Developmental modulation of *Fab-7* boundary function

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Summary

The *Fab-7* boundary functions to ensure the autonomous activity of the *iab-6* and *iab-7* cis-regulatory domains in the *Drosophila* Bithorax Complex from early embryogenesis through to the adult stage. Although *Fab-7* is required only for the proper development of a single posterior parasegment, it is active in all tissues and stages of development that have been examined. In this respect, *Fab-7* resembles conventional constitutive boundaries in flies and other eukaryotes that act through ubiquitous cis-elements and trans-acting factors. Surprisingly, however, we find that the constitutive activity of *Fab-7* is generated by combining sub-elements with developmentally restricted boundary function. We provide in vivo evidence that the *Fab-7* boundary contains separable regions that function at different stages of development. These findings suggest that the units (domains) of genetic regulation that boundaries delimit can expand or contract by switching insulator function off or on in a temporally regulated fashion.

Key words: Fab-7, *Drosophila*, Bithorax, Boundary, Insulator, Abd-B

Introduction

Eukaryotic chromosomes are subdivided into functionally autonomous chromatin domains. Crucial to ensuring autonomy are special cis-acting elements called boundaries or insulators (Kellum and Elgin, 1998; Udvardy, 1999; Gerasimova and Corces, 2001; West et al., 2002; Schedl and Broach, 2003). These elements define the limits of each chromosomal domain and establish independent units of genetic activity by shielding genes or regulatory elements within the domain from the regulatory influences of adjacent domains. Most of the boundaries that have been identified in multicellular organisms appear to be constitutive and are active independent of developmental stage or tissue type. Moreover, the proper functioning of these boundaries depends upon trans-acting factors that are ubiquitously expressed.

Several constitutively active boundary elements have been found in the *Drosophila* Antennapedia (ANT-C) and bithorax (BX-C) complexes, and these elements are crucial for the developmental functions of the homeotic genes in each complex (Gyurkovics et al., 1990; Mihaly et al., 1998; Zhou and Levine, 1999; Barges et al., 2000; Belozerov et al., 2003). BX-C contains three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), which are responsible for specifying segment identity in the posterior parasegments (PS) 5-14 of the fly. Parasegment identity depends upon which of these homeotic genes is activated and upon its precise pattern of expression. Transcriptional activity is controlled by a large ~300 kb *cis*-regulatory region that is subdivided into nine parasegment specific cis-regulatory domains: *abx/bx*, *bxd/pbx* and *iab2-iab8* (Duncan, 1996; Mihaly et al., 1998). Each cis-regulatory domain directs the expression of one of the BX-C homeotic genes in a pattern appropriate for specifying a particular parasegment. The domains are sequentially activated going from anterior to posterior parasegments. For example, in PS11, *Abd-B* expression is controlled by the *iab-6* domain. Although *iab-6* is active in this parasegment, the adjacent domain, *iab-7* and its neighbor *iab-8*, are silenced. In PS12, *iab-7* is activated and it directs *Abd-B* expression, while *iab-8* remains silent. The activity state of the BX-C *cis*-regulatory domains is set early in embryogenesis by the products of the gap and pair-rule genes. The gap and pair-rule gene products are only transiently expressed and by stage 11 of embryogenesis, BX-C regulation switches from the initiation to the maintenance phase. Maintenance depends upon *trithorax group* (trxG) and *Polycomb group* (PcG) genes (Simon, 1995; Simon and Tamkun, 2002). Genes in the *trx* group are required to maintain the homeotic genes in their active state, whereas genes in the *Pc* group function to silence homeotic gene expression.

The most thoroughly characterized of the BX-C boundary elements is *Fab-7*. It is located in between the *iab-6* and *iab-7* *cis*-regulatory domains (Fig. 1A) and, like the other boundaries in BX-C, it functions to ensure the genetic autonomy of the two flanking *cis*-regulatory domains. Mutations that inactivate *Fab-7* lead to the fusion of the *iab*-6 and *iab*-7 domains, and this disrupts the specification of PS11 (Gyrukovics et al., 1990; Galloni et al., 1993; Mihaly et al., 1997). In most *Fab-7* mutant PS11 cells, positive regulatory elements in *iab-6* inappropriately activate the *iab-7 cis*-regulatory domain. As a consequence, *Abd-B* expression in these cells is driven by *iab-7* not *iab-6*, and they assume a PS12 identity. In the remaining mutant PS11 cells negative elements in *iab-7* inappropriately silence *iab-6* (and *iab-7*). When *iab-6* is silenced *Abd-B* expression is driven by *iab-5* and the cells assume a PS10 identity.

Although the normal role of *Fab-7* is to prevent crosstalk

between the *iab-6* and *iab-7 cis*-regulatory domains, it also has the ability to insulate promoters from the regulatory effects of nearby enhancers or silencers. Like other known boundaries, the insulating activity of the *Fab-7* element does not appear to be restricted to specific enhancer-promoter combinations, nor is it stage or tissue specific. In the context of BX-C, the endogenous *Fab-7* boundary insulates the *Ubx* and *rosy* promoters carried by '*bluetail*' (*blt*), a transposon inserted into the *iab-7* domain, from the regulatory effects of the *iab-6* and *iab-5* domains (Galloni et al., 1993). This insulating activity is observed from early embryogenesis through the adult stage. In transgene assays in embryos, the *Fab-7* boundary blocks the *fushi tarzu* (*ftz*) stripe (UPS) and neurogenic (NE) enhancers from activating the *hsp70* promoter in the embryonic ectoderm and CNS, respectively (Hagstrom et al., 1996). It also blocks *eve*, *hairy*, *iab-5*, *rhomboid* and *twist* enhancers from activating *eve* and *white* promoters in the embryo (Zhou et al., 1996). In the adult, *Fab-7* blocks the *white* eye and testes enhancers from activating the *mini-white* promoter (Hagstrom et al., 1996).

The minimal *Fab-7* boundary defined in the *ftz:hsp70-lacZ* and w_{EN}*:mini-white* enhancer blocking assays is 1.2 kb in length. As shown in Fig. 1A, it extends from the minor nuclease hypersensitive site (*) on the proximal side to the *iab-7* PRE (which corresponds HS3) (Hagstrom et al., 1997; Mishra et al., 2001) on the distal side and includes two major chromatin-specific nuclease hypersensitive regions, HS1 and HS2 (Karch et al., 1994). The largest hypersensitive region, HS1, contains six consensus GAGA factor binding sites arranged in three pairs, 1-2, 3-4 and 5-6. The ubiquitously expressed GAGA factor is encoded by the *Trithorax-like* (*Trl*) gene (Farkas et al., 1994), and it is thought to function in the formation and/or maintenance of the nucleosome free regions of chromatin associated with a variety of *cis*-acting elements in flies, including enhancers, promoters, Polycomb Response Elements (PRES) and boundaries (Lehmann, 2004). Chromatin immunoprecipitation experiments demonstrate that GAGA is associated with the *Fab-7* boundary in vivo (Strutt et al., 1997). Moreover, the GAGA-binding sites in HS1 are important for boundary function. In previous studies, we found that the enhancer blocking activity of the minimal 1.2 kb boundary is compromised in both the embryo and adult when GAGA sites 1-5 are mutated (Schweinsberg et al., 2004). Although this finding indicates that GAGA (or another protein that recognizes the GAGA consensus) is required for *Fab-7* boundary activity throughout development, the GAGA sites are not functionally equivalent. We found that when only the centromere proximal pair, 1-2, are mutated, blocking of the *ftz* UPS stripe enhancer in the ectoderm of early embryos by the minimal *Fab-7* boundary is weakened, but there is no apparent effect on the blocking of either the *ftz* NE enhancer in the CNS of older embryos or the *w* enhancer in adults. By contrast, mutation of the central pair, 3-4, weakens blocking of the *w* enhancer in the eye, but has little effect on the blocking of the *ftz* enhancers in embryos.

One interpretation of these results is that the constitutive boundary activity of the *Fab-7* element is generated by sub-elements whose activities are developmentally restricted. In the studies reported here, we have tested this hypothesis. We show that, unlike other well characterized boundaries, the constitutive activity of *Fab-7* is generated by combining a series of subelements that function at different stages of development. This unexpected finding indicates that chromatin domains are not always static units, but instead may be redefined by inactivating or activating a boundary element such that the chromatin domain can expand to include new genes or regulatory sequences, or alternatively contract eliminating genes or regulatory sequences.

Materials and methods

P-element constructs

All PCR fragments were amplified from a 3.35 kb *Hin*dIII-to-*Xba*I *Fab-7* genomic fragment inserted into BlueScript as the template (described by Hagstrom et al., 1996). The primers used were SES23C (TGC*GGATCC*GTGGCAAAAGCTGGCAAAG), SES23Xho (TGC-*CTCGAG*TGGCAAAAGCTGGCAAAG), SES24C (TGC*AGATCT*-GCGTTGATATGCCCCAATG), SES24Sal (TGC*GTCGAC*GCGT-TGATATGCCCCAATG), SES27Sal (TGC*GTCGAC*TTTCCCCC-GCCACACAGC), SES28Xho (TGC*CTCGAG*CTGTGTGGCGGG-GGAAAG), SES29Xho (TGC*CTCGAG*CATTGGGGCATATCAA-CGC), SES30Sal (TGC*GTCGAC*GAACGGCAACTGAATTCC), SES33Xho (TGC*CTCGAG*AACCGCACGCACACCACCGC) and SES34Sal (TGC*GTCGAC*GCGGTGGTGTGCGTGCGGTTCTC). Restriction enzyme sites are indicated in italics. All PCR fragments were confirmed by sequencing. The pHS1 fragment was amplified using SES23C and SES24C and was cloned in four tandem copies into the *Bam*HI and *Bgl*II restriction sites of Litmus29. The *Bam*HI/*Bgl*II fragment was then excised and cloned into the *Bam*HI site of Bluescript. The pHS1A, pHS1B, dHS1, dHS1A and dHS1B fragments were amplified using the SES23Xho/SES27Sal, SES28Xho/SES24Sal, SES29Xho/SES30Sal, SES29Xho/SES34Sal and SES33Xho/SES30Sal, respectively. The resulting PCR products were cloned in four tandem copies into the *Xho*I and *Sal*I restriction sites of Bluescript. *Xho*I-*Not*I fragments of pHS1, pHS1A, dHS1, dHS1A and dHS1B were excised from Bluescript and inserted into the *white-enhancer: miniwhite* vector (XN vector) in between the *white* enhancer and the *miniwhite* gene or upstream and into the *ftz enhancer: hsp70-LacZ* vector (pCfhL vector) in between the UPS/NE enhancers of *fushi-tarazu* (*ftz*) and the *hsp70* promoter or upstream of UPS (Hagstrom et al., 1996). Embryos in each set of experiments in Fig. 1, Fig. 2, Fig. 4 and Table 1 were stained in parallel as described in Hagstrom et al. (Hagstrom et al., 1996) for a direct comparison of staining intensities. We tested all *ftz:hsp70-lacZ* transgenic lines for silencing effects of either the *Fab-7* subfragment or chromosomal position by determining whether the *hsp70* promoter was heat inducible and gave a uniformly high level of *lacZ* expression after a brief heat shock. The few lines showing anomalous *lacZ* expression after heat induction were not included in the assays.

Results

The effects of mutations in the GAGA sites 1-2 suggested that sequences on the proximal side of HS1 may be important for *Fab-7* boundary activity in the early embryo, but not at later stages of embryogenesis or in the adult. To explore this idea further, we re-examined the insulating activity of several boundary deletions of the endogenous *Fab-7* element that were generated by 'hopping' the *bluetail* (*blt*) transposon (Mihaly et al., 1997). As shown in Fig. 1B, *blt* is inserted just within the *iab-7 cis*-regulatory domain in between the *Fab-7* boundary and the *iab-7* PRE. The *Fab-7* boundary insulates the *Ubx-LacZ* and *rosy* genes in the *blt* transposon from the *iab-6* and *iab-5 cis*-regulatory domains, and the activity of both genes is controlled exclusively by the *iab-7* cis-regulatory domain. As a consequence, both *lacZ* (Fig. 1B) and *rosy* (Galloni et al.,

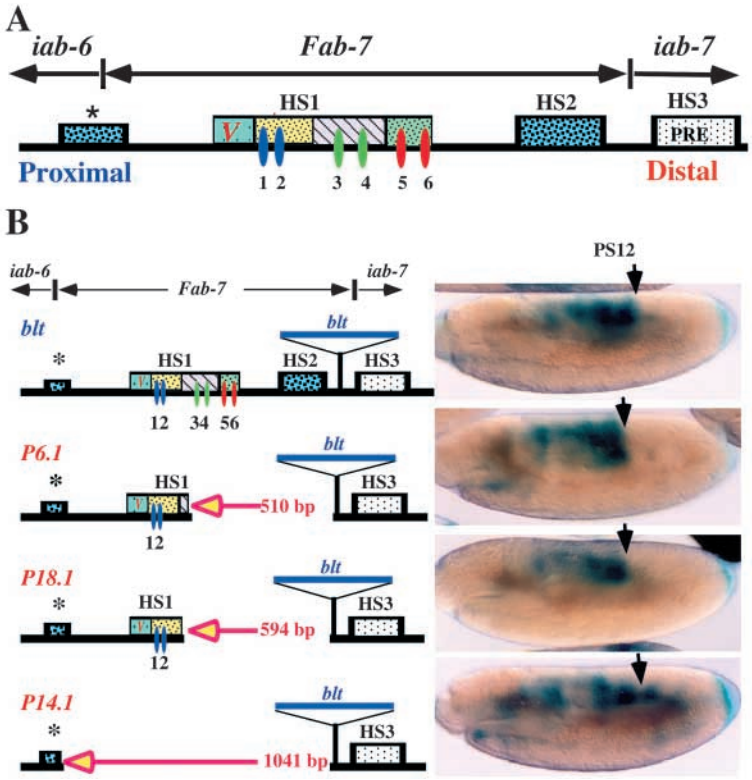


Fig. 1. (A) Map of the *Fab-7* region showing the minor nuclease hypersensitive site (*), HS1, HS2 and HS3. GAGA sites in HS1 are indicated by colored ovals and numbers 1-6. (B) Left: map of *iab-7*^{bluetail}, *iab6,7*$^{P6.1}$, *iab6,7*$^{P18.1}$ and *iab6,7*$^{P14.1}$ mutations showing the *bluetail* transposon inserted at the distal edge (right) of *Fab-7* in between HS2 and HS3. The size of the *Fab-7* deletion is indicated for each mutant. Right: *lacZ* expression in representative germband extended embryos heterozygous for *iab-7*^{bluetail}, *iab6,7*$^{P6.1}$, *iab6,7*$^{P18.1}$ and *iab6,7*$^{P14.1}$. Arrow in each embryo marks the anterior edge of PS12.

1993) are expressed in PS12 and more posterior parasegments. Three imprecise 'excisions' (*iab6,7*$^{P6.1}$, *iab6,7*$^{P14.1}$ and *iab6,7*$^{P18.1}$) that delete *Fab-7* boundary sequences but retain an intact *blt* transposon were recovered by Mihaly et al. (Fig. 1B). When they examined β-galactosidase expression in the CNS of germband retracted embryos carrying these three imprecise excisions, Mihaly et al. found that the anterior limit of β-galactosidase expression was PS11, not PS12. Further support for the conclusion that these three deletions disrupt *Fab-7* comes from an analysis of derivative alleles, *Fab-7*$^{P6.1}$, *Fab-7*$^{P18.1}$ and *Fab-7*$^{P14.1}$ in which the *blt* transposon was precisely excised. All three derivatives give cuticular phenotypes in the adult that would be expected for mutations that completely inactivate the *Fab-7* boundary (Mihaly et al., 1997).

Although all three deletions share the same distal breakpoint, they differ in their proximal breakpoints. The DNA segment removed in the largest deletion, *P14.1*, is more than 1 kb in length (Fig. 1B) and it corresponds closely to the minimal *Fab-7* boundary defined in transgene assays. By contrast, the *P6.1* and *P18.1* deletions are only 510 bp and 594 bp, respectively. As both of the smaller deletions still have HS1 GAGA sites 1-2, as well as more proximal *Fab-7* sequences, we wondered whether they retained an ability to block the *iab-6 cis*-regulatory domain from activating the *Ubx-lacZ* reporter in early embryos. As this earlier time point had not been examined in the studies of Mihaly et al. (Mihaly et al., 1997), we decided to compare the pattern of β-galactosidase expression in germband extended *blt* embryos and in the various deletion mutant embryos. As illustrated by representative embryos in Fig. 1B, the anterior limit of β-galactosidase expression in *P6.1* and *P18.1* embryos is PS12, just like the parental *blt* control that has an intact *Fab-7* boundary. By contrast, the anterior limit of β-galactosidase expression in the larger *P14.1* deletion is PS11. As reported by Mihaly et al. (Mihaly et al., 1997) for the activity of the *Ubx-lacZ* reporter in the CNS, when we examined β-galactosidase expression in the ectoderm of older germband retracted embryos, we found that the anterior limit for *P6.1* and *P18.1* embryos was PS11, just like the larger deletion *P14.1* (not shown). These findings indicate that the sequences retained in *P6.1* and *P18.1*, which include the GAGA site pair 1-2, are sufficient to confer boundary activity during the initiation phase of BX-C regulation, but not later in development when regulation has switched to the maintenance mode. It should be pointed out that in our experiments and those of Mihaly et al., β-galactosidase expression in PS11 of germ band retracted *P6.1* and *P18.1* embryos is not as robust as it is in the *PS14.1* deletion. As all three deletions have indistinguishable *Fab-7* mutant phenotypes in the adult, we presume that the smaller deletions have lower levels of β-galactosidase expression in PS11 in germband retracted embryos because the early boundary activity of *P6.1* and *P18.1* is lost gradually rather than abruptly, perhaps reflecting a depletion of some maternal product.

Proximal HS1 has boundary function in the early embryo but not later in development

The results described above suggest that sequences in the proximal part of *Fab-7* have boundary function during the early stages of embryogenesis, but not later in embryogenesis or in subsequent stages of development. To investigate this possibility further, we tested whether sequences from the large HS1 nuclease hypersensitive region that are retained in both the *P6.1* and *P18.1* deletions have boundary activity in transgene assays. For this purpose, we tetramerized a 235 bp HS1 sub-fragment called pHS1. As illustrated in Fig. 2, the proximal end of pHS1 corresponds to the proximal edge of the HS1 nuclease hypersensitive region while the distal end corresponds to the proximal breakpoint of the 594 bp deletion *P18.1*. This fragment includes GAGA sites 1-2 plus a 100 bp sequence that is 95% identical between *D. melanogaster* and *D. virilis* (*V* in Fig. 2). We placed the pHS1x4 tetramer either in the blocking position in between the *ftz* enhancers and the *hsp70* promoter or, as a control, in the non-blocking position upstream of the enhancers. Transgenic *ftz:hsp70-lacZ* embryos were stained with X-gal and compared with control transgenes carrying either the minimal 1.2 kb *Fab-7* fragment, five binding sites for the Su(Hw) insulator protein, or a random DNA control fragment with no enhancer blocking activity (Fig. 2).

In the *ftz:hsp70-lacZ* assay (Fig. 2B), the ability of the

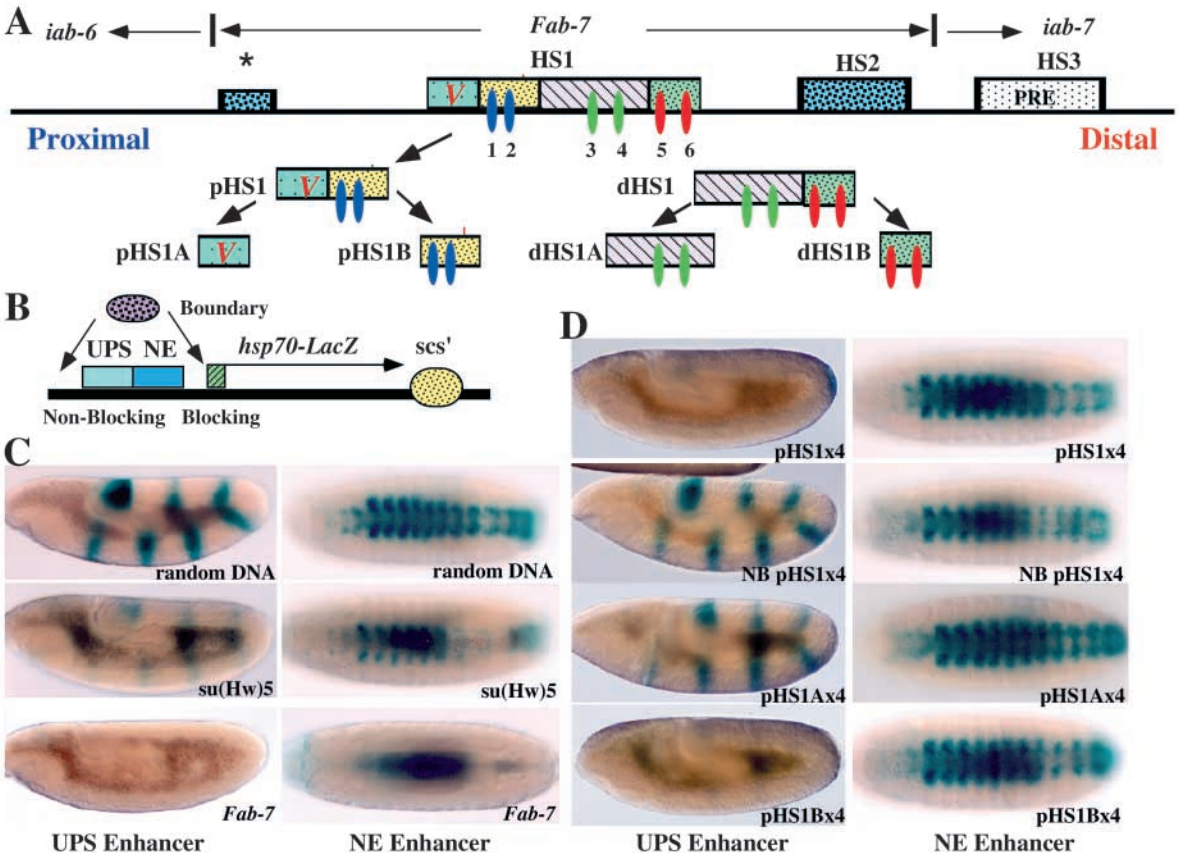


Fig. 2. (A) Map of the *Fab-7* boundary and the various HS1 subfragments used in the transgene assays. The *virilis* homology region is indicated by *V*. (B) Map of the *ftz:hsp70-lacZ* reporter construct. Inserts placed upstream of the *ftz* UPS enhancer are in the non-blocking position (NB), while inserts placed between the NE enhancer and the *hsp70* promoter are in the blocking position. (C,D) *lacZ* expression in embryos from representative lines homozygous for *ftz:hsp70-lacZ* transgenes carrying different boundary elements. (C) Random DNA, five binding sites for Su(Hw) and 1.2kb *Fab-7*. (D) Tetramerized pHS1 fragments: pHS1x4 in the blocking position, pHS1x4 in the non-blocking position (NB), pHS1Ax4 and pHS1Bx4. All embryos in Fig. 2 (and also in Fig. 4) were stained in parallel as described elsewhere (Hagstrom et al., 1996) for a direct comparison of staining intensities.

minimal 1.2 kb *Fab-7* boundary to block the stripe (UPS) and the neurogenic (NE) enhancers is intermediate between that of an element containing 5 (Fig. 2C) and 12 (not shown) (see Hagstom et al., 1996) binding sites for the ubiquitously expressed insulator protein, Suppressor of Hairywing, Su(Hw). As illustrated in Fig. 2D, the pHS1x4 tetramer has no effect on β-galactosidase expression when placed in the non-blocking position (NB pHS1x4) upstream of the two enhancers and the level of β-galactosidase stripe and CNS expression resembles that observed in the random DNA control. By contrast, in the blocking position, pHS1x4 insulates the *hsp70* promoter from the *ftz* UPS enhancer about as well as the 1.2 kb *Fab-7* boundary, and little if any stripe β-galactosidase expression is observed (Fig. 2D; Table 1). This result indicates that sequences derived from the proximal side of HS1 can confer boundary function during the early stages of embryogenesis. As boundary function is lost in the *Fab-7 P6.1* and *P18.1* deletions at later stages in embryogenesis, we anticipated that pHS1x4 would be less effective in blocking the *ftz* NE enhancer in the CNS of germband retracted embryos. This is the case. As shown in Fig. 2D and Table 1, blocking of the NE enhancer by pHS1x4 is reduced compared with the minimal 1.2 kb *Fab-7* boundary (Fig. 2C,D; Table 1). In three of the pHS1X4 lines, the level of β-galactosidase in the CNS is the same as the random DNA control, while in the three other lines it is comparable with (or higher than) the *ftz* transgene carrying 5 su(Hw)-binding sites in the blocking position.

We also examined the enhancer blocking activity of pHS1x4 in the w_{EN}*:mini-white* assay. The minimal 1.2 kb *Fab-7* boundary blocks the *w* enhancer when interposed between the enhancer and *w* promoter; however, unlike other fly boundaries, including the BX-C boundary *Fab-8*, *Fab-7* blocking activity in this assay is sensitive to chromosomal position effects and is observed in only ~50% of the lines (Hagstrom et al., 1996). The reason for this position dependence is not currently understood. Unlike the minimal boundary, pHS1x4 has no apparent boundary function in the w_{EN}*:mini-white* assay and out of almost 40 independent transgenic lines, only two were classified as 'blocking' (Fig. 3). This result is consistent with the *Fab-7* mutant phenotypes seen in *Fab-7*$^{P6.1}$ and *Fab-7*$^{P18.1}$ flies, and suggests that as was the case in the CNS of germband retracted embryos, pHS1 has little if any boundary function in adults.

To further localize the UPS-enhancer blocking activity, we subdivided pHS1 into a 133 bp fragment (pHS1A) containing the *virilis* homology region (*V*) and a 121 bp fragment (pHS1B)

Table 1. Summary of the blocking activity in *ftz:hsp70-lacZ* transgenic lines with the different boundary elements in Figs 2 and 4

	UPS enhancer			NE enhancer		
Transgene	L	M	D	L	M	D
1.2 kb *Fab-7*	10	1	0	6	2	0
pHS1x4	5	1	0	0	3	3
NB pHS1x4	0	0	3	0	0	3
pHS1Ax4	0	3	8	0	0	11
pHS1Bx4	4	2	5	1	0	10
dHS1x4	4	12	0	10	5	0
dHS1Ax4	2	7	7	8	4	3
dHS1Bx4	3	4	3	3	5	2

lacZ expression in the tetramerized boundaries is scored relative to the controls, random DNA, the su(Hw) 5 element and the 1.2 kb *Fab-7* boundary, and is subdivided into three classes based on staining intensity. L, light; M, medium; D, dark. The typical 1.2 kb *Fab-7* control gives light stripe and CNS staining (L). The typical 5 su(Hw) control gives medium stripe and CNS staining (M), while random DNA gives dark (D) staining. Number of lines in each category is indicated.

containing the two GAGA-binding sites (Fig. 2A). pHS1Ax4 has significantly reduced UPS-enhancer blocking activity and, as expected, is unable to block either the NE or the *w* enhancers (Fig. 2D; not shown). Although pHS1Bx4 retains UPS-enhancer blocking activity (Fig. 2D), this activity is insertion site dependent. It gives full UPS-specific boundary activity in only 4/11 lines and shows weak UPS-specific boundary activity in 2/11 lines (Table 1). Therefore, full UPS-specific enhancer blocking activity requires elements in both pHS1A and B; however, the pHS1B fragment contains the majority of insulating activity.

Distal HS1 has boundary function in the CNS and in adults

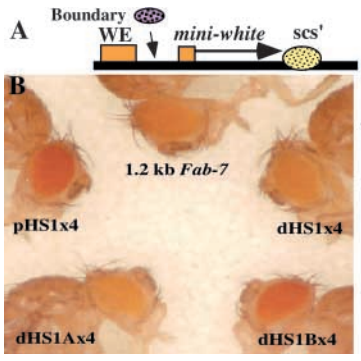
We next tested a tetramerized 291 bp fragment that corresponds roughly to the distal half of HS1 (dHS1) (Fig. 4). Though dHS1x4 blocks the UPS enhancer, its insulating activity is reduced compared with either pHS1x4 or the 1.2 kb *Fab-7* fragment. Most of the dHS1x4 transgenic lines (Table 1) have blocking activity approximately equivalent to that of Su(Hw)5 (Fig. 2C, Fig. 4). However, the boundary activity of dHS1x4 in the embryonic CNS is close to that of the intact *Fab-7* element and most of the lines show a substantial reduction in β-galactosidase expression in the CNS (Table 1). We also examined the boundary activity of dHS1x4 in the w_{EN}*:mini-white* assay. Although the intact *Fab-7* element blocks shows blocking in only about 50% of the lines, blocking is observed in over 80% of the dHS1x4 lines (Fig. 3). These findings demonstrate that the distal region of HS1 is able to strongly block the NE and *white* enhancers but is significantly compromised in its ability to block the UPS enhancer.

We next divided dHS1 into a 198 bp fragment, dHS1A, that contains the central pair of GAGA sites (3-4) and a 114 bp fragment, dHS1B, that contains the most distal GAGA sites (5-6). The boundary activity of dHS1Ax4 in the embryo resembles but is not quite as strong as the larger dHS1x4 tetramer. Thus, unlike dHS1x4, many of the dHS1Ax4 lines exhibit neither UPS nor NE enhancer blocking activity (Fig. 4). However, dHS1Ax4 retains the very strong *w* enhancer blocking activity (91%) observed with dHS1x4 (Fig. 3). These findings map an element that functions to block the *w* enhancer to dHS1A. Moreover, the strong *w* enhancer blocking activity of dHS1Ax4 would be consistent with the deleterious effects of mutations in GAGA sites 3-4 on the boundary activity of the 1.2 kb *Fab-7* fragment in the *mini-white* assay (Schweinsberg et al., 2004).

A very different result is obtained for dHS1Bx4. Like pHS1, dHS1Bx4 has little if any enhancer blocking activity in the w_{EN}*:mini-white* assay. However, in the embryo, dHS1Bx4 exhibits a weak position dependent enhancer blocking activity for both the UPS and NE enhancers (Fig. 4; Table 1). These findings indicate that sequences on the distal edge of HS1 have boundary activity during embryogenesis, but not at later stages.

Discussion

The *Fab-7* boundary functions to prevent crosstalk between the BX-C *iab-6* and *iab-7* cis-regulatory domains. When *Fab-7* is deleted, these two domains fuse into a single cis-regulatory domain and this leads to the misregulation of *Abd-B* and a failure to properly specify parasegment identity in PS11. Evidence from transgene assays and from the pattern of expression of the *Ubx-lacZ* and *rosy* genes in the *blt* transposon indicate that the *Fab-7* boundary is active in a wide range of cell types and tissues from early embryogenesis through the adult stage. In the studies reported here, we have investigated the basis for this constitutive activity. Unexpectedly, we have found that constitutive activity is generated by combining subelements whose function is developmentally restricted. Thus, a fragment, pHS1, from the proximal half of the major *Fab-7* nuclease hypersensitive region, HS1, can block the *ftz* UPS stripe enhancer in early embryos. However, this same fragment only has residual boundary activity in the CNS of older embryos and no detectable boundary activity in the adult eye. The opposite result is obtained with a fragment containing



C

	Blocking			No Blocking		
Transgene	Y	Y-O	O	O/R-R	Total	% Blocking
vector only	0	0	0	6	6	0%
1.2kb *Fab-7*	9	8	7	27	51	48%
pHS1x4	0	1	1	37	39	5%
dHS1x4	2	7	6	3	18	83%
dHS1Ax4	7	11	13	3	34	91%
dHS1Bx4	0	0	1	7	8	13%

Fig. 3. (A) Map of w_{EN}:*mini-white* construct containing the *white* enhancer (w_{EN}), a *mini-white* reporter gene, and the *scs'* boundary element to block 3′ position effects.
(B) Representative adult males homozygous for transgenes containing the 1.2 kb *Fab-7* element, pHS1x4, dHS1x4, dHS1Ax4 or dHS1Bx4.
(C) Summary of the blocking activity seen in w_{EN}*:mini-white* transgenic lines with the different boundary elements shown in Figs 2 and 4.

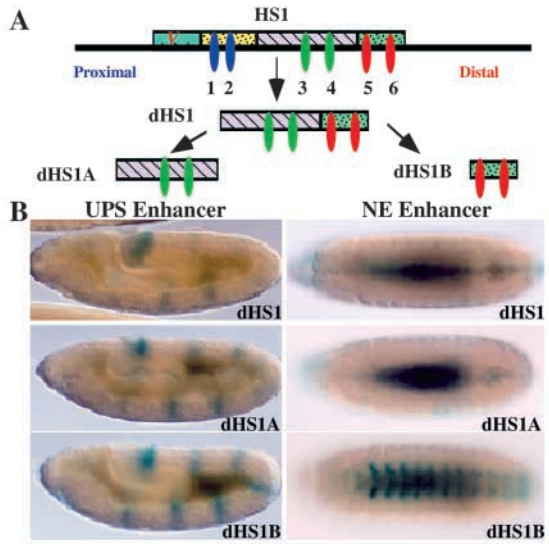


Fig. 4. (A) Map of the distal half of HS1 and the subfragments dHS1A and dHS1B. (B) *lacZ* expression in embryos of representative lines homozygous for *ftz:hsp70-lacZ* transgenes carrying different boundary elements: dHS1x4, dHS1Ax4 and dHS1Bx4.

the remainder of the HS1, dHS1. Unlike, pHS1, dHS1 can function as a boundary element in the adult eye. In fact, a multimerized version of dHS1 is more effective in blocking the *white* enhancer than the intact *Fab-7* boundary. In embryos, dHS1 is nearly as effective in blocking the *ftz* NE enhancer in the CNS as is *Fab-7*. By contrast, dHS1 is comparatively ineffective in blocking the *ftz* UPS enhancer in the early embryo, functioning about as well as 5 su(Hw)-binding sites. Further subdivision of dHS1 localize boundary function in the *mini-white* assay to dHS1A, a subfragment that is derived from the center of HS1. Like dHS1, the multimerized HS1A fragment is more effective in blocking the *mini-white* enhancer than the intact *Fab-7* boundary. dHS1A also retains an ability to block the *ftz* NE enhancer in the CNS, though it is less effective than the larger dHS1 fragment. Finally, on the distal side of HS1, dHS1B can block, albeit weakly, both the UPS and NE enhancers in the *ftz:hsp70-lacZ* assay. However, like pHS1, it has little or no blocking activity in the w_{EN}*:mini-white* assay. It should be noted that although the various sub-elements in HS1 seem to function most effectively at different stages of development, there is clearly some overlap in their activities (e.g. between pHS1 and dHS1B). It seems likely that this overlap is important in that it would allow the subelements (which in the endogenous locus are present in only single copies) to collaborate with each other to generate a functional *Fab-7* boundary.

The idea that the constitutive boundary function of *Fab-7* depends upon combining subelements whose activity is developmentally restricted is supported by our analysis of three *Fab-7* deletions generated by imprecise excisions of *blt* that retain an intact transposon. The largest of these, *P14.1*, removes a DNA segment closely corresponding to the minimal *Fab-7* element defined in enhancer blocking transgene assays. This deletion has no discernable boundary activity at any stage of development and the *blt Ubz-lacZ* reporter is active in PS11 from early embryogenesis onwards. The two smaller deletions, *P6.1* and *P18.1*, retain all of the sequences in pHS1 (plus sequences proximal to pHS1, which are important for the boundary function of the minimal 1.2 kb *Fab-7* element). Because the pHS1 sequence (when multimerized) confers boundary activity in transgene assays during the early stages of embryogenesis, one might expect that these two smaller deletions will retain at least some boundary function in early embryos, and indeed they do. In both deletions, the anterior limit of *Ubx-lacZ* expression is initially PS12 just like wild-type *Fab-7*. However, as these two deletions lack sequences on the distal side of HS1 that confer enhancer blocking activity in the embryonic CNS and the adult eye in transgene assays, they might be expected to have little boundary function at later stages of development. Indeed, Mihaly et al. (Mihaly et al., 1997) and we have found that *lacZ* expression from the *blt* transposon in both deletion mutants spreads into PS11 in germband retracted embryos. In addition, the *Fab-7* mutant phenotype of the two smaller deletions in adult flies is indistinguishable from that of the larger deletion. These findings indicate that although functionally autonomous *iab-6* and *iab-7* cis-regulatory domains can be established by the *P6.1* and *P18.1* mutants, the *Fab-7* boundary sequences remaining in these mutants are unable to sustain autonomy as development proceeds. This would suggest that the process of establishing an autonomous domain is not irreversible and that boundary elements must remain continuously active in order to maintain independent units of genetic activity. Conversely, the properties of dHS1 or dHS1A would suggest that functionally independent domains can be established de novo by activating a previously inactive boundary element.

A number of models could potentially account for the developmentally restricted activity of the different subelements from *Fab-7*. One idea is that the boundary function of each subelement is enhancer and/or promoter specific. Although we can not exclude this possibility, we note that the *Fab-7* boundary itself shows no evidence of enhancer or promoter specificity. In transgene assays and also in the context of BX-C itself, the boundary is able to block a wide range of enhancer-promoter combinations in many different tissues and cell types from early embryogenesis through to the adult (Galloni et al., 1993; Hagstrom et al., 1996; Zhou et al., 1996). Another idea is that the subelements have target sequences for DNA-binding proteins and/or accessory factors whose expression or activity is developmentally restricted. In this model, the boundary function of the pHS1 multimer, the two deletions *P6.1* and *P18.1*, and perhaps also dHS1B in early embryos would depend upon factors that are either deposited in the egg during oogenesis or expressed only in early stages of embryogenesis. In this case, one would expect that boundary activity would be lost when the complement of these factors is depleted as the embryo develops. Consistent with the idea that pHS1 function depends upon maternal factors, we have found that UPS blocking by the 4xpHS1 multimer is compromised in progeny of mothers heterozygous for several 3rd chromosome deficiencies (A. DeBourcy, C. Summers and S.E.S., unpublished). Conversely, because blocking by dHS1 (or dHS1A) is weak in early embryos, but then becomes stronger, it would be reasonable to think that its boundary activity depends more crucially upon factors that are zygotically expressed rather than of maternal origin. In this context, it is interesting to note that the interval in

which the pHS1 subelement is active as a boundary corresponds roughly to the initiation phase of BX-C regulation, while it is not active once regulation switches to the maintenance mode. The converse seems to be true for the dHS1 subelement, which appears to become activated as BX-C regulation switches from initiation to maintenance.

These overlapping patterns of activity suggest that one reason why *Fab-7* might be composed of different subelements is that this would permit the use of boundary factors that are specialized with respect to their interactions with, in one case, initiation phase gap and pair-rule transcription factors, and, in the other case, with the maintenance phase trithorax and Polycomb group proteins. More generally, the fact that the boundary activity of the *Fab-7* subelements is developmentally restricted suggests a hitherto unexpected plasticity in boundary function. This plasticity indicates that the activity of some boundary elements is likely to be subject to tissue or stage-specific regulation. If this is the case, the genes and regulatory elements included within a chromosomal domain, which is the unit of autonomous genetic activity, could change from one tissue or stage to the next by turning boundary function on or off. This would afford a novel mechanism of high order genetic regulation.

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