Fab-7 functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila* bithorax complex

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Fab-7 deletions in the bithorax complex have a novel gain-of-function phenotype, typically transforming parasegment 11 (PS11) into PS12 identity. Genetic analysis indicates that removal of the *Fab-7* element results in the fusion of the *iab-6* (PS11) and *iab-7* (PS12) *cis*-regulatory domains into a single regulatory domain that inappropriately regulates *Abdominal-B* in PS11. This has led to the hypothesis that *Fab-7* is a chromatin domain boundary that normally functions to ensure the autonomous activity of the *iab-6* and *iab-7* *cis*-regulatory domains. We use several different enhancer blocking assays to demonstrate that *Fab-7* has the insulating properties expected of a domain boundary. We define a minimal fragment of *Fab-7* sufficient for enhancer blocking, and demonstrate that it is completely distinct from an adjacent *Polycomb*-dependent silencer. We compare *Fab-7* to the *su(Hw)* insulator element, and show that *Fab-7* enhancer blocking activity is intermediate between that of five and twelve reiterated binding sites for the Su(Hw) protein. These results support the model that *Fab-7* functions as a domain boundary within the context of the bithorax complex, making *Fab-7* one of the first boundary elements that is known to have an essential function in vivo.

[Key Words: Bithorax complex; *Fab-7*; chromatin domain boundary; insulator; suppressor of *Hairy-wing*]

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Body patterning in many organisms involves the development of segments whose unique identities are specified by homeotic genes. The precise expression patterns of the homeotic genes are crucial for generating a normal body plan, and the misregulation of these genes can result in dramatic transformations of one body segment into another. In *Drosophila*, the identity of parasegments in the posterior of the fly is controlled by the three homeotic genes of the bithorax complex (BX-C), *Ultra-bithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) (Lewis 1978; Duncan 1987). The parasegment-specific expression patterns of these three genes 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 regulatory elements or domains, *abx/bx*, *bxl/pbx*, *iab-2*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7*, and *iab-8* (Lewis 1978; Karch et al. 1985; Celniker et al. 1990; Sanchez-Herrero 1991). Each of these domains directs the expression of one of the three BX-C homeotic genes in a specific parasegment. For example, the *iab-5*, *iab-6*, and *iab-7* *cis*-regulatory domains direct *Abd-B* expression in parasegments (PS) 10, 11, and 12, respectively (Karch et al. 1985).

As illustrated in Figure 1, these *cis*-regulatory domains are located downstream of the *Abd-B* transcription unit, and as is the case for the other BX-C *cis*-regulatory domains, their proximal-distal order along the chromosome corresponds to the anterior-posterior order of the parasegments they specify.

Bithorax complex gene regulation can be divided into two phases, initiation and maintenance. The choice of parasegment identity is made during the initiation phase, and depends upon the combinatorial action of the gap and pair-rule gene products found in each parasegment (Müller and Bienz 1992; Shimell et al. 1994; Casares and Sanchez-Herrero 1995). These gap and pair-rule proteins sequentially activate the BX-C *cis*-regulatory domains in successively more posterior parasegments. For example, the set of regulatory proteins present in PS11 activates *iab-6* in this parasegment, but not *iab-7*, whereas the regulatory proteins present in PS12 activate *iab-7*. Because the products of the gap and pair-rule genes are present only transiently in the early embryo, the activity state selected during the initiation phase must be 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*) genes (for review, see Paro 1990; Kennison 1993; Simon 1995). The products of the *Pc-G* genes function as negative regulators, maintaining the

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Figure 1. Schematic of BX-C *cis*-regulatory domains controlling *Abd-B*. The three genes of the BX-C are controlled by a large *cis*-regulatory region that is organized into a series of parasegment-specific domains. The *cis*-regulatory domains *iab-5* to *iab-8* (ovals) control the *Abd-B* gene (large arrow) to specify the identity of parasegments (PS) 10 through 13, respectively (Karch et al. 1985). Domain boundary elements (boxes) are thought to delimit the domains and allow their autonomous function by insulating the regulatory information in one domain from the influence of regulatory elements in the neighboring domain. The regions defined by the *Mcp* and *Fab-7* mutations have been proposed to be such domain boundaries (Gyurkovics et al. 1990; Galloni et al. 1993; Vazquez et al. 1993).

inactive state of the homeotic genes, whereas the products of the *trx-G* function as positive regulators, maintaining the active state. The *Pc-G* proteins appear to exert their regulatory effects by interacting with specific elements in each *cis*-regulatory domain, called polycomb response elements (PREs) (Simon et al. 1990; Busturia and Bienz 1993; Simon et al. 1993; Chan et al. 1994; Gindhart and Kaufman 1995). There may be equivalent or overlapping elements (TREs) for the *trx-G* proteins. Whether the programming of the maintenance system in each *cis*-regulatory domain involves the direct interaction of gap and pair-rule gene products with components of the PREs (and TREs) or requires some other intermediary factors is not known (Bienz 1992).

Mutations that inactivate one of the nine parasegment-specific regulatory domains transform the parasegment specified by that domain into a copy of the parasegment immediately anterior. Paralleling this transformation in segmental identity, the normal temporal and spatial pattern of the relevant BX-C homeotic gene is replaced by the expression pattern found in the parasegment immediately anterior. For example, a mutation that deletes much of the *iab-7* *cis*-regulatory domain (*iab-7^{Sz}*) transforms PS12 into a duplicated copy of PS11 (Galloni et al. 1993). As expected from this loss-of-function phenotype, the *Abd-B* expression pattern normally seen in PS12 is replaced by a PS11 pattern, which is generated by the adjacent *iab-6* *cis*-regulatory domain.

Whereas most mutations in the BX-C *cis*-regulatory domains cause a loss-of-function phenotype, the *Fab-7* alleles are exceptional in that they have a gain-of-function phenotype. *Fab-7* was first defined by *Fab-7¹*, a 4-kb deletion that removes sequences between the *iab-6* and *iab-7* *cis*-regulatory domains (Gyurkovics et al. 1990; see Figs. 1 and 2). Subsequently, smaller deletions within the same region have been generated (Galloni et al. 1993; Karch et al. 1994). All of the *Fab-7* alleles are dominant and lead to the transformation of PS11 into a duplicate copy of PS12. This phenotypic 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-pattern in PS11 (Galloni et al. 1993).

Why does a deletion that removes a region between *cis*-regulatory domains result in the ectopic expression of *Abd-B*? One possibility is that the sequences deleted by the *Fab-7* mutations contain a parasegment-specific repressor that normally prevents the PS12-specific activators in *iab-7* from acting on *Abd-B* in PS11 (Busturia and Bienz 1993; Zink and Paro 1995). However, the results of reversion analysis argue against this model. The *Fab-7* phenotype can be reverted not only by second site mutations that inactivate *iab-7* but also by mutations that inactivate *iab-6* (Gyurkovics et al. 1990). These results indicate that both *iab-6* and *iab-7* contribute to the mutant phenotype and suggest that the deletion of the *Fab-7* region causes inappropriate interactions between regulatory elements in the two domains. Thus an alternative hypothesis is that the *Fab-7* region contains a chromatin domain boundary that functions to insulate the two domains (originally proposed in Gyurkovics et al. 1990). In the absence of the *Fab-7* boundary element, *iab-6* and *iab-7* no longer behave as autonomous units but instead fuse into a single *cis*-regulatory domain.

The idea that *Fab-7* corresponds to a domain boundary is supported by studies on the *Bluetail* transposon (Galloni et al. 1993). This transposon contains a *Ubx/lacZ* reporter and is inserted on the distal side of *Fab-7*, just within the *iab-7* *cis*-regulatory domain. *Bluetail* is expressed in an *Abd-B*-like pattern in PS12, indicating that it is subject to control elements in the *iab-7* *cis*-regulatory domain. However, because it is inactive anterior to PS12, the transposon apparently is insulated from control elements in the immediately adjacent *iab-6* *cis*-regulatory domain (and also from elements in *iab-5*). Thus, like other elements that have been proposed to function as chromatin domain boundaries, *Fab-7* appears to restrict enhancers from acting outside of their normal domain.

Although these findings suggest that sequences from the *Fab-7* region act as a boundary within the context of the BX-C, it is important to establish whether these sequences can function as an autonomous boundary element in assays that are independent of other regulatory components from the BX-C. In the studies reported here, we use three different enhancer blocking assays to demonstrate that *Fab-7* has boundary activity. We evaluate the strength of the *Fab-7* boundary in different enhancer-promoter combinations, and in comparison with previously characterized boundaries. We also define a minimal *Fab-7* fragment that can function as a boundary and show that it is distinct from an adjoining DNA segment that has silencer activity and appears to be the *iab-7* PRE.

Results

Fab-7 shows boundary function in an enhancer blocking assay

Whereas it is thought that the normal function of *Fab-7*

Figure 2. Organization of the *Fab-7* region and summary of enhancer blocking results obtained with *white enhancer:mini-white* transgenes. (A) The *Fab-7* region of the BX-C is depicted by the black bar between the PS11 regulatory domain *iab-6* at left, and the PS12 regulatory domain *iab-7* at right. This 3.35 *HindIII*-*XbaI* fragment includes one minor and three major nuclease hypersensitive sites (HS1, HS2, and HS3) whose limits are indicated by boxes (Galloni et al. 1993; Karch et al. 1994). Half-ovals represent the presumed position of nucleosomes. The dotted lines above indicate the extent of three *Fab-7* deletions: *Fab-7¹* is the original deletion and transforms PS11 to PS12, whereas *Fab-7²* and *Fab-7³* give a slightly less complete transformation (Gyurkovics et al. 1990; Galloni et al. 1993). The insertion site of the *Blue-tail* transposon, which is subject to control by *iab-7* but not *iab-6*, is shown (Galloni et al. 1993). Results presented here and elsewhere suggest that the two bracketed regions are the *Fab-7* boundary and the *iab-7* PRE (this work; K. Hagstrom, M. Muller, and P. Schedl, in prep.). (H3) *HindIII*; (Nco) *NcoI*; (Pst) *PstI*; (Nsi) *NsiI*; (Apa) *ApaI*; (Aat) *AatII*; (Eae) *EaeI*; (Xba) *XbaI*. (B–D) The basic P-element construct contains a *white* enhancer fragment that drives expression in the eye and testes (gray box), the *mini-white* eye color gene (arrow), and *scs'* to minimize position effects from this side (open box) (Levis et al. 1985; Pirrotta 1988; Kellum and Schedl 1992). The extent of the *Fab-7* fragment (black bar) in the test constructs is indicated by restriction sites that correspond to those in A. In all constructs the distance between the *white* enhancer and *mini-white* depends on the size of the inserted fragment (thin line does not indicate preserved spacing). At right are shown the number of independent transgenic lines with an eye color lighter than that of the basic w#2 control over the total number of lines generated for each construct.

is to insulate the *iab-6* and *iab-7* domains, studies on the *Blue-tail* transposon indicate that *Fab-7* can also prevent enhancer–promoter interactions (Galloni et al. 1993). Thus it seemed appropriate to use an enhancer-blocking assay (Kellum and Schedl 1992). This assay tests whether a putative boundary element is able to impede enhancer–promoter communication when interposed between an enhancer and a reporter gene. DNA fragments containing a boundary or insulator element should block enhancer–promoter interactions, and reduce reporter-gene expression. If the fragment does not contain a boundary, expression should be equivalent to that observed when only the enhancer and reporter are present. A boundary or insulator element can be distinguished from an ele-

ment that acts as a silencer by its position dependence. A boundary element can only block enhancer–promoter interactions when placed between the enhancer and promoter; when placed upstream of the enhancer, it should have no effect on enhancer–promoter interactions. In contrast, a silencer is expected to attenuate enhancer and/or promoter activity even from an upstream position.

For the enhancer blocking assay we used a *mini-white* reporter gene and an enhancer from the *white* locus that drives expression in the eye and testes (Levis et al. 1985; Pirrotta 1988). In the first series of constructs (Fig. 2B) we tested a fragment of *Fab-7* that spans most of the 4-kb region deleted in the original *Fab-7¹* mutation. Previous

chromatin mapping studies identified a set of three strong and several weak nuclease hypersensitive sites within the region deleted in *Fab-7*¹ (Galloni et al. 1993). Two of the strong and one of the more prominent weak hypersensitive sites are located centromere proximal to the *Bluetail* transposon, whereas the third strong hypersensitive site is distal to the transposon (see Fig. 2A). Because we suspected that the *Fab-7* boundary might be associated with this unusual chromatin structure, we selected a 3.3-kb *HindIII*–*XbaI* fragment that includes all of these nuclease hypersensitive sites for our initial experiments. The experimental construct in the series (w#4) contains this 3.3-kb *Fab-7* fragment between the *white* enhancer and the *mini-white* reporter. Control constructs w#1 and w#2 allowed us to determine the level of *mini-white* expression with or without the enhancer. Construct w#3 controls for the possible effects of distance by inserting a random 2.6-kb DNA fragment between the enhancer and reporter. Finally, control construct w#5 distinguishes between boundary and silencer activity by placing the 3.3-kb *Fab-7* fragment upstream of the enhancer.

Figure 3 shows the eye color phenotype of a representative transgenic line for each construct. As expected from previous studies on *mini-white* (Kellum and Schedl 1991), transgenic lines that carry the *mini-white* gene but no enhancer have a light yellow eye color (Fig. 3, w#1). When the *white* enhancer is included in the construct, the expression of *mini-white* is stimulated and the eye color is orange/red in all 5 of the lines (Fig. 3, w#2; Fig. 2B). The same orange/red eye color is observed when a random DNA fragment is inserted between the *white* enhancer and the *mini-white* gene (Fig. 3, w#3; Fig. 2B). Hence, it would appear that a modest increase in distance has no effect on enhancer–promoter interaction in this reporter system.

Figure 3. Eye colors of representative transgenic lines containing *white* enhancer:*mini-white* constructs w#1–5. Eye colors of heterozygous 1-day-old females are shown. (w#1) With no *white* enhancer, eyes have the yellow color characteristic of basal *mini-white* expression. (w#2) The *white* enhancer elevates *mini-white* expression to give an orange/red color. (w#3) An insert of random DNA between the enhancer and promoter has no effect and gives the same orange/red eye color as w#2. (w#4) The entire *HindIII*–*XbaI* fragment of *Fab-7* between the enhancer and promoter reduces eye color to nearly the same yellow as with no enhancer. (w#5) *Fab-7* cloned upstream of the enhancer gives an orange/red eye color like the control.

A different result was obtained when the *Fab-7* DNA fragment was inserted between the enhancer and promoter (w#4). For this construct, 12 of the 24 transgenic lines have reduced *mini-white* expression and have an eye color lighter than the control constructs w#2 and w#3 that contain the *white* enhancer (Fig. 3). Similar effects on *white* expression were observed in the testes (not shown). A reduction in *white* expression in the eye and testes is only found when the *Fab-7* fragment is placed between the enhancer and promoter. When the *Fab-7* fragment is placed upstream of the *white* enhancer (w#5), the eye color phenotype in most of the transgenic lines (10/11) was the same as that observed in the construct with just the *white* enhancer (Fig. 2B; Fig. 3).

Taken together these findings indicate that the 3.3-kb *Fab-7* fragment is able to interfere with enhancer–promoter interactions when interposed between the *white* enhancer and the *mini-white* reporter but has no effect on *mini-white* expression when placed upstream of the *white* enhancer. There were, however, two troubling observations.

The first is the observation that only half of the construct w#4 lines had the eye and testes pigmentation phenotype expected for an insulator element. At least two factors could account for this result. One would be position effects arising from regulatory elements located downstream of the *mini-white* transgene (c.f. Roseman et al. 1993). Although *scs'* is located 3' of *mini-white* in our vector, this element is not able to insulate completely against chromosomal position effect (Vazquez and Schedl 1994). The other factor may be the relative strength of the *Fab-7* element. Incomplete enhancer blocking activity has been observed in the *white* assay system for boundary elements whose activity has been partially compromised by deletion (Vazquez and Schedl 1994). This issue is considered further below.

The second troubling observation came when we compared the eye color of animals heterozygous and homozygous for the *Fab-7* transgenes w#4 and w#5. Normally the eye color of animals homozygous for a *mini-white* transgene are darker than the eye color of heterozygous animals. However, in about 10% of the lines the eye color is lighter, not darker, in homozygous animals. Moreover, in some cases, regions of the eye are completely white indicating that even the basal activity of the *mini-white* promoter has been shut off. This phenomenon is called pairing-sensitive repression, and has been observed when PREs are included in *mini-white* constructs (Kassis et al. 1991; Fauvarque and Dura 1993; Chan et al. 1994; Gindhart and Kaufman 1995). Consistent with the presence of a PRE in the 3.3-kb *HindIII*–*XbaI* fragment, we found that the pairing-sensitive repression of *mini-white* requires *Pc-G* genes (K. Hagstrom, M. Muller, and P. Schedl, in prep.). Other workers have also detected a PRE-like silencer in constructs containing large fragments from the *Fab-7* region of the BX-C (Busturia and Bienz 1993; Zink and Paro 1995). Although this PRE silencing activity would not seem to account for the fact that *Fab-7* fragments block the action of the *white* enhancer only when interposed be-

tween the enhancer and the *mini-white* reporter, it would nevertheless complicate interpretation of our experiments.

Silencing activity maps to the distal side of the Bluetail transposon

One way to resolve the question of whether *Fab-7* is a boundary or a silencer is to determine whether these two activities can be separated. For this purpose we divided the 3.3-kb *Fab-7* fragment into three smaller fragments and introduced them into the *mini-white* reporter vector (Fig. 2C, also see K. Hagstrom, M. Muller, and P. Schedl, in prep.). The first of these (w#6) extends from the *HindIII* site at the proximal end of the 3.3-kb fragment to just beyond the minor nuclease hypersensitive site; the second (w#7) spans the first two major proximal nuclease hypersensitive sites, and the third (w#8) contains the third major nuclease hypersensitive site just beyond the site of insertion of the *Bluetail* transposon. Of these three subfragments only w#8 shows pairing sensitive silencing in our *mini-white* reporter system. About a third of the w#8 lines had reduced *mini-white* expression when the transgene is homozygous, and, like the 3.3-kb *HindIII*–*XbaI* fragment, this silencing depends upon *Pc-G* proteins. In studies described in detail elsewhere we have shown that fragment w#8 most likely contains the PRE for the *iab-7* cis-regulatory domain (K. Hagstrom, M. Muller, and P. Schedl, in prep.). It should be noted that, whereas fragment w#8 has silencing activity, it does not have enhancer-blocking activity (Fig. 2C). Although fragments w#6 and w#7 had no detectable silencing activity, they also showed no evidence of boundary function (Fig. 2C).

Fab-7 boundary activity maps to the hypersensitive sites on the proximal side of the Bluetail transposon

In previous studies two other *Fab-7* alleles, *Fab-7²* and *Fab-7³*, were generated by imprecise excision of the *Bluetail* transposon (Galloni et al. 1993). Both of these deletions extend proximally from the *Bluetail* transposon, and remove the major hypersensitive sites HS1 and HS2, but leave HS3 (and the *iab-7* PRE) intact (Fig. 2A). The DNA sequence removed in the smaller deletion, *Fab-7²*, corresponds closely to the *NsiI*–*EaeI* fragment contained in construct w#7. Consequently, we had anticipated that this fragment would have enhancer blocking activity; however, as described in the previous section it did not. This suggests that the sequences removed by *Fab-7²* may be essential, but not sufficient, for boundary activity. If this hypothesis is correct, then we might be able to reconstitute boundary activity by including additional proximal sequences.

For this purpose we tested a fragment which extends from *HindIII* to *Apal*, and covers the DNA sequences removed in the slightly larger *Fab-7³* deletion (w#9). We found that this *HindIII*–*Apal* fragment has enhancer-blocking activity (see Fig. 2D), and, as was observed for the 3.3 *Fab-7* fragment (w#4), ~50% of the transgenic lines had reduced eye pigmentation. However, unlike

the 3.3-kb fragment, the *HindIII*–*Apal* fragment does not show pairing-sensitive repression of *mini-white*. This finding confirms the localization of the PRE to distal side of the *Bluetail* transposon and demonstrates that boundary and silencing activities are distinct.

To delimit further the DNA sequences that are sufficient to confer boundary activity, we generated two additional constructs (Fig. 2D). Construct w#10 has a fragment that includes the major hypersensitive sites HS1 and HS2, plus the minor hypersensitive site. The *PstI*–*Apal* fragment in w#11 includes all of HS1 and HS2, but only about half of the minor hypersensitive site. As indicated in Fig. 2D, enhancer blocking activity roughly equivalent to that of the entire 3.3-kb fragment was observed for both of these smaller *Fab-7* fragments. Moreover, neither of these fragments showed any evidence of silencer activity. Thus, the *PstI*–*Apal* fragment in construct w#11 defines the maximum limits of the *Fab-7* element sufficient for enhancer blocking in this assay.

Fab-7 functions as a boundary in a ftz:hsp70/lacZ enhancer blocking assay

Although the results described in the previous sections indicate that the *Fab-7* element can block enhancer–promoter communication, it could be argued that our *mini-white* system measures boundary activity at stages of development and/or in cell types that are not especially relevant to BX-C regulation. Consequently, it was important to determine whether *Fab-7* also has enhancer-blocking activity at developmental stages and in cell types in which the element would normally be required. For this purpose, we chose the *fushi tarazu* (*ftz*):*hsp70/lacZ* reporter diagrammed in Fig. 4A. This reporter has two enhancers from *ftz*, the upstream element (UPS) that activates the *hsp70* promoter in even-numbered parasegments of germband-extended embryos, and the neurogenic element (NE) that activates the *hsp70* promoter in the central nervous system (CNS) of germband-retracted embryos (Hiromi et al. 1985; Hiromi and Gehring 1987). The *ftz:hsp70/lacZ* reporter is also advantageous because it can be used to ask whether the activity of the *Fab-7* boundary is modulated along the anterior–posterior axis (c.f. Peifer et al. 1987).

We generated four constructs with the *ftz:hsp70/lacZ* vector (Fig. 4A). The first, fh#1, controls for distance effects in this assay system and has a random DNA fragment inserted between the *ftz* enhancers and the *hsp70* promoter. The second, fh#2, tests the enhancer-blocking function of *Fab-7*. It has a *Fab-7* fragment (*NcoI*–*Apal*) that includes the boundary but not the silencer. Finally, in constructs fh#3 and fh#4 we have inserted a constitutively active insulator, the *suppressor of Hairy-wing* (*su(Hw)*) element from the *gypsy* transposon, between the *ftz* enhancers and the *hsp70* promoter (Roseman et al. 1993; Scott and Geyer 1995). Besides providing a control for any parasegmental regulation of *Fab-7* boundary activity, these two constructs were designed to address an issue raised by the *mini-white* assay, namely the strength of *Fab-7* enhancer blocking activity. It is thought that the blocking activity of the *su(Hw)* element

Figure 4. *ftz* enhancer:*hsp70/lacZ* reporter construct design and enhancer-blocking results. (A) The basic *ftz:hsp70/lacZ* P-element construct contains two enhancers from the *ftz* gene. The UPS (lightly shaded box) drives expression in seven stripes in germ band-extended embryos and the NE (darkly shaded box) drives expression in the CNS of germband-retracted embryos (Hiromi et al. 1985; Hiromi and Gehring 1987). The reporter is an *hsp70*(-194) promoter fragment, which includes the HSE upstream sequences required for heat shock induction, fused to the *lacZ* gene. The fragments tested between the enhancers and promoter are as follows: (fh#1) 1.4-kb random DNA control fragment from *Bic-D* (Suter et al. 1989); (fh#2) 1.7-kb *Fab-7* *Apal*-*NcoI* boundary fragment; (fh#3) 390-bp fragment from *gypsy* (and pUC18) with only 5 *su(Hw)* binding sites; (fh#4) 440-bp fragment from *gypsy* with all 12 *su(Hw)* binding sites (Spana et al. 1988). (B) The relative levels of β -galactosidase staining for each of the fh constructs were scored as none, faint, light, moderate, and dark. All lines were scored simultaneously and compared relative to selected control lines. Each individual transgenic line is shown as one bar above the name of the construct, and the relative level of staining of each line is indicated by the height of the bar. All lines that gave no staining were treated with heat shock, and only the *su(Hw)*12 line could be induced, suggesting that only in this line is the lack of staining attributable to strong enhancer blocking.

depends upon the number reiterated binding sites for the Su(Hw) protein (Peifer and Bender 1988; Smith and Corcos 1992). Construct fh#3 has five Su(Hw) protein-binding sites, whereas construct fh#4 has twelve binding sites, the number of sites typically found in *gypsy* transposons. The pattern of β -galactosidase expression in transgenic embryos from a line representative of each of the four *ftz:hsp70/lacZ* constructs is shown in Figure 5.

For the random DNA control, fh#1, we observe seven darkly stained β -galactosidase stripes in germband-extended embryos. The level of β -galactosidase expression in these embryos is indistinguishable from that observed in embryos transgenic for the starting *ftz:hsp70/lacZ*

vector (not shown). This would suggest that the activation of the *hsp70* promoter by the *ftz* UPS enhancer is not significantly perturbed by a small increase in distance. This also appears to be true for the *ftz* NE enhancer element. This enhancer drives high levels of β -galactosidase expression in the CNS of germband-retracted fh#1 embryos. As indicated in the graphs in Figure 4B, seven of the ten fh#1 transgenic lines had high levels of stripe and CNS β -galactosidase expression like that observed in the representative embryos shown in Figure 5 (dark in Fig. 4B). For the three remaining lines, one had an intermediate level of β -galactosidase expression (moderate, like that observed for fh#3 embryos in Fig. 5), whereas no expression could be detected in the other two lines (none). The lack of expression in these two lines is likely to be attributable to some type of position effect (see below).

The level of β -galactosidase expression is reduced relative to the control in embryos transgenic for the construct containing five binding sites for the Su(Hw) protein, fh#3. As can be seen for one of the fh#3 transgenic lines in Figure 5, the intensity of β -galactosidase staining in the seven stripes and also in the CNS is decreased compared to that observed for the transgenic line carrying the random DNA control, fh#1. The results for all of the fh#3 transgenic lines are summarized in Figure 4B. The level of stripe and CNS β -galactosidase expression in 8 of the 14 lines resembles the fh#3 embryos shown in Figure 5, and these fall into the moderate class. Five lines had levels of expression equivalent to the fh#1 control and these are in the dark class, whereas the one remaining line had only a low level of expression and is in the light class. Thus a *su(Hw)* 5 mer has a weak enhancer-blocking activity.

Much stronger enhancer blocking is observed for the construct containing twelve *su(Hw)* binding sites, fh#4. As illustrated in Figure 5, the *su(Hw)* 12 mer causes a substantial decrease in the level of β -galactosidase expression. The characteristic stripe and CNS staining patterns generated by the UPS and NE enhancers are mostly eliminated, and only some residual staining is evident. Four of the five fh#4 lines have stripe and CNS expression patterns like those shown in Figure 5 (faint), whereas β -galactosidase expression is reduced even further in the fifth line. These findings demonstrate that the enhancer-blocking activity of the *su(Hw)* element depends upon the number of *su(Hw)* binding sites.

The level of β -galactosidase expression in embryos transgenic for the *Fab-7* construct, fh#2, is in between that observed for the constructs containing five and twelve *su(Hw)* binding sites. As shown in Figure 5, the *Fab-7* boundary substantially reduces both stripe and CNS staining. Although the level of stripe or CNS expression is not precisely the same in all parasegments of fh#3 embryos, equivalent parasegmental-specific differences are observed for the control constructs containing the constitutively active *su(Hw)* boundary or random DNA (Fig. 5). Thus, these parasegmental differences most likely reflect some intrinsic property of the *ftz* UPS and NE enhancer elements, rather than a parasegmental

Figure 5. β -galactosidase staining of representative lines transgenic for *ftz:hsp70/lacZ* constructs fh#1–4. The UPS enhancer-driven seven-stripe staining pattern in germband-extended embryos is shown at *left*, and the NE enhancer-driven CNS staining pattern in germband-retracted embryos is shown at *right*. Embryos were stained simultaneously, and a representative of the average level of staining for each fh construct is shown. Anterior is at *left*, and posterior at *right*.

specificity of *Fab-7*. As indicated in Figure 4B, β -galactosidase expression in six of the eight *Fab-7* transgenic lines resembles the line shown in Figure 5 (light). Of the two remaining lines, one showed a moderate level of β -galactosidase expression much like that observed for constructs containing five Su(Hw) protein binding sites (see Fig. 5), whereas no β -galactosidase expression could be detected in the other line (see below). These results demonstrate that *Fab-7* can block enhancer–promoter interactions in a second independent assay system, and that the relative enhancer-blocking activity of the *Fab-7* boundary is in between that generated by five and twelve copies of the Su(Hw) protein binding site.

The Fab-7 boundary does not repress intrinsic promoter or enhancer activity

One criterion that distinguishes a boundary or insulator element from a silencer is whether it has any effect on the intrinsic activity of the promoter or the enhancer. A boundary element is expected to reduce reporter gene expression by impeding enhancer–promoter communication. In contrast, silencers exert their effects on reporter gene expression either by repressing the promoter, or by inactivating the enhancer. The *hsp70* promoter fragment used in our *ftz* reporter construct contains not only the sequences required for basal promoter activity (the GAGA binding sites, the TATA box, and the transcription start site) but also the upstream HSE elements that activate the promoter in response to heat shock (Simon et al. 1985). Hence we can use heat induction to test whether the *Fab-7* element has any inhibitory effect on the activity of either the basal *hsp70* promoter or the HSE elements.

As illustrated in Figure 6, the *hsp70* promoter in the construct containing the *Fab-7* boundary is efficiently induced by heat shock. The level of induction in the

Fab-7 (fh#2) transgenic embryos appears to be equivalent to that observed in embryos transgenic for either the random DNA control (fh#1) or the *su(Hw)* fragment (fh#4). This is true for both germ band-extended embryos (Fig. 6), in which *Fab-7* blocks *hsp70* promoter activation by the *ftz* UPS enhancer, and for germ band-retracted embryos (not shown), in which *Fab-7* blocks *hsp70* promoter activation by the *ftz* NE enhancer. Because the *hsp70* promoter can be induced efficiently, the enhancer-blocking activity of *Fab-7* in the *ftz* assay cannot be attributed to the silencing of the basal *hsp70* promoter. Similarly, the fact that the HSE elements in the fh#2 constructs appear to be fully active would suggest that the *Fab-7* fragment is unlikely to function by directly inhibiting enhancers.

We also tested whether the *hsp70* promoter can be activated by heat shock in the three exceptional transgenic lines (two control fh#1 lines, and one *Fab-7* fh#2 line) that showed little stripe and CNS β -galactosidase expression. We found that β -galactosidase could not be induced by heat shock in either of these control lines (fh#1) or in the *Fab-7* (fh#2) line (not shown). This would suggest that the basal promoter of these particular transgenes is inactivated by some type of position effect.

Blocking activity depends upon the enhancer–promoter combination

The *ftz* reporter used in the blocking assays described above combines the *ftz* enhancers with a heterologous *hsp70* promoter. We wondered whether the *Fab-7* and *su(Hw)* boundaries would have an equivalent blocking activity when assayed in a construct that instead combines the *ftz* enhancers with the homologous *ftz* promoter (ff#1). As shown in Figure 7A, we placed the *Fab-7* (ff#2) and the *su(Hw)* 12 mer (ff#3) boundaries between the NE and UPS enhancers and the *ftz* promoter in this *ftz:ftz/lacZ* reporter. This reporter differs from the

Figure 6. The *Fab-7* boundary does not inactivate the *hsp70* promoter or the HSE elements. Embryos transgenic for *ftz:hsp70/lacZ* constructs containing random DNA, the *Fab-7* *ApaI-NcoI* fragment, and the *su(Hw)*12 binding site fragments are shown with (right) and without (left) heat shock at 37° for 1 hr. In all constructs heat shock treatment induces equivalent high levels of β -galactosidase expression.

ftz:hsp70/lacZ reporter in that it also contains the *ftz* Zebra element that is located immediately adjacent to the *ftz* basal promoter (Hiromi et al. 1985; Hiromi and Gehring 1987). In pre-germ band-extended embryos, the Zebra element directs β -galactosidase expression in seven stripes, and this makes it difficult to determine whether a boundary element is able to block the stripe expression generated later in development by the UPS enhancer. For this reason, we only examined the effects

of the boundary elements on the NE-dependent CNS expression.

The results obtained with the *ftz:ftz/lacZ* reporter are somewhat different from the *ftz:hsp70/lacZ* reporter (Fig. 7B). First, the level of β -galactosidase activity observed in the CNS of embryos transgenic for the starting *ftz:ftz/lacZ* reporter appeared to be somewhat less than that found in embryos transgenic for the *ftz:hsp70lacZ* reporter. Second, although the *Fab-7* boundary is able to

Figure 7. *ftz:ftz* promoter/*lacZ* construct design and comparison of enhancer blocking to *ftz:hsp70* promoter/*lacZ* constructs. (A) The basic *ftz:ftz/lacZ* reporter construct contains the same UPS and NE enhancers as in the *ftz:hsp70/lacZ* construct (shaded boxes; also see Fig. 4). The promoter fused to *lacZ* (arrow), however, is derived from the *ftz* upstream region and contains basal promoter elements as well as the zebra element enhancer (striped box) that drives a seven-stripe pattern in early embryos (Hiromi et al. 1985; Hiromi and Gehring 1987). Because of this additional stripe enhancer, we only assayed blocking of the NE enhancer. (B) The β -galactosidase pattern generated by the NE is shown in germband-extended embryos transgenic for *ftz* promoter/*lacZ* constructs (left) or *hsp70* promoter/*lacZ* constructs (right). Without inserts (top), the basic *ftz* promoter construct has slightly weaker staining than the basic *hsp70* promoter construct. The insertion of the *ApaI-NcoI* *Fab-7* fragment (middle) causes a slight reduction of NE driven staining in the *ftz* promoter construct, and a greater reduction in the *hsp70* promoter construct. The insertion of 12 *su(Hw)* binding sites (bottom) causes nearly complete loss of staining in both, although this reduction is slightly more pronounced in the *hsp70* promoter construct.

block NE-*ftz* promoter interactions, the boundary does not appear to function as effectively in the *ftz* promoter construct as it does when the NE enhancer is combined with the heterologous *hsp70* promoter. This can be seen by comparing CNS expression in the control and experimental embryos in Figure 7B. Third, the *su(Hw)* insulator also appears to be slightly less effective in the *ftz:ftz/lacZ* reporter (Fig. 7B), although the difference in *su(Hw)* activity in the *hsp70* and *ftz* promoter constructs is less pronounced than it is for *Fab-7*.

Proteins that influence su(Hw) enhancer blocking do not affect Fab-7 blocking

Two *trans*-acting factors have been implicated in the blocking activity of the *su(Hw)* element. One of these is encoded by the *su(Hw)* locus, and loss-of-function mutations in this gene reduce or eliminate blocking (Modolell et al. 1983; Roseman et al. 1993). The other is encoded by the *mod(mdg4)* locus. Mutations in *mod(mdg4)* have been reported to have an unusual effect on the enhancer-blocking activity of a *gypsy* transposon inserted in the *yellow* locus (the *y²* allele) (Gerasimova et al. 1995). In the *mod(mdg4)* background the *su(Hw)* element within the *gypsy* transposon mimics a bidirectional silencer and interferes with the activity of *yellow* enhancers located both upstream and downstream of the transposon insertion.

We used the *ftz:hsp70/lacZ* reporter to test whether either of these genes is also important for *Fab-7* boundary function. As expected, we found that mutations in *su(Hw)* relieve the blocking activity of reiterated *su(Hw)* binding sites (compare wt with *su(Hw)*⁻ in Fig. 8). For example, the 12 mer largely eliminates β -galactosidase expression in a wild-type background; however, in a *su(Hw)* mutant background, stripe expression is restored to a level equivalent to that of a *ftz:hsp70/lacZ* construct containing random DNA (not shown; note that levels of expression appear lower than in other figures because embryos in Fig. 8 are heterozygous for the transgene). Because studies on *y²* suggest that the *mod(mdg4)*

mutation causes the *gypsy* element to mimic a silencer, we expected that the mutation might silence the *ftz* enhancers in reporters containing the reiterated *su(Hw)* binding sites, accentuating the apparent blocking activity (Gerasimova et al. 1995). However, we obtained the opposite result. Mutations in *mod(mdg4)* restore β -galactosidase expression in the *su(Hw)* lines (Fig. 8). This finding suggests that the enhancer-blocking activity of the reiterated *su(Hw)* binding sites in the *ftz:hsp70/lacZ* reporter requires the *mod(mdg4)* protein. Unlike the *su(Hw)* binding sites, the enhancer blocking activity of *Fab-7* is not noticeably affected by mutations in either *su(Hw)* or *mod(mdg4)* (Fig. 8). This suggests that a different set of proteins are required for the boundary function of *su(Hw)* and *Fab-7*.

Discussion

The Fab-7 mutation disrupts a domain boundary

A growing body of evidence indicates that eukaryotic chromosomes are subdivided into structurally and functionally distinct domains. In addition to providing a mechanism for organizing and compacting the large eukaryotic chromosomes inside the nucleus, the domain organization of the chromatin fiber can have a significant impact on gene regulation. For example, the parasegment-specific expression of the homeotic genes in the bithorax complex of *Drosophila* appears to depend upon the subdivision of the complex into a series of functionally independent *cis*-regulatory domains. This subdivision into autonomous domains is illustrated by the expression patterns of enhancer trap transposons integrated in different domains of the complex (Galloni et al. 1993; McCall et al. 1994). These enhancer traps are subject to regulatory elements located within the same domain, but are insensitive to regulatory elements in adjacent domains.

What ensures the autonomy of each domain? One mechanism may be the insulation of the domains by elements that can function as chromatin boundaries. Previous genetic studies have suggested that the *Fab-7*

Figure 8. Mutations in the *su(Hw)* and *mod(mdg4)* proteins relieve enhancer blocking by *su(Hw)* binding sites, but have no effect on *Fab-7* blocking activity. β -galactosidase staining patterns of germband-extended embryos heterozygous for *ftz:hsp70/lacZ* constructs containing 12 *su(Hw)* binding sites (fh#4, top row), five *su(Hw)* binding sites (fh#3, middle row), and the *Fab-7* boundary (fh#2, bottom row). In a wild-type background (wt, left column), these fragments show various degrees of staining attributable to enhancer blocking (as shown for homozygous embryos in Fig. 5). In a *su(Hw)* loss-of-function mutant background (*su(Hw)*⁻, middle column), and in a *mod(mdg4)* mutant background (*mod*⁻, right column) β -galactosidase levels increase in constructs containing *su(Hw)* fragments. Neither mutation affects staining of embryos containing *Fab-7* (bottom row, last two columns).

region of the bithorax complex is likely to contain such a boundary element (Gyurkovics et al. 1990; Galloni et al. 1993; Vazquez et al. 1993). This putative boundary appears to be required to separate the *iab-6* and *iab-7* cis-regulatory domains and small deletions in the *Fab-7* region result in the fusion of these two domains into a single regulatory unit. This fused domain inappropriately regulates the *Abd-B* homeotic gene in PS11, and typically causes a phenotypic transformation of PS11 into a copy of PS12.

In the studies reported here we have used enhancer-blocking assays to determine whether the *Fab-7* region actually contains an element that can act as an autonomous chromatin domain boundary. We have found that a *Fab-7* sequence spanning two major chromatin-specific nuclease-hypersensitive sites can function as a domain boundary in this assay, and block enhancer–promoter interactions. This same DNA sequence was implicated previously in the boundary activity of the *Fab-7* region within the context of the bithorax complex (Galloni et al. 1993; Karch et al. 1994). The mapping of boundary function in the bithorax complex has been extended in more recent studies on the *lacZ* expression pattern in mutants that delete *Fab-7* boundary sequences but retain the *Bluetail* transposon (J. Mihaly, I. Hogga, H. Gyurkovics, J. Gausz, and F. Karch, in prep.). This *in situ* analysis defines a minimal element for boundary function in the bithorax complex that corresponds almost precisely to the sequences required for enhancer blocking in our transgene assays.

As has been observed for other insulators, enhancer-blocking activity depends upon the position of the *Fab-7* element relative to the enhancer and promoter. The *Fab-7* element can prevent promoter activation only when interposed between the enhancer and the promoter, but has no effect on activation when the order is reversed and it is placed upstream of the enhancer and promoter. We have also found that the blocking activity of *Fab-7* can not be attributed to some repressive or inhibitory effect of the element on either the promoter or the enhancer. Like other generic boundaries, blocking activity is not restricted to a specific enhancer or promoter, but can be detected in several different enhancer–promoter combinations. Similarly, the *Fab-7* element is able to function as a boundary in a variety of cell and tissue types, and at different stages of development.

The results reported here demonstrate that *Fab-7* has many properties expected for an element that functions as a boundary of higher order chromatin domains. Whereas numerous other elements of this type have been identified, for the most part the biological functions of these putative domain boundaries have not yet been clearly established (Kellum and Schedl 1991; Chung et al. 1993; Roseman et al. 1993; for review, see Schedl and Grosfeld 1995). In this respect, *Fab-7* is an exceptional case as genetic studies have demonstrated that deletion of the *Fab-7* boundary has profound consequences on the regulation of the homeotic *Abd-B* gene. As such, *Fab-7* would be one of the first examples of an element whose boundary activity is known to be essential *in vivo*.

The Fab-7 boundary is distinct from an adjacent PRE silencer

Two previous studies on transgenes containing a large DNA fragment from the *Fab-7* region uncovered a potent negative regulatory activity (Busturia and Bienz 1993; Zink and Paro 1995). This finding led to the suggestion that *Fab-7* is a gap and *Pc-G* gene dependent repressor rather than a boundary element (Busturia and Bienz 1993). Although we have also detected a *Pc-G* dependent silencer in the *Fab-7* region, our experiments indicate that this silencer is completely distinct from the *Fab-7* boundary. As shown in Figure 2A, we have found that the silencer is contained in a 0.8-kb *AatI*–*Xba* I restriction fragment located just distal to the site of insertion of the *Bluetail* transposon. In studies described in detail elsewhere (K. Hagstrom, M. Muller, and P. Schedl, in prep.) we have shown that this silencer corresponds to a PRE for the *iab-7* cis-regulatory domain. Although we have not yet defined the precise limits of this *iab-7* PRE, it seems likely that nuclease hypersensitive site III (see Fig. 2A) is critical for silencing activity as it contains a conserved sequence found in PREs from other BX-C cis-regulatory domains (Karch et al. 1994; unpubl.). Whereas the *iab-7* PRE maps to the distal side of the *Bluetail* insertion site, we show here that sequences critical for boundary function in enhancer blocking assays are located to the proximal side of the transposon insertion site. The minimal *Fab-7* boundary element defined by our assays includes two strong nuclease hypersensitive sites (HS1 and HS2), plus part of a minor hypersensitive site (Fig. 2).

Our functional dissection of the *Fab-7* region into a boundary element and a PRE provides a plausible explanation for why the phenotype of the original *Fab-7*¹ is slightly different from the two deletions, *Fab-7*² and *Fab-7*³, generated by excision of the *Bluetail* transposon (Gyurkovics et al. 1990; Galloni et al. 1993). In *Fab-7*¹ the *iab-7* cis-regulatory domain is ectopically activated in all PS11 cells and, in adults, this causes a complete transformation of abdominal segment A6 into A7. Although *iab-7* is also ectopically activated by the *Fab-7*² and *Fab-7*³ mutations, this activation does not always occur and A6 has small clones of cells in which *iab-7* remains off (Galloni et al. 1993; J. Mihaly, I. Hogga, H. Gyurkovics, J. Gausz, and F. Karch, in prep.).

As indicated in Figure 2A, the deletion in *Fab-7*¹ removes not only the *Fab-7* boundary but also the *iab-7* PRE. The loss of the boundary element results in the fusion of the *iab-6* and *iab-7* cis-regulatory domains. Because the *iab-7* PRE is also missing, the fused domain is primed to ectopically activate *iab-7* in PS11, leading to the complete transformation of PS11 into a copy of PS12 (A6 to A7). In contrast, the deletions in both *Fab-7*² and *Fab-7*³ extend proximally from the *Bluetail* insertion site and remove the *Fab-7* boundary, but leave the *iab-7* PRE intact. Like *Fab-7*¹, the removal of the boundary element results in a fusion of the *iab-6* and *iab-7* cis-regulatory domains. However, because the *iab-7* PRE is still present in these mutants, the interactions between

positive and negative initiation elements in the fused domain need not lead exclusively to the permanent activation of *iab-7* in PS11. Instead, there is a competition between ectopic activation of *iab-7* by positive elements in *iab-6* and ectopic silencing of *iab-6* by negative elements in *iab-7* (J. Mihaly, I. Hogga, H. Gyurkovics, J. Gausz, and F. Karch, in prep.).

Properties of the *Fab-7* boundary

In general, the properties of the *Fab-7* element evident in the enhancer-blocking assays closely resemble those of classically defined insulators such as *su(Hw)*. In fact, the only major difference apparent in our experiments is that *Fab-7* does not require either the *su(Hw)* or the *mod-(mdg4)* proteins. There are, however, some subtle differences that may ultimately be relevant to understanding how the *Fab-7* boundary functions in the regulation of BX-C gene expression. The most clear-cut is the relative strength of the blocking activity. This is best illustrated in the experiments with the *ftz:hsp70/lacZ* reporter where we compared the blocking activity of the *Fab-7* element with *su(Hw)* elements containing five and twelve binding sites. We found that the enhancer-blocking activity of the *Fab-7* element is not as strong as the *su(Hw)* 12 mer but is stronger than the *su(Hw)* 5 mer. The intermediate strength of the *Fab-7* boundary might also account for the position-dependent blocking in the *white* assay system (Vazquez and Schedl 1994). A second difference was noted in our analysis of the enhancer-blocking activity of *Fab-7* in the CNS using the *ftz:ftz/lacZ* reporter. Whereas the *Fab-7* element reduced CNS β -galactosidase expression, it was clearly less effective in blocking the action of the *ftz* NE enhancer in this reporter construct than it was in the *ftz:hsp70/lacZ* construct. By contrast, the enhancer-blocking activity of the *su(Hw)* 12 mer seemed to be relatively unaffected by the switch from the heterologous *hsp70* to the homologous *ftz* promoter. This finding could indicate that the potency of the *Fab-7* element depends upon the specific enhancer-promoter combination.

Domain boundaries in the BX-C

Although the *Fab-7* boundary can block enhancer-promoter communication much like other insulators, this is not the normal function of *Fab-7* in the bithorax complex. Rather, *Fab-7* ensures the autonomous activity of the *iab-6* and *iab-7* cis-regulatory domains (Gyurkovics et al. 1990; Galloni et al. 1993). Early in development when parasegment identity is initially determined, the *Fab-7* boundary must prevent adventitious interactions between gap and pair-rule gene products associated with each cis-regulatory domain. In the absence of the boundary, these adventitious interactions can lead to the inappropriate activation or inactivation of the cis-regulatory domains, and the improper regulation of *Abd-B* expres-

sion. As part of this process the *Fab-7* boundary must also ensure that the maintenance system in each domain, the PRE (or TRE), is properly programmed. For example, in PS11, *Fab-7* must prevent the gap and pair-rule gene products associated with the *iab-6* cis-regulatory domain from inappropriately inactivating the *iab-7* PRE. (A misprogrammed *iab-7* PRE would not keep the *iab-7* cis-regulatory domain silent in PS11, leading to the ectopic expression of *Abd-B*.) Once BX-C regulation switches to the maintenance mode, the main function of the boundary is presumably to restrict PRE silencing (or TRE activation) to the appropriate domain. Thus, in PS11 the *Fab-7* boundary must prevent the *Pc-G* proteins associated with the *iab-7* PRE in the silent *iab-7* cis-regulatory domain from ectopically silencing the adjacent *iab-6* domain.

An obvious expectation of this model is that the BX-C contains additional *Fab-7*-like boundaries that block adventitious interactions between positive and/or negative regulatory elements in adjacent domains (see Fig. 1). In addition to their insulation function, these *Fab-7*-like boundaries could provide a mechanism for building functionally autonomous regulatory units with distinct parasegment specificity. The parasegmental specificity of each unit would be dictated by the particular combination of gap and pair-rule responsive elements that are included within the DNA segment delimited by a pair of *Fab-7*-like boundaries. Whereas the existence of other *Fab-7*-like boundary elements in BX-C has not been established, there are several potential candidates. These include the *Mcp* region that is located between *iab-4* and *iab-5* (Karch et al. 1985) and an element (M. Galloni, S. Barges and F. Karch, pers. comm.) between *iab-7* and *iab-8* that has enhancer-blocking activity in the *mini-white* assay system (M. Muller, unpubl.).

Whereas *Fab-7* ensures the independent activity of the *iab-6* and *iab-7* cis-regulatory domains, its ability to block enhancer-promoter communication when interposed between an enhancer and a promoter poses a problem. Why does this enhancer-blocking activity not prevent the more proximal *iab-5* and *iab-6* cis-regulatory domains from controlling the expression of the distal *Abd-B* gene (see Fig. 1) in PS10 and PS11 (especially because *Fab-7* can block these cis-regulatory domains from activating the *Bluetail* transposon)? One factor may be the strength of the *Fab-7* boundary. The intermediate enhancer-blocking activity of the *Fab-7* element might be sufficient to prevent cross-talk between the cis-regulatory domains, but insufficient to obstruct the proximal cis-regulatory domains from interacting with *Abd-B*. Another factor is suggested by the response of the *Fab-7* and *su(Hw)* boundaries to different enhancer-promoter combinations. There may be specialized proteins that facilitate long-distance communication between these cis-regulatory domains and the *Abd-B* promoter. Whereas a generic boundary like *su(Hw)* might be able to interfere efficiently with these specialized proteins, the *Fab-7* boundary may be designed so that it does not. Further studies will clearly be required to resolve this question.

Materials and methods

Cloning of P-element constructs

white enhancer: mini-white The basic CaSpeR P-element construct is derived from the pRW vector described in Vazquez and Schedl (1994). It was modified to contain a minimal white enhancer fragment that drives expression in the eye and testes, and extends only from *Bam*HI to *Hind*II (bp 4433–5540 in O'Hare et al. 1984). Unique cloning sites were introduced upstream (*Kpn*I) and downstream (*Not*I and *Xho*I) of the enhancer. At the 3' end of the mini-white gene is a 450-bp *Eco*RI to *Bam*HI fragment of *scs*' to minimize position effects (Kellum and Schedl 1992). However, this *scs*' fragment is not a strong enhancer blocker and may not completely insulate the 3' end from position effects (Vazquez and Schedl 1994).

The *Fab-7* fragments tested in constructs w#3-12 were isolated from a 3.35-kb *Hind*III to *Xba*I *Fab-7* fragment inserted in *Bluescript*. This *Fab-7* region was originally isolated from phage λ 8053 (Karch et al. 1985) and spans bp 163–3517 as indicated in (Karch et al. 1994). The *Fab-7* restriction fragments were cloned in a variety of ways; generally, if ends were not compatible they were modified with linkers to allow cloning into the unique sites.

The random DNA tested in w#3 is a 2.3-kb *Not*I–*Xho*I fragment of *Bic-D* cDNA (from plasmid pc15a+ described in Suter et al. 1989).

ftz enhancers: hsp70 promoter/lacZ The basic CaSpeR P-element construct contains two enhancers from the *ftz* regulatory region: the UPS and the NE. The enhancers were derived from within a 10-kb *Kpn*I fragment that contains the *ftz* gene and its cis-regulatory regions (Hiromi et al. 1985). The upstream element is a 2.7-kb *Kpn*I–*Xba*I fragment, and the neurogenic enhancer is a *Xba*I–*Bal*I fragment. The *hsp70/lacZ* reporter gene fusion is as described in Simon et al. (1985). The *hsp70* promoter fragment begins at –194 (thus including regulatory elements such as the heat shock elements, the GAGA factor binding sites, and the basal promoter) and extends to include the first 37 amino acids of the *hsp70* ORF (Simon et al. 1985). This fragment is fused to the *lacZ* ORF. At the unique *Sac*I site within *lacZ* is a fusion to the remainder of *lacZ* and an *hsp70* trailer sequence (as found in plasmid pryFGH3 from Hiromi et al. 1985). A 450-bp *Eco*RI–*Bam*HI fragment of *scs*' is located just beyond the trailer sequences of the *hsp70/lacZ* reporter to minimize position effects (see above). The mini-white gene of CaSpeR is located at the 5' end of the construct as a marker for transformation.

Various test fragments were cloned into this vector using unique *Not*I and *Xho*I sites between the enhancers and the promoter. The random DNA in fh#1 is a 1.4-kb *Not*I–*Hind*III fragment of *Bic-D* cDNA (from plasmid pc15a+ described in Suter et al. 1989). The *Fab-7* insert in fh#2 is a 1.7-kb *Apa*I–*Nco*I fragment (Fig. 2) that has enhancer blocking, but not silencing, function in the white enhancer:mini-white assay. The *su(Hw)*12 fragment in fh#4 is derived from a *Xmn*I–*Bsp* 1286 fragment of the gypsy transposon [position 670–1041 in Spana et al. (1988) that includes 12 binding sites for the Su(Hw) protein]. The *su(Hw)*5 fragment in fh#3 includes only five Su(Hw) protein binding sites from within the same fragment (position 670–830 in Spana et al. 1988) but in addition contains 195 bp of pUC18 sequences.

ftz enhancers:ftz promoter/lacZ This construct is identical to the *ftz:hsp70/lacZ* vector, except that the *hsp70/lacZ* reporter is replaced by the *ftz/lacZ* reporter. The *ftz* promoter is originally derived from within a 10-kb *Kpn*I *ftz* gene fragment (Hi-

romi et al. 1985). In addition to basal promoter elements, the *ftz* promoter includes the zebra element, which in this construct extends to a *Hind*III site, 281 bp further 5' than the zebra element described in Hiromi et al. (1985). This promoter fragment extends to amino acid position 270 of the *ftz* ORF, includes four newly introduced amino acids, and is then fused to the fifth amino acid of *lacZ*, and again ends with trailer sequences from *hsp70*. The *Fab-7* and *su(Hw)* 12 fragments tested in this vector are the same as those tested in the *ftz:hsp70/lacZ* vector.

P-element transformation

Of each P-element construct, 0.5 mg/ml was injected along with P-turbo helper plasmid (pUCHspD2-3wc) into *w*¹ embryos, and transformants were selected by rescue of the *w*[–] eye color. Crosses to marked balancer chromosomes were performed to generate balanced stocks and to determine the chromosome of insertion for each line.

Scoring reporter gene expression

mini-white To compare the relative levels of mini-white expression between different lines, homozygous transgenic lines were crossed back to *w*¹, and heterozygous progeny of the same sex and age were examined. (Heterozygous females that were 1 day old allowed the most sensitive detection of differences in eye color.) The eye color of lines heterozygous for transgenes carrying the various fragments of *Fab-7* were scored relative to the eye color of a control containing the basic construct (w#2). All flies were raised at 22°.

β -galactosidase Embryos were collected at 0–15 hr and stained overnight for β -galactosidase activity according to the protocol in (Bellen et al. 1989) except that embryos were fixed in one part PBS, one part 50% glutaraldehyde, and two parts heptane. To facilitate comparison of relative levels, stainings were done simultaneously in the same dish, and the same positive and negative control lines were included each time staining was performed.

Heat shock of the *ftz:hsp70/lacZ* transgenic lines was performed by collecting 0–14 hr embryos, transferring a portion of them to a prewarmed apple juice plate, incubating at 37° for 1 hr, then staining as described.

Testing su(Hw) and mod(mdg4) mutant backgrounds

The mutant strains used were: *ywf;su(Hw)*^v, *bx^{34e}/TM6*, *Ubx*, *su(Hw)*^f (a null over a hypomorph), and *mod(mdg4)^{u1}/mod(mdg4)^{u1}* (nature of allele unknown) (Gerasimova et al. 1995). Males homozygous for fh enhancer blocking constructs were crossed to *w*¹ females as a control or to mutant females, and β -galactosidase stainings of the resulting progeny were compared.

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References

- Bellen, H.J., C. O'Kane, C. Wilson, U. Grossniklaus, R.K. Pearson, and W.J. Gehring. 1989. P-element mediated enhancer detection: A versatile method to study development in *Drosophila*. *Genes & Dev.* **3**: 1288–1300.
- Bienz, M. 1992. Molecular mechanisms of determination in *Drosophila*. *Curr. Opin. Cell Biol.* **4**: 955–961.
- Busturia, A. and M. Bienz. 1993. Silencers in *Abdominal-B*, a homeotic *Drosophila* gene. *EMBO J.* **12**: 1415–1425.
- Casares, F. and E. Sanchez Herrero. 1995. Regulation of the infraabdominal regions of the bithorax complex of *Drosophila* by gap genes. *Development* **121**: 1855–1866.
- Celniker, S.E., S. Sharma, D.J. Keelan, and E.B. Lewis. 1990. The molecular genetics of the bithorax complex of *Drosophila*: cis-regulation in the *Abdominal-B* domain. *EMBO J.* **9**: 4277–4286.
- Chan, C.S., L. Rastelli, and V. Pirrotta. 1994. A Polycomb response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *EMBO J.* **13**: 2553–2564.
- Chung, J.H., M. Whiteley, and G. Felsenfeld. 1993. A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* **74**: 505–514.
- Duncan, I. 1987. The bithorax complex. *Annu. Rev. Genet.* **21**: 285–319.
- Fauvarque, M.O. and J.M. Dura. 1993. *polyhomeotic* regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in *Drosophila*. *Genes & Dev.* **7**: 1508–1520.
- Galloni, M., H. Gyurkovics, P. Schedl, and F. Karch. 1993. The *bluetail* transposon: Evidence for independent cis-regulatory domains and domain boundaries in the bithorax complex. *EMBO J.* **12**: 1087–1097.
- Gerasimova, T.I., D.A. Gdula, D.V. Gerasimov, O. Simonova, and V.G. Corces. 1995. A *Drosophila* protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. *Cell* **82**: 587–597.
- Gindhart, J.G., Jr. and T.C. Kaufman. 1995. Identification of Polycomb and trithorax group responsive elements in the regulatory region of the *Drosophila* homeotic gene *Sex combs reduced*. *Genetics* **139**: 797–814.
- Gyurkovics, H., J. Gausz, J. Kummer, and F. Karch. 1990. A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. *EMBO J.* **9**: 2579–2585.
- Hiromi, Y. and W.J. Gehring. 1987. Regulation and function of the *Drosophila* segmentation gene *fushi tarazu*. *Cell* **50**: 963–974.
- Hiromi, Y., A. Kuriowa, and W.J. Gehring. 1985. Control elements of the *Drosophila* segmentation gene *fushi tarazu*. *Cell* **43**: 603–613.
- Karch, F., B. Weiffenbach, M. Peifer, W. Bender, I. Duncan, S. Celniker, M. Crosby, and E.B. Lewis. 1985. The abdominal region of the bithorax complex. *Cell* **43**: 81–96.
- Karch, F., M. Galloni, L. Sipos, J. Gausz, H. Gyurkovics, and P. Schedl. 1994. *Mcp* and *Fab-7*: Molecular analysis of putative boundaries of cis-regulatory domains in the bithorax complex of *Drosophila melanogaster*. *Nucleic Acids Res.* **22**: 3138–3146.
- Kassiss, J.A., E.P. VanSickle, and S.M. Sensabaugh. 1991. A fragment of *engrailed* regulatory DNA can mediate transvection of the *white* gene in *Drosophila*. *Genetics* **128**: 751–761.
- Kellum, R. and P. Schedl. 1991. A position-effect assay for boundaries of higher order chromosomal domains. *Cell* **64**: 941–950.
- . 1992. A group of scs elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* **12**: 2424–2431.
- Kennison, J.A. 1993. Transcriptional activation of *Drosophila* homeotic genes from distant regulatory elements. *Trends Genet.* **9**: 75–78.
- Levis, R., T. Hazelrigg, and G.M. Rubin. 1985. Separable cis-acting control elements for expression of the *white* gene of *Drosophila melanogaster*. *EMBO J.* **4**: 3489–3499.
- Lewis, E.B. 1978. A gene complex controlling segmentation in *Drosophila*. *Nature* **276**: 565–570.
- McCall, K., M.B. O'Connor, and W. Bender. 1994. Enhancer traps in the *Drosophila* bithorax complex mark parasegmental domains. *Genetics* **138**: 387–399.
- Modolell, J., W. Bender, and M. Meselson. 1983. *D. melanogaster* mutations suppressible by the suppressor of *Hairy-wing* are insertions of a 7.3 kb mobile element. *Proc. Natl. Acad. Sci.* **80**: 1678–1682.
- Müller, J. and M. Bienz. 1992. Sharp anterior boundary of homeotic gene expression conferred by the *fushi tarazu* protein. *EMBO J.* **11**: 3653–3661.
- O'Hare, K., C. Murphy, R. Levis, and G. Rubin. 1984. DNA sequence of the *white* locus of *Drosophila melanogaster*. *J. Mol. Biol.* **180**: 437–455.
- Paro, R. 1990. Imprinting a determined state into the chromatin of *Drosophila*. *Trends Genet.* **6**: 416–421.
- Peifer, M. and W. Bender. 1988. Sequences of the gypsy transposon of *Drosophila* necessary for its effects on adjacent genes. *Proc. Natl. Acad. Sci.* **85**: 9650–9654.
- Peifer, M., F. Karch, and W. Bender. 1987. The bithorax complex: Control of segmental identity. *Genes & Dev.* **1**: 891–898.
- Pirrotta, V. 1988. Vectors for P-mediated transformation in *Drosophila*. In *Vectors: A survey of molecular cloning vectors and their uses* (ed. R.L. Rodriguez and D.T. Denhardt), pp. 437–456. Butterworth, Boston, MA.
- Roseman, R., V. Pirrotta, and P. Geyer. 1993. The *su(Hw)* protein insulates expression of the *Drosophila melanogaster white* gene from chromosomal position effects. *EMBO J.* **12**: 435–442.
- Sanchez-Herrero, E. 1991. Control of the expression of the bithorax complex genes *abdominal-A* and *abdominal-B* by cis-regulatory regions in *Drosophila* embryos. *Development* **111**: 437–449.
- Schedl, P. and F. Grosfeld. 1995. Boundaries and domains. In *Chromatin structure and gene expression* (ed. S.C.R. Elgin), pp. 172–196. IRL Press, New York, NY.
- Scott, K. and P. Geyer. 1995. Effects of the *su(Hw)* insulator protein on the expression of the divergently transcribed *Drosophila* yolk protein genes. *EMBO J.* **14**: 6258–6279.
- Shimell, M.J., J. Simon, W. Bender, and M.B. O'Connor. 1994. Enhancer point mutation results in a homeotic transformation in *Drosophila*. *Science* **264**: 968–971.
- Simon, J. 1995. Locking in stable states of gene expression:

- Transcriptional control during *Drosophila* development. *Curr. Opin. Cell Biol.* **7**: 376–385.
- Simon, J.A., C.A. Sutton, R.B. Lobell, R.L. Glaser, and J.T. Lis. 1985. Determinants of heat shock induced chromosomal puffing. *Cell* **40**: 805–817.
- Simon, J., M. Peifer, W. Bender, and M. O'Connor. 1990. Regulatory elements of the bithorax complex that control expression along the anterior–posterior axis. *EMBO J.* **9**: 3945–3956.
- Simon, J., A. Chiang, W. Bender, M.J. Shimell, and M. O'Connor. 1993. Elements of the *Drosophila* bithorax complex that mediate repression by Polycomb group products. *Dev. Biol.* **158**: 131–144.
- Smith, P.A. and V.G. Corces. 1992. The *su(Hw)* binding region is required for gypsy mutagenesis. *Mol. Gen. Genet.* **233**: 65–70.
- Spana, C., D.A. Harrison, and V.G. Corces. 1988. The *Drosophila melanogaster* suppressor of Hairy-wing protein binds to specific sequences of the gypsy retrotransposon. *Genes & Dev.* **2**: 1414–1423.
- Suter, B., L. Romberg, and R. Steward. 1989. *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry: Localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. *Genes & Dev.* **3**: 1957–1968.
- Vazquez, J. and P. Schedl. 1994. Sequences required for enhancer blocking activity of scs are located within two nuclease-hypersensitive regions. *EMBO J.* **13**: 5984–5993.
- Vazquez, J., G. Farkas, M. Gaszner, A. Udvardy, M. Muller, K. Hagstrom, H. Gyurkovics, L. Sipos, J. Gausz, M. Galloni, et al. 1993. Genetic and molecular analysis of chromatin domains. *Cold Spring Harbor Symp. Quant. Biol.* **58**: 45–54.
- Zink, D. and R. Paro. 1995. *Drosophila* Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. *EMBO J.* **14**: 5660–5671.

Fab-7 functions as a chromatin domain boundary to ensure proper segment specification by the *Drosophila* bithorax complex.

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