1 fragment has boundary activity.

The second fragment is a ~1.4 kb EcoRI-AflII fragment, which spans HS2 and is expected to contain the iab-8 PRE (see...
Fig. 1. (A) Distal part of the BX-C. The thin horizontal line represents the genomic DNA of the distal region of BX-C marked off in kilobases. The only class A Abd-B transcript that is required for morphogenesis in PS10 to 13 is shown below the DNA line (Zavortink and Sakonju 1989; Celniker et al., 1990). The horizontal brackets below the DNA indicate the extents of iab-5, iab-6, iab-7 and iab-8 (the beginning of the iab-8 domain is placed according to the data presented in this paper). The Fab-7 boundary is indicated above the DNA line. The 8 kb Xho1-SalI fragment (8XS) used in this study is drawn below the DNA line. Proximal points towards the centromere, while distal indicates the direction of the telomere. (B) The 8XS fragment and its derivatives. The 8XS fragment is marked every 500 bp. Restriction sites used to generate the 8XS derivatives are indicated with their coordinate in the BX-C sequence of Martin et al. (1995; see Materials and Methods). The two nuclease hypersensitive regions, HS1 and HS2, are shown by the red and yellow boxes, respectively. While the fragments used for the enhancer blocking and PRE assays are indicated by the boxes above the 8XS restriction map, the 8XS derivatives used with the Ubx-lacZ reporters are drawn below. At the bottom is a colored version of 8XS that indicates the different DNA segments in this fragment; this colored version is used for reference in several of the subsequent figures. The insertion site for the P[ry lacZ] transposon in fs(3)5649 is indicated (the insertion point was sequenced by Sue Celniker and Ed Lewis, personal communication). (C) Fab-8 boundary deletions in the context of the BX-C. The extent of the Fab-8 deletions recovered after mobilization of the fs(3)5649 transposon are indicated at the same scale as in Fig. 1B. In Fab-8^64, a 6 kb DNA fragment from the transposon remains; it contains the lacZ reporter gene and part of ry^+.
The Fab-8 boundary separates iab-7 and iab-8 initiation elements

The results described in the previous section are consistent with the hypothesis that the HS1 fragment corresponds to the Fab-8 boundary, while the HS2 fragment corresponds to an iab-8 PRE. If this model is correct, then sequences in 8XS located proximal to the Fab-8 boundary (see Fig. 1) should be derived from iab-7, and hence might contain regulatory elements that are responsible, at least in part, for specifying the PS12 expression pattern of Abd-B. To test this prediction, we asked whether sequences on each side of the boundary will drive expression of a Ubx promoter-lacZ reporter construct in the expected parasegmental pattern. On the proximal side we used a 3.6 kb XhoI-HindIII fragment (3.6XH), while on the distal side, we used a 2.1 kb PstI fragment (2.1PP) which is located just beyond HS2. The b-galactosidase expression pattern observed for each transgene indicates that the Fab-8 boundary is flanked on the proximal side by elements which can function as iab-7 initiators and on the distal side by elements that can function as iab-8 initiators.

During the initiation phase the 3.6XH Ubx-lacZ transgenic lines typically express low levels of b-galactosidase in PS12 and in PS 14. As can be seen in the germband extended embryo.
in Fig. 4A, this iab-7-like pattern is superimposed upon the basal activity of the Ubx promoter, which gives β-galactosidase expression in the head and in stripes in the lateral side of the epidermis (Simon et al., 1990). PS12 expression in the epidermis is not maintained at later stages of embryogenesis and disappears by the completion of germband retraction. At the same time, relatively high levels of β-galactosidase appear in a group of cells in the CNS, in cells along the line of the dorsal closure and in group of cells that form the tracheal placode (Fig. 4A). These results argue that the 3.6XH fragment contains iab-7 initiators and cell/tissue-specific enhancers, but no functional PRE.

The initial pattern of β-galactosidase expression for the 2.1PP Ubx-lacZ transgenic lines differs in two respects from that of the 3.6XH Ubx-lacZ lines. First, the 2.1PP drives much higher levels of β-galactosidase expression. Second, and more importantly, the anterior limit is PS13 not PS12. At the early stage shown in Fig. 4A, the β-galactosidase expression is observed in the ectoderm and mesoderm of PS13 and in more posterior parasegments. Like the 3.6XH transgene, the parasegment-specific β-galactosidase expression pattern is not properly maintained, and either spreads anteriorly or fades away altogether during the maintenance phase. Thus, the 2.1PP fragment contains iab-8 initiators, but no PRE.

The regulatory activities of the 8XS fragment depend upon its orientation relative to the Ubx-lacZ reporter

Since the iab-7 and iab-8 initiators in the 8XS fragment are separated by the Fab-8 boundary (and the iab-8 PRE), we reasoned that the 8XS fragment might generate different parasegmental expression patterns depending upon its orientation relative to the Ubx-lacZ reporter gene. To test this possibility, we inserted 8XS into the Ubx-lacZ reporter so that either the proximal iab-7 (the 8XS:iab-7 transgene in Fig. 4B) or the distal iab-8 (the 8XS:iab-8 transgene in Fig. 4B) sequences are located close to the promoter. As anticipated, the pattern of β-galactosidase expression in transgenic embryos depends upon which initiator elements are next to the promoter.

When the iab-7 sequences are adjacent to the Ubx promoter (8XS:iab-7), the expression pattern is indistinguishable from that observed for the 3.6XH transgene (compare Fig. 4A and 4B). During the initiation phase, the 8XS:iab-7 transgene gives β-galactosidase expression in PS12 (and PS14), however, in spite of the fact that the 8XS fragment contains iab-8 initiators, these regulatory elements do not seem to activate the Ubx reporter and no expression is observed in PS13. As was found for the 3.6XH transgene, the iab-7-like pattern fades during the maintenance phase, and is replaced by β-galactosidase expression in the CNS and trachea. This result implies that the iab-8 PRE (present in this 8XS fragment) is unable to sustain the β-galactosidase expression pattern initiated by the iab-7 regulatory elements in PS12.

When the iab-8 sequences from 8XS are adjacent to the Ubx promoter (8XS:iab-8), the expression pattern during the initiation phase is the same as that observed for the 2.1PP transgene; β-galactosidase is expressed at high levels in PS13, while there is no expression in PS12. However, the 8XS:iab-8 transgene differs from the 2.1PP transgene in expression as it is maintained in PS13 and more posterior parasegments at later stages of development (compare Fig. 4A and B). This difference presumably reflects the activity of the iab-8 PRE, which is missing from the 2.1PP fragment. To confirm that the maintenance activity of the 8XS:iab-8 transgene is due to the presence of a PRE, we examined the effects of a Polycomb mutation on β-galactosidase expression. We found that the PS13-limited expression pattern is not maintained in the absence of Pc function, and β-galactosidase expression spreads into the anterior of the embryo (data not shown).

The Fab-8 boundary confers the orientation dependence of the 8XS fragment

A plausible explanation for the different parasegmental expression patterns of the 8XS:iab-7 and 8XS:iab-8 transgenes is that only regulatory elements proximal to the Fab-8 boundary in each transgene are allowed to interact with the Ubx-lacZ reporter, while interactions with regulatory elements distal to the boundary are blocked. If this explanation is correct, then this orientation dependence should disappear when the Fab-8 boundary is removed. Alternatively, the distal regulatory elements in each orientation may simply be too far from the Ubx promoter to control its activity. To distinguish between these possibilities, we generated two different types of 8XS transgenes. In the first, 8XSΔHS1-2, we deleted a 2 kb fragment spanning HS1 and HS2; this deletion, 8XSΔHS1-2, is expected to remove both the Fab-8 boundary and the iab-8 PRE (see Fig. 1). The 8XSΔHS1-2 deletion fragment was then inserted in either orientation upstream of the Ubx-lacZ reporter to give 8XSΔHS1-2:iab-7 or 8XSΔHS1-2:iab-8. In the second type of transgene, 8XSλ, we replaced the 2 kb HS1-2 fragment with a 2 kb fragment from bacteriophage λ. The 8XSλ fragment was also inserted in either orientation upstream of the Ubx-lacZ reporter to give 8XSλ:iab-7 and 8XSλ:iab-8.

If the Fab-8 boundary, and not the distance, is responsible for the orientation dependence, then the expression pattern of all four of these transgenes should be identical. This is the case. As shown in Fig. 5A for the 8XSλ pair, the initial expression pattern is the same as that observed for 8XS:iab-8 (see Fig. 4B). There is no expression in PS12, instead, high levels of β-galactosidase are found in the epidermis and mesoderm of PS13 and PS14 (compare 8XSλ:iab-7 and 8XSλ:iab-8). This result indicates that the initial activation of the Ubx-lacZ reporter is under the control of the iab-8 and not the iab-7 initiators. During the maintenance phase, all four transgenes also give the same expression patterns; however, in contrast to the early pattern, the late pattern is reminiscent of that seen for the 8XS:iab-7 transgene (or for 3.6XH), and not the 8XS:iab-8 transgene. First, the PS13-specific anterior border of expression is lost, indicating that these four transgenes do not have functional PRE. This was expected since the fragment containing HS2, which had PRE activity, is deleted from both 8XSΔHS1-2 and 8XSλ. Second, β-galactosidase is expressed in the tracheal placode, along the dorsal closure and in cells in the CNS, suggesting that, in the absence of the boundary and the PRE, the regulation of the Ubx-lacZ reporter switches from the early initiators in the iab-8 DNA segment to the late cell/tissue-specific enhancers in the iab-7DNA segment.

Although these results are consistent with the model in which the Fab-8 boundary confers orientation dependence on 8XS fragment by blocking the action of distal regulatory elements, there is one potential caveat. The deletion in both 8XSΔHS1-2 and 8XSλ removes not only the boundary but also...
the iab-8 PRE. Hence, it was important to examine the orientation dependence of a deletion, 8XS\textDelta HS1, that removes only HS1 and should eliminate just Fab-8 boundary function. Fig. 5B shows the β-galactosidase expression pattern of the 8XS\textDelta HS1:iab-7 and 8XS\textDelta HS1:iab-8 transgene pair. Like the 8XS\textDelta HS1-2 and the 8XS transgene pairs, the 8XS\textDelta HS1 fragment drives β-galactosidase expression with an anterior limit of PS13 irrespective of its orientation relative to the Ubx-lacZ reporter. Thus when just the boundary is removed, the iab-8 initiators in 8X control the initial activation of the Ubx promoter even when they are farther from the promoter than the iab-7 initiators. The two 8XS\textDelta HS1 transgenes differ from the transgenes carrying the larger deletions 8XS\textDelta HS1-2 and 8XS\textDelta in that they retain maintenance activity (compare Fig. 5A and B). For both 8XS\textDelta HS1 transgenes, the anterior limit of PS13 set during the initiation phase is maintained later in development. This result confirms our mapping of the iab-8 PRE to the fragment containing HS2 and demonstrates that this PRE is not responsible for the orientation dependence of the 8XS fragment. In addition, the late cell type-specific expression of the Ubx-lacZ reporter in the trachea and CNS observed in the two larger deletions 8XS\textDelta HS1-2 and 8XS\textDelta is suppressed in 8XS\textDelta HS1 (compare Fig. 5A and 5B). This result indicates that these late iab-7 cell/tissue enhancers are inactivated by the iab-8PRE when the boundary is deleted.

The Fab-8 boundary restricts the realm of action of the iab-8 PRE

Besides demonstrating that Fab-8 boundary blocks distal initiators, the results described in the previous sections suggest that the boundary may also restrict the action of the iab-8 PRE. First, when the Fab-8 boundary is present, the PRE is unable to maintain the PS12 expression pattern initiated by the 8XS:iab-7 transgene. Second, the boundary prevents the PRE from inactivating the cell/tissue-specific enhancers in the iab-7 3.6XH DNA segment. To test this suggestion further, we inverted the restriction fragment in 8XS, which contains the Fab-8 boundary and the iab-8 PRE (8XS-IN in Fig. 5C). We reasoned that this inversion should alter the realm of action of the iab-8PRE so that it now functions to maintain the Ubx-lacZ expression pattern activated by the iab-7 instead of the iab-8 initiators. Effectively, at early stages, the pattern of expression of the 8XS-IN:iab-7 and 8XS-IN:iab-8 transgenes is the same as their parental counterparts 8XS:iab-7 and 8XS:iab-8 (compare Fig. 4B with 5C). However, the PS12 expression pattern initiated by iab-7 elements in the 8XS-IN:iab-7 transgene is maintained, while the PS13 pattern initiated by the iab-8 elements in 8XS-IN:iab-8 is not maintained (see Fig. 5C). Additionally, as was the case for the boundary deletion, the late cell/tissue-specific expression in the trachea and CNS is repressed in the 8XS-IN:iab-7 transgene.

The Fab-8 boundary blocks PS12 repressors in the iab-8 initiator

One unexpected finding was that the iab-7 initiators in the 8XS fragment appear to be inactivated when the boundary (or the boundary plus the PRE) is deleted (see Fig. 5A and B). In fact, even when the iab-7 initiators adjoin the Ubx-lacZ reporter, all of the 8XS boundary deletion constructs drive expression in PS13 but not in PS12. This suggests that negative regulatory elements or repressors in the iab-8 initiator can inactivate the iab-7 initiator at a distance when the Fab-8 boundary is deleted. This model predicts that PS12 expression should be restored in the boundary deletion, 8XS\textDelta HS1, by removing the distal DNA segment containing the iab-8 initiator. This prediction is correct. As can be seen in Fig. 5D, the double deletion, 8XS\textDelta HS1:\textDelta iab-8 drives β-galactosidase expression in PS12 (and PS14) early in development. Since the double deletion retains the DNA segment containing the HS2- iab-8 PRE, β-galactosidase expression should be maintained with an anterior limit of PS12 later in development. This is also the case (see Fig. 5D).

Isolation of Fab-8 mutations in the context of BX-C

The transgene assays described above provide strong evidence that the 8XS fragment spans the junction of the iab-7 and iab-8 cis-regulatory domains and contains not only parasegment-specific initiators but also the Fab-8 boundary and an iab-8PRE. However, it was important to confirm the conclusions drawn from these transgene experiments by examining the functions of this DNA segment in the context of BX-C itself. For this purpose we took advantage of an enhancer trap, fs(3)5649 isolated by L. Cooley which is inserted into the BX-C near the proximal edge of HS1, and hence should be located just within the iab-7 cis-regulatory domain (see Fig. 6). According to our model, the fs(3)5649 enhancer trap should behave like the previously characterized Bluetail transposon, which is inserted on the opposite (proximal) side of the iab-7 cis-regulatory domain (Galloni et al., 1993); it should be subject to regulatory elements located within iab-7, but not to regulatory elements in the adjacent cis-regulatory domains. Like Bluetail, the fs(3)5649 transgene should be insulated from the iab-6 regulatory elements by the Fab-7 boundary. Similarly, on the distal side, it should be protected from regulatory elements in iab-8 by the Fab-8 boundary. Fig. 6 shows the expression pattern of the fs(3)5649 enhancer trap line. During the initiation phase, high β-galactosidase levels are observed in PS12 and in the posterior part of PS14. There is also expression in a row of cells localized laterally in PS13. At later stages of development the PS12 pattern is maintained while expression in more posterior parasegments is lost.

We next mobilized the fs(3)5649 transposon to generate imprecise excisions that remove the Fab-8 boundary and/or other flanking sequences. Two imprecise excisions extending distally from the transposon insertion site were characterized. The first, Fab-8\textsuperscript{fs(3)5649} removes the Fab-8 boundary but not the iab-8 PRE. As was observed for deletions in the Fab-7 region, which remove the Fab-7 boundary, but not the iab-7 PRE, the Fab-8\textsuperscript{fs(3)5649} deletion exhibits a mixture of gain-of-function and loss-of-function phenotypes. In adult mutant females, the gain-of-function phenotypes of A7 to A8 can be seen on the dorsal side by the partial absence of tergite in A7 (see Fig. 7A). This segmental transformation is that expected for the ectopic activation of iab-8 in PS12/A7, where it would direct Abd-B expression in a pattern appropriate for PS13. The loss-of-function transformation of A7 into A6 can be seen on the ventral side by the shape of the hairs of the sternite. This mixture of gain- and loss-of-function phenotypes can be explained by a fusion of the iab-7 and iab-8 cis-regulatory domains. In PS12/A7 there is a competition between ectopic activation of the fused domain by positive regulatory elements in iab-7 and silencing of the fused domain by negative
The larger deletion, *Fab-8*Δ64, extends from a site within the *fs(3)5649* transposon to 250 bp near the distal edge of the *8X* restriction fragment (see Fig. 1), removing the *Fab-8* boundary (HS1) *iab-8* PRE (HS2) and much of the *iab-8* initiator in 8X (though there must be other *iab-8* initiators distal to the breakpoint that remain intact: see in Gyurkovics et al., 1990). Like deletions in the *Fab-7* region that remove both the *Fab-7* boundary and the *iab-7* PRE, *Fab-8*Δ64 exhibits only a gain-of-function phenotype. The transformation of PS12 into PS13 can be seen on both the ventral and dorsal sides of the first instar (Fig. 7B) and third instar larval ectoderm (Fig. 7C) and in the cuticle of adult females (see Fig. 7A). Though a competition between ectopic activation and silencing in the fused *iab-7*-*iab-8* regulatory domain probably still occurs in this deletion, the balance is apparently shifted towards activation. This could be due, at least in part, to the removal of the repressors that seem to be associated with the *iab-8* initiators in the 8X fragment (see above). In addition, it is also likely that the silenced state cannot be properly maintained in the absence of the *iab-8* PRE.

Further evidence that the insulating activity of the *Fab-8* boundary is responsible for ensuring the functional autonomy of the *iab-7* and *iab-8* cis-regulatory domains comes from an analysis of the β-galactosidase expression pattern of the *Fab-8*Δ64 deletion. While the *ry* marker is lost in the *Fab-8*Δ64 deletion, it retains a functional lacZ gene. As shown in Fig. 6, the deletion alters the pattern of β-galactosidase expression pattern. Unlike the parental transposon, the *Fab-8*Δ64 deletion variant expresses high levels of β-galactosidase not only in PS12 and PS14, but also in PS13. Moreover, expression in PS12 and PS13 is maintained at a high level at late stages of development. This is the result expected if the lacZ reporter is subject to the regulatory influences of the *iab-8* cis-regulatory domain in the *Fab-8*Δ64 deletion mutant.

**DISCUSSION**

In the studies reported here, we have investigated the mechanisms that ensure the functional autonomy of two BX-C cis-regulatory domains, *iab-7* and *iab-8*. These two domains regulate the *Abd-B* gene and are responsible for specifying segmental identity in PS12 and PS13, respectively. We show that *iab-7* and *iab-8* are separated by a chromatin domain boundary element, *Fab-8*. In transgene assays, the *Fab-8* boundary can block enhancer-promoter interactions and restrict the realm of action of the *iab-8*PRE. We demonstrate that *Fab-8* is essential in BX-C for the proper specification of segmental identity in PS12. When the boundary is deleted, the *iab-7* and *iab-8* cis-regulatory domains fuse into a single domain resulting in the misspecification of cell identity in PS12 as either PS13 or PS11. Our conclusions are supported by a recent independent study of Zhou et al. (1999) who have used completely different transgene assays to identify *iab-7* and *iab-8* regulatory elements and the *Fab-8* boundary.

While *Fab-8* marks the distal end of the *iab-7* domain, the proximal end is defined by a previously identified boundary element *Fab-7* (Hagstrom et al., 1996; Zhou et al., 1996; Mihaly et al., 1997). Like *Fab-8*, *Fab-7* has an essential insulator function in vivo and is required to protect *iab-7* from
Fab-8 boundary defines \textit{iab-7} domain

the adjacent proximal domain, \textit{iab-6}. Together, the \textit{Fab-7} and the \textit{Fab-8} boundaries delimit the \textit{iab-7} domain, ensuring its functional autonomy by shielding the domain from the regulatory influences of the neighboring \textit{iab-6} and \textit{iab-8} domains. Thus, \textit{iab-7} represents the first example of a genetically defined higher order chromatin domain in a multicellular eukaryote.

Recent studies in yeast have shown that the transcriptionally repressed HMR locus is flanked by elements that function to delineate a silenced chromatin domain (Donze et al., 1999). These elements block the spread of silenced chromatin, protecting neighboring genes from repression by the SIR silencing complex assembled at HMR. The \textit{Fab-7} and \textit{Fab-8}

Fig. 5. \textit{Fab-8} boundary deletions lead to the loss of orientation dependence of \textit{lacZ} expression of 8XS. (A) The structure of the 8XS fragment in which the central \textit{HindIII}-\textit{PstI} fragment is replaced by a fragment of similar size from bacteriophage \textit{\lambda} is shown on top of the figure (see text and Fig. 4 legend). Similar patterns are observed in three out of three transformant lines obtained with 8XS\textit{\lambda}:\textit{iab-7} and all four lines obtained with 8XS\textit{\lambda}:\textit{iab-8}. (B) Fragment 8XS\textit{HS1} in which the \textit{Fab-8} boundary is deleted is shown on top. All three transformant lines obtained with 8XS\textit{HS1}:\textit{iab-7} show the same expression pattern. With 8XS\textit{HS1}:\textit{iab-8}, one line out of three shows the strong maintenance in the CNS. (C) The structure of 8XS\textit{IN} in which the central \textit{HindIII}-\textit{PstI} fragment containing the \textit{Fab-8} and \textit{iab-8PRE} has been inverted is drawn on top. These patterns are observed in all four transformant lines recovered with 8XS\textit{IN}:\textit{iab-7} and all eight transformant lines recovered with 8XS\textit{IN}:\textit{iab-8}. (D) The structure of the fragment 8XS\textit{HS1}:\textit{iab-8} carrying the deletion of the region containing the \textit{PS13} initiator is shown on top. The same expression pattern is observed in all four transformant lines recovered with this construct.

Fig. 6. \textit{\beta}-galactosidase expression in \textit{fs(3)5649} and \textit{Fab-8}^{64}. The \textit{\beta}-galactosidase expression patterns driven by the P\textit{[ry+ lacZ]} transposon in \textit{fs(3)5649} and \textit{Fab-8}^{64} are shown in early and late embryos (I and M).
Fig. 7. Larval and adult phenotypes of Fab-8 mutants. (A) Since A7 and A8 do not contribute to any visible cuticle after metamorphosis in males, there is no visible phenotype in adult males homozygous for the Fab-8 deletion. The A7-to-A8 transformation is, however, clearly visible in females where A7 develops. Female 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 5th, 6th and 7th abdominal segments (numbered) are visible on the right of each panel, as well as the genitalia at the bottom (A8 is represented by a rudimentary plate of hard cuticle called the tergite). 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 the pleura. On the ventral midline of the 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 7th sternite can be easily distinguished from the more anterior sternites by its different shape and by the few bristles that are pointed towards the midline (arrows). While there is no sign of an 8th sternite, its characteristic bristles is absent. Instead, we occasionally observe the appearance of thorn bristles, normally found on the vaginal plate, in the 7th sternite. On the dorsal side, they have A8 identity as revealed by the absence of the 8th tergite. On the ventral side is a sternite. The organization and orientation of the sternites in this sternite resemble that normally found in more anterior sternites. (B) Ventral surface of the posterior parts of a WT and Fab-864 first instar larva. Each abdominal parasegment (numbered) is covered by rows of seta belts of trapezoidal shape. The number of rows as well as their length increases in more posterior parasegments to reach the largest trapeze in PS12 (the PS12 and PS13 seta belts are indicated by arrows). PS13 can be distinguished from more anterior parasegments, because of the rectangular shape of the setal belt. In Fab-864 homozygous larvae, the transformation of PS12 into PS13 is visible by the appearance of a setal belt of rectangular shape in PS12. (C) Dorsal sides of a WT and Fab-864 homozygous 3rd instar larvae. In the posterior part of PS13 of WT larvae, a number of sensory organs specific to this parasegment are present (arrowheads). The transformation of PS12 into PS13 in Fab-864 is revealed by the presence of these sensory organs in the posterior part of PS12.

Boundaries perform an analogous function, restricting the action of Pc-G silencing complexes; however, they not only prevent silencing complexes assembled in the iab-7 domain from spreading to adjacent domains, they also protect the iab-7 domain from the effects of silencing complexes in adjacent domains.

Boundary functions of Fab-8 in transgene assays

In the context of BX-C, the primary role of the Fab-8 boundary is to prevent adventitious interactions between regulatory elements (parasegment initiators/repressors, cell/tissue-specific enhancers and PREs) in adjacent cis-regulatory domains. Except for enhancer trap insertions like fs(3)5649, the boundary does not normally function to block interactions between these regulatory elements and promoters. However, our transgene assays, as well as those of Zhou et al. (1999) reveal that the boundary activities of Fab-8 closely resemble those described for other insulators (Gerasimova and Corces 1996; Geyer 1997). When interposed between an enhancer and a promoter, the Fab-8 boundary blocks enhancer/promoter interactions. This insulating activity is not specific to regulatory elements derived from BX-C; the Fab-8 boundary can block interactions between enhancer/promoter combinations that are derived from other completely unrelated loci. The insulating activity also appears to be 'constitutive'; it can be detected both in early blastoderm embryos and in adult flies and exhibits no apparent stage or tissue specificity. In addition to blocking the action of positive regulatory elements, the Fab-8 boundary can restrict the activity of elements that function as repressors or silencers. These negative elements include a silencer associated with the iab-8 initiator that inactivates the iab-7 initiator, and a Polycomb Response Element, the iab-8PRE. Finally, like other insulators, the Fab-8 boundary is associated with a nuclease hypersensitive region.

Boundary functions of Fab-8 during the BX-C initiation phase

During the initiation phase, the products of the gap and pair-rule genes select parasegmental identity by setting the activity state of the individual BX-C cis-regulatory domains. For example, the combination of gap and pair-rule gene products present in cells that give rise to PS12 set the iab-7 domain in the active state, while they set the iab-8 domain in the inactive state. To properly specify parasegmental identity, the cis-regulatory domains must function autonomously during this phase and there must be no cross-talk between initiator elements in adjacent domains. Our results indicate that the Fab-8 boundary plays a critical role in establishing the functional
independence of the iab-7 and iab-8 cis-regulatory domains, blocking adventitious interactions between the initiators in these two domains. This conclusion is supported by several lines of evidence.

The first comes from studies on the regulatory activities of the iab-7 and iab-8 initiators in the 8XS restriction fragment. When the Fab-8 boundary is present, the regulatory activities of the 8XS fragment are determined by its orientation relative to the Ubx-lacZ reporter; only the initiator proximal to the boundary is able to activate the reporter, while the initiator distal to the boundary can not. When the boundary is removed from the 8XS fragment, orientation dependence is lost. The iab-7 initiator drives Ubx-lacZ expression in PS13 and represses the iab-7 initiator in PS12. Second, deletion of the Fab-8 boundary in BX-C changes the pattern of β-galactosidase expression of the fs(3)5649 enhancer trap. When the boundary is present, the enhancer trap is under the control of the iab-7 cis-regulatory domain and is expressed at high levels in PS12 during the initiation phase. When the boundary is deleted, high levels of β-galactosidase expression are observed not only in PS12 but also throughout PS13. Finally, the proper specification of PS12 identity depends upon the Fab-8 boundary. When the boundary is deleted positive elements in iab-7 can ectopically activate iab-8 in PS12, transforming identity from PS12 to PS13. Conversely, negative elements in iab-8 can ectopically repress iab-7 in PS12. In this case, PS12 identity is transformed to PS11 because only iab-6 remains active in the presumptive PS12 cells.

Boundary functions of Fab-8 during the BX-C maintenance phase

In addition to preventing cross talk between regulatory elements in iab-7 and iab-8 during the initiation phase, our results indicate that the Fab-8 boundary functions to restrict the realm of action of PREs during the maintenance phase. This activity is most clearly demonstrated by the experiment in which we inverted a fragment within 8XS that contains both the Fab-8 boundary and the iab-8 PRE. In its normal orientation, the iab-8 PRE maintains the β-galactosidase expression pattern initiated by the iab-8 initiators in PS13, while it is unable to maintain the expression pattern activated by the iab-7 initiators in PS12. However, when the iab-8 PRE is placed on the same side of the boundary as the iab-7 initiators, it maintains the PS12 β-galactosidase expression pattern induced by these elements, but fails to maintain the pattern activated by the iab-8 initiators. Further evidence that the Fab-8 boundary restricts the spread of Pc-G silencing complexes comes from a comparison of the phenotypes of a deletion that removes just the boundary with a deletion that removes both the boundary and the PRE. Whereas the former exhibits a mixture of gain- and loss-of-function transformations in PS12, the latter exhibits only a gain-of-function transformation of PS12 into PS13.

Organization and functioning of the Abd-B cis-regulatory domains

The studies reported here taken together with previous work on Fab-7 indicate that the iab-7 cis-regulatory domain spans a DNA segment of approximately 18 kb. The proximal side of this domain is delimited by the Fab-7 boundary, while the distal side is marked by the Fab-8 boundary. Located immediately adjacent to the Fab-7 boundary, just within the proximal edge of the iab-7 domain is an iab-7 PRE. A similar organization of boundary and PRE is seen on the other side of iab-7; just beyond the distal edge of the Fab-8 boundary is an iab-8 PRE. In addition to this similarity in organization of boundary and PRE, our results suggest that the functional properties of the corresponding elements are remarkably similar. Deletions that remove Fab-7 fuse the iab-7 cis-regulatory domain with iab-6, while deletions that remove Fab-8 fuse iab-7 with iab-8. In both cases, the fused domains misfunction in the specification of parasegmental identity, PS11 for Fab-7 deletions and PS12 for Fab-8 producing a mixture of gain- and loss-of-function phenotypes in the affected parasegment. This mixed phenotype appears to arise from the ectopic activation of the more distal (posterior) cis-regulatory domain (iab-7 for Fab-7 and iab-8 for Fab-8) or the ectopic silencing of the more proximal (anterior) cis-regulatory domain (iab-6 for Fab-7 and iab-7 for Fab-8). Finally deletions that remove both the boundary and the PRE exhibit only the gain-of-function phenotype; in the case of the Fab-7:iab-7 PRE deletion, a PS11-to-PS12 transformation while in the case of the Fab-8:iab-8 PRE deletion, a PS12-to-PS13 transformation.

The identification of a second boundary element in the Abd-B regulatory region of BX-C, besides suggesting that there may be similar elements elsewhere in the complex, poses the question of how the boundaries block cross-talk between adjacent domains without preventing these domains from interacting with the Abd-B promoter. This problem can be illustrated for the iab-6 cis-regulatory domain, which controls Abd-B in PS11 (see Fig. 1). The Fab-7 and Fab-8 boundaries are interposed between iab-6 and the Abd-B gene, and thus might be expected to prevent regulatory interactions between this domain and the Abd-B promoter. Moreover, there is ample evidence that both Fab-7 and Fab-8 are capable of blocking enhancer:promoter interactions not only in transgenes assays but also in the context of BX-C itself. One good example is the parasegmental expression pattern of the Bluetail transposon which is inserted between the Fab-7 boundary and the iab-7 PRE. When Fab-7 is present, it insulates Bluetail from regulatory interactions with iab-6; however, when the boundary is deleted, Bluetail is activated in PS11 (Mihaly et al., 1997). The fs(3)5649 enhancer trap on the distal side of iab-7 also appears to be insulated from regulatory interactions with either iab-6 or iab-8.

A number of factors might enable the Fab-7 and Fab-8 boundaries to function selectively, insulating the cis-regulatory domains from each other, but having little or no effect on their interactions with the Abd-B promoter. One factor is the strength of the boundary. The blocking activity of both Fab-7 (see Hagstrom et al., 1996) and Fab-8 in transgene assays is clearly not as strong as an intact sat(Hw) insulator, which contains twelve binding sites for the Su(Hw) protein. A second factor may be the presence of special tethering elements upstream of the Abd-B promoter, which are not present in the promoters of the transposons. Recent studies by Sipos et al. (1998) on transvection in the Abd-B domain of BX-C have shown that there is an extensive region upstream of the Abd-B promoter that mediates long distance interactions with cis-regulatory domains. It is possible that these special tethering elements help overcome the insulating activities of boundaries like Fab-7 and Fab-8.
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