Boundary swapping in the *Drosophila* Bithorax complex

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Although the boundary elements of the *Drosophila* Bithorax complex (BX-C) have properties similar to chromatin insulators, genetic substitution experiments have demonstrated that these elements do more than simply insulate adjacent cis-regulatory domains. Many BX-C boundaries lie between enhancers and their target promoter, and must modulate their activity to allow distal enhancers to communicate with their target promoter. Given this complex function, it is surprising that the numerous BX-C boundaries share little sequence identity. To determine the extent of the similarity between these elements, we tested whether different BX-C boundary elements can functionally substitute for one another. Using gene conversion, we exchanged the Fab-7 and Fab-8 boundaries within the BX-C. Although the Fab-8 boundary can only partially substitute for the Fab-7 boundary, we find that the Fab-7 boundary can almost completely replace the Fab-8 boundary. Our results suggest that although boundary elements are not completely interchangeable, there is a commonality to the mechanism by which boundaries function. This commonality allows different DNA-binding proteins to create functional boundaries.

**KEY WORDS**: Bithorax, Chromatin, Boundaries, Insulator

**INTRODUCTION**

The large cis-regulatory region of the BX-C is divided into nine parasegment-specific chromatin domains that control the expression of the three BX-C homeotic genes along the anteroposterior (AP) axis (*Ubx, abd-A* and *Abd-B*) (for reviews, see Duncan, 1987; Maeda and Karch, 2006). The precise parasegment-specific expression pattern of these genes determines the segmental identity of each of the segments of the posterior two-thirds of the fly. Each domain is kept separate and autonomous by specialized elements known as domain boundaries (Barges et al., 2000; Gyurkovics et al., 1990; Karch et al., 1994; Mihaly et al., 1997). In transgenic constructs, these boundary elements behave as insulators, blocking enhancer activity when placed between the enhancer and its target promoter (Barges et al., 2000; Gruzdeva et al., 2005; Hestrom et al., 1996; Zhou et al., 1996). However, within their native context, they are often found between an enhancer and its target promoter. How BX-C enhancers bypass intervening boundaries is still a topic of contention.

Boundary deletions indicate that these elements are required to provide functional autonomy to the enhancers and silencers within the large cis-regulatory region. The Fab-7 boundary element, for example, is normally found separating the *iab-6* and *iab-7* cis-regulatory domains (see Fig. 1A). The *iab-6* enhancer region controls the level of *Abd-B* expression in parasegment 11 (PS11) and determines the identity of segment A6. The *iab-7* region, however, controls the level of *Abd-B* expression in PS12 and determines the identity of segment A7 (Celniker et al., 1990; Galloni et al., 1993; Mihaly et al., 2006; Sanchez-Herrero, 1991). When *Fab-7* is deleted, the *iab-6* and *iab-7* domains become fused into a single domain, allowing both the *iab-6* and *iab-7* enhancers or silencers to become active in PS11 and PS12. In most cells in PS11, the *iab-7* enhancers are activated by *iab-6* initiation elements, resulting in a homeotic transformation of PS11/A6 into PS12/A7. However, in other cells of PS11, the *iab-6* initiators fail to activate the fused domain before *iab-7* Polycomb Response Elements (PRE) silence the domain, causing these cells to take on a PS10/A5 identity (Galloni et al., 1993; Gyurkovics et al., 1990; Mihaly et al., 1997).

Previously, we have shown that insulators such as *gypsy* (Geyer and Corces, 1992) or *scs* (Kellum and Schedl, 1992) cannot substitute for *Fab-7* within the BX-C (Hogga et al., 2001). Both of these insulators block interactions between the distal *Abd-B* enhancers and the *Abd-B* promoter. To test whether the boundaries of the BX-C can functionally replace each other, we used gene conversion to exchange the *Fab-7* and *Fab-8* boundaries within the BX-C. Although these two boundaries perform similar functions, they share little sequence identity. Surprisingly, we find that the *Fab-7* boundary is almost completely capable of replacing the *Fab-8* boundary, indicating that there is a similarity in the mechanism of boundary function that cannot be predicted through modern sequence analysis.

**MATERIALS AND METHODS**

**Fab-7 replacement by Fab-8**

The *Fab-8* boundary element is an *Alul-MscI* 659 bp fragment (3R:12745503-12744844) cloned into an *Nsi* site of a P-CaSpER-based plasmid containing the genomic region surrounding *Fab-7* (Hogga and Karch, 1995). This construct was injected into white flies. Third chromosome inserts were recombined with the bluetail insertion (Galloni et al., 1993). Convertants were obtained and verified as described previously (Hogga and Karch, 2001).

**Fab-8 replacement by Fab-7**

The genomic region surrounding the *Fab-8* deletion (3R:12745801-12744797) was generated by PCR using Pfu polymerase (*Promega*) and the following primers: 5′-TCTAGAAGCTTCACTTGCGGGGG-3′ and 5′-CTCGAGTTCGGATTTCTGCTTTCTGAGC-3′ for the proximal region, and 5′-TCTAGACATAAAGGGAAGCGGAGGC-3′ and 5′-CTCGAGGTTTATTTCATTGCTCTC-3′ for the distal region. The Fab-7 boundary (a 0.8 kb fragment) was generated by PCR using 5′-CTCGAGGCGGCAAGTTTGAGGAAAA-3′ and 5′-CTCGAGGCCAGAAACAAAGGCCGACG-3′, and was inserted between the two break points of the *Fab-8* deletion. Transgenic flies were made as above and recombined onto a chromosome carrying the fs(3)5649 P-element insertion. In trans to this chromosome, we placed the Df(3R)R59 chromosome.

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Accepted 9 October 2008
carrying the Δ2-3 transposase and a Tp(3;1)bxd111 duplication to rescue the sterility of these dysgenic males. Putative conversion events were screened as above.

**Antibody staining**

Embryos were stained as previously described (Karch et al., 1990), using mouse monoclonal antibodies against Abd-B obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

**Preparation of abdominal cuticles**

Abdominal cuticles were prepared as described previously (Mihaly et al., 1997).

**RESULTS AND DISCUSSION**

**The Fab-7 boundary can substitute for the Fab-8 boundary**

Three reasons dictated our choice in converting Fab-8 to Fab-7 (F8→F7). First, Fab-7 and Fab-8 perform similar functions yet share almost no sequence similarity. Second, we wanted to test whether a BX-C cis-regulatory domain could interact with the Abd-B promoter over a boundary element that it generally never encounters. As Fab-7 is located on the promoter distal side of iab-7, iab-7 enhancers are never faced with the challenge of bypassing the Fab-7 boundary (see Fig. 1A). And third, recent data have suggested that BX-C boundaries are regulated along the AP axis (Cléard et al., 2006). From these data, it seems that boundaries interact with the Abd-B promoter until the neighboring (probably more posterior) domain becomes active (see also Maeda and Karch, 2007). If this regulated association is responsible for boundary function and the association is controlled by the boundary element itself, then a substitution of Fab-8 by Fab-7 should result in the inactivation of the boundary one parasegment too anterior. The expected phenotype resulting from such an event would be a homoeotic transformation of A7 to A8 (much like a boundary deletion).

In the gene conversion, the Fab-8 region was removed and replaced by a minimal Fab-7 boundary element (Chen et al., 2005), inserted, in separate constructs, in each orientations. In order to completely remove the Fab-8 boundary without removing potentially important iab-7 or iab-8 sequences, we deleted the region around Fab-8 that is removed in the Fab-8 deletion. The Fab-8 deletion is the smallest characterized Fab-8 deletion that displays a complete Fab-8 phenotype; homozygous adult females are sterile and the A7 segment disappears due to an A7 to A8 transformation (Fig. 2B). Both the iab-8PRE (Barges et al., 2000) and most of the promoter targeting sequence 7 (PTS7) element (Zhou and Levine, 1999) are left intact in the Fab-8 deletion. As convertants for both orientations display identical phenotypes, we will simply call them F8→F7.

Given the simple nature of the experiment, we expected one of three outcomes: that the Fab-7 boundary would act as a simple insulator and block iab-7 from interacting with the Abd-B promoter (like an iab-7 deletion); that Fab-7 would not be functional in replacing Fab-8 and behave as an Fab-8 deletion mutation; or that Fab-7 would substitute for Fab-8. Scoring females homozygous for either F8→F7 conversion showed that Fab-7 can almost completely substitute for Fab-8. Almost all F8→F7 flies are wild-type appearance and are fertile (Fig. 2). In rare cases, we do observe homozygous flies displaying evidence of slight Abd-B misexpression. Patches of cells in A7 occasionally take on an A6 or A8 identity. To characterize this phenotype more carefully, we looked at F8→F7 hemizygous flies. F8→F7/Df(3R)P9 flies have features reminiscent of Fab-8 homozygotes (Fig. 3), indicating that although Fab-8 can mostly substitute for Fab-7, the boundary system in F8→F7 flies is less robust, occasionally allowing the iab-7 domain to be influenced by neighboring cis-regulatory domains. However, in a non-sensitized background, this effect is quite mild, affecting <5% of the flies scored.

Abd-B antibody staining confirms our results. In the embryonic CNS of wild-type flies, Abd-B is expressed in a step gradient pattern that noticeably increases parasegmentally from PS10 to PS13 (Fig. 2E). In Fab-8 deletion mutants, that pattern changes such that PS12 expression levels mimic those found in PS13. Meanwhile in iab-7 mutants, PS12 expression drops to the level of PS11. In F8→F7 conversion lines, we observe a staining pattern that is similar to that found in wild-type embryos.

This result was quite surprising. The fact that Fab-7 can substitute for Fab-8 means that everything required to restore Fab-8 function is present in the Fab-7 fragment inserted. However, at the DNA sequence level, the Fab-7 and Fab-8 boundaries share almost no similarity. A detailed analysis of the two sequences using dot-plot and Markov analysis found little in common between the two elements other than GAGA factor-binding sites (six in Fab-7 and two in Fab-8). The GAGA factor binding sites have previously been shown to be important for Fab-7 enhancer blocking activity in transgenic contexts (Schweinsberg et al., 2004). However, the role of the GAGA factor in Fab-8 enhancer blocking activity is still unknown. Thus far, the only factor shown to be important for Fab-8 function is the dCTCF factor. Previously, it has been shown that deleting the dCTCF-binding sites in Fab-8 impaired its insulator function in transgenic insulinator assays (Moon et al., 2005). Moreover, dCTCF mutants display phenotypes reminiscent of Fab-8
mutants (Mohan et al., 2007). As Fab-7 was shown to be one of the few BX-C boundaries to which dCTCF does not bind (Holohan et al., 2007), our results show that dCTCF is not absolutely required for Fab-8-like function.

One explanation for this substitution could be that there are different ways to make a functional boundary. It has been reported that the GAGA factor, which binds to both boundaries, is a protein involved in nucleosome remodelling (Tsukiyama et al., 1994). Given that each of the BX-C boundaries has been isolated as a characterized deletion that completely removes Abd-B. PS12 increases to the level normally observed in PS13. (G) F8 substitution. (E-G) Abd-B expression in the embryonic CNS. (F) Wild-type: Abd-B expression level in PS12 increases to the level normally observed in PS13. (G) F8 embryos show a restoration of the wild-type expression pattern of Abd-B.

**The Fab-8 boundary cannot fully substitute for the Fab-7 boundary**

In the F8→F7 flies, the iab-7 enhancers are able to bypass Fab-7 even if, in the wild-type situation, they are never faced with the challenge of bypassing it. Because Fab-7 could substitute for Fab-8, we wondered whether all BX-C boundaries are capable of substituting for each other. We, therefore, decided to replace the Fab-7 boundary with the Fab-8 boundary.

For this gene conversion, we replaced the Fab-7 boundary with a minimal Fab-8 boundary element (in both orientations) (Fig. 1C). To do this, we removed a Fab-7 fragment identical to that deleted in the Fab-7 deletion. The Fab-7 deletion is the smallest characterized deletion that completely removes Fab-7 boundary function; Fab-7 homozygous adult flies primarily show an A6 towards A7 transformation (Fig. 4) (Galloni et al., 1993; Mihaly et al., 1997). Previous genetic and molecular analysis indicates that the nearby iab-7PRE (Mihaly et al., 1997) and Pts6 element (Chen et al., 2005) are left intact in the Fab-7 deletion. Again, we isolated conversants for each Fab-8 orientation. Although the two conversions differ slightly in their intensity, for the most part, they display similar phenotypes. Therefore, we will simply call the mutants F7→F8, indicating, when necessary, where the two orientations differ.

Although Fab-8 can restore the autonomy of the iab-7 domain (freeing it from ectopic activation by iab-6), surprisingly, F7→F8 homozygous flies show a transformation of A6 towards A5. This means that there is a loss of Abd-B activation by iab-6 (Fig. 4). Abd-B antibody staining confirms these results (Fig. 4E-G). Instead of the normal stepwise gradient seen in the wild-type embryonic CNS, F7→F8 embryos display PS10-like Abd-B expression in PS11 (Fig. 4G). This phenotype is reminiscent of the phenotype obtained by substituting a minimal scs insulator for Fab-7 (Hogga et al., 2001). For that substitution, it was believed that the loss of iab-6 function was due to the blocking of iab-6 by the intervening insulator. A second possible explanation for this phenotype is that iab-6 is somehow being silenced in the F7→F8 substitution by the nearby iab-7PRE. This hypothesis is presented because Fab-7 functions, not only to prevent the inappropriate activation of adjacent cis-regulatory domains, but also to prevent the inappropriate silencing of adjacent domains. In Fab-7 mutants, for example, one sees a clonal mixture of both ectopic activation and ectopic silencing (Mihaly et al., 1997). The balance between these two clonal populations is sensitive to mutations in Polycomb group genes. We, therefore, crossed F7→F8 flies to the Polycomb-group mutant, Pcl. Because the phenotype of F7→F8 flies does not change upon the introduction of a Pcl/+ mutation (data not shown), we believe that Fab-8 is acting like a short-range insulator at this locus, blocking iab-6 enhancers from interacting with the Abd-B promoter.

As mentioned above, there is a slight orientation effect with the F7→F8 substitution. Lines with Fab-8 placed in the wild-type orientation (F7→F8') (relative to the Abd-B gene) display a slightly
less-severe transformation than lines with Fab-8 placed in the opposite orientation (F7–F8'). The difference in phenotype can be seen by looking at the trichome pattern in the transformed A6 segment. In F7–F8' flies, trichomes cover most of the transformed segment (A5-like), whereas in F7–F8'' flies, trichomes primarily cover the ventral-anterior region of the transformed segment (more A6-like) (see Fig. S1 in the supplementary material). In all other assays, the two transformants behave identically (Fig. 4).

In the case of the F8–F7 conversion, we found that the iab-7 cis-regulatory domain was capable of bypassing a boundary element that it never has to bypass but in the case of the F7–F8 conversion, we found that iab-6 is partially blocked by a boundary element that it must normally bypass (Fab-8 is located between iab-6 and the Abd-B promoter). One possible explanation is discrepancy is that the Fab-8 fragment inserted lacked a specific element required for insulator bypass. Although this is a possibility, we do not believe this to be the case. Both the Fab-7 and Fab-8 regions have been extensively scanned for elements allowing insulator bypass. In these attempts, elements called promoter-targeting sequences (PTSs) have been identified that allow enhancers to bypass insulator elements on reporter transgenes (Chen et al., 2005; Zhou and Levine, 1999). In our experiments, we replaced the smallest characterized boundary deletions with the smallest characterized insulator fragments. In both cases, molecular data suggest that the fragments we introduced were separated from any PTS-type activity, but were capable, in transgenic contexts, of being bypassed by known PTS elements. Conversely, the deletions we created were chosen to be clean boundary deletions; as much as possible, all known nearby elements, including PTS elements, were left intact. In the F7–F8 substitution, for example, the entire PTS-6 element that was capable of bypassing the identical Fab-8 insulator fragment is still present.

Therefore, if no PTS-type elements were deleted, the main difference between the cases tested is context. For example, in the wild-type situation, Fab-8 is located between the iab-7 and iab-8 cis-regulatory domains, whereas in F7–F8, Fab-8 is placed between the iab-6 and iab-7 cis-regulatory domains. We have recently found that the Fab-7 boundary seems to be regulated along AP axis (Cléard et al., 2006). If we assume that all boundaries behave in a similar manner, then Fab-8 would also be regulated along the AP axis. As this regulation does not seem to come from the boundary element itself (see above), it must come through specific interactions with the nearby cis-regulatory domains. Previous work has pointed to PTS elements as the mediators of this function. However, based on our data and because PTS deletions have little phenotype when deleted, we believe that there must be something more that inactivates boundary elements (Mihaly et al., 2006; Zhou and Levine, 1999). For now, the identity of these elements remains a mystery.

We thank Annick Mutero and Jean-Michel Gentet for critically reading this manuscript, and Eve Favre and Jorge Faustino for excellent technical assistance. C.I., F.C., R.K.M. and F.K. were supported by grants from the State of Geneva, the Swiss National Foundation and the Swiss National Center of Competence in Research. H.G. is supported by grants from OTKA and by the NIH as a subcontractor.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/24/3983/DC1

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