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Dissecting the regulatory landscape of the *Abd-B* gene of the bithorax complex

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The three homeotic genes of the bithorax complex (BX-C), *Ubx*, *abd-A* and *Abd-B* control the identity of the posterior thorax and all abdominal segments. Large segment-specific cis-regulatory regions control the expression of *Ubx*, *abd-A* or *Abd-B* in each of the segments. These segment-specific cis-regulatory regions span the whole 300 kb of the BX-C and are arranged on the chromosome in the same order as the segments they specify. Experiments with *lacZ* reporter constructs revealed the existence of several types of regulatory elements in each of the cis-regulatory regions. These include initiation elements, maintenance elements, cell type- or tissue-specific enhancers, chromatin insulators and the promoter targeting sequence. In this paper, we extend the analysis of regulatory elements within the BX-C by describing a series of internal deficiencies that affect the *Abd-B* regulatory region. Many of the elements uncovered by these deficiencies are further verified in transgenic reporter assays. Our results highlight four key features of the *iab-5*, *iab-6* and *iab-7* cis-regulatory region of *Abd-B*. First, the whole *Abd-B* region is modular by nature and can be divided into discrete functional domains. Second, each domain seems to control specifically the level of *Abd-B* expression in only one parasegment. Third, each domain is itself modular and made up of a similar set of definable regulatory elements. And finally, the activity of each domain is absolutely dependent on the presence of an initiator element.

KEY WORDS: Bithorax, Hox gene, Abd-B, Chromatin boundary, Insulator, PRE, PTS, *Drosophila*

INTRODUCTION

The three homeotic genes of the *Drosophila* bithorax complex (BX-C), *Ubx*, *abd-A* and *Abd-B*, specify the identity of the 3rd thoracic segment (T3) and all abdominal segments (A1 to A8) of the fly (Lewis, 1978; Sanchez-Herrero et al., 1985). These segments derive from parasegments (PS) 5 to 14 that form during early embryogenesis. The expression of *Ubx*, *abd-A* and *Abd-B* is regulated by a complex cis-regulatory region covering 300 kb of DNA. Molecular genetic analysis has subdivided this large cis-regulatory region into nine segment/parasegment-specific subregions [*abd/bx*, *bxd/pbx*, *iab-2* to *iab-8* (Lewis, 1978; Bender et al., 1983; Karch et al., 1985; Peifer et al., 1987; Duncan, 1987; Celniker et al., 1990; Sanchez-Herrero, 1991) (for a review, see Maeda and Karch, 2006)]. Remarkably, the genes and their regulatory regions are aligned on the chromosome in the order of the body segments they specify. Genetic studies using chromosomal rearrangement breaks have shown that when cis-regulatory regions are removed from the complex, specific parasegments are transformed into copies of more anterior parasegments. The clustering of mutations with similar phenotypes along the chromosome has led to a model in which each regulatory region is important in specifying homeotic gene expression in one parasegment. For example, *iab-5* is thought to control the expression of *Abd-B* in PS10/A5, while *iab-6* is thought to control expression of *Abd-B* in PS11/A6.

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Although it is clear that each specific cis-regulatory region is important for the parasegment specific expression of a BX-C homeotic gene, it is still unclear if each regulatory region works autonomously in this function. *Abd-B*, for example, is expressed in PS10-13 in a stepwise gradient from anterior to posterior. Its specific expression pattern is determined by the four cis-regulatory regions *iab-5* to *iab-8*. Because of the stepwise increase in *Abd-B* expression and the fact that mutations affecting the cis-regulatory regions transform parasegments into a copy of the parasegment immediately anterior to them, it has been thought that the pattern of *Abd-B* expression is the result of the additive effect of sequential activation of the cis-regulatory domains (Lewis, 1978; Celniker et al., 1990).

How individual cis-regulatory domains work also remains a mystery. And although many elements have been identified within the BX-C using transgenic assays [such as initiation elements, maintenance elements (ME) cell type- or tissue-specific enhancers, chromatin insulators and the Promoter Targeting Sequence (PTS)], there is still a surprising lack of appropriate mutations to determine how all of these elements interact and function within their native environment (for a review, see Maeda and Karch, 2006). Many of the mutations used to map the cis-regulatory regions are chromosomal rearrangements breaks. Such breaks often separate more than one regulatory region from its relevant target promoter, resulting in homeotic transformations of multiple segments. Rearrangement breaks also introduce foreign DNA next to the sequences of the BX-C, and so position effects are observed. Because of these limitations, it has been very difficult to confirm the function in the context of the BX-C of elements studied in transgenes.

By mobilization of three P-element enhancer trap lines, we have isolated a number of internal deficiencies that affect the *iab-3* to *iab-8* cis-regulatory regions. Among the deficiencies isolated are ones that remove initiator elements, ones that remove cell-type specific enhancers, ones that remove insulators and two deficiencies that cleanly removes two PTSs. Combining our genetic analysis with results from transgenic reporter constructs allowed us to define two

Table 1. Names and positions of fragments inserted upstream of the *Ubx-lacZ* reporter

Fragment name	Distal breakpoint	Proximal breakpoint
13.8EcI	100694	114508
7.5H	95533	102973
11.9X	92665	104569
7.8H	87737	95552
2.8XN	89883	92665
4.4E	84115	88503
11X	70098	81163
4.4H	75077	79512
3H	79513	82553
3.4E	67074	70507
3.6XH	64584	68235
2.1PP	60451	62541

new initiators (IAB6 and IAB7a), a new ME and various cell type-specific enhancers. Overall, our work provides compelling functional evidence that the *Abd-B* region of the BX-C is modular, with the expression of *Abd-B* in each parasegment being primarily controlled by a single cis-regulatory domain. Our work also provides strong genetic evidence for a temporal transition in cis-regulation, in which the initiator elements dictate the later activity state for an entire cis-regulatory domain.

MATERIALS AND METHODS

Plasmid constructions

Overlapping fragments from the walk (Karch et al., 1985) have been inserted upstream from the *Ubx-lacZ* reporter gene in the Casper*Ubx-lacZ* P-element vector (Qian et al., 1991). All cloning details are available upon request. Germline transformation was performed essentially as described by Mihaly et al. (Mihaly et al., 1997). Positions of the constructs are given in nucleotide numbering along the BX-C sequence of Martin et al. (Martin et al., 1995). The coordinates of each of the fragments used for the transgenic assays is listed in Table 1.

Generating mutations

Internal deficiencies

The *iab-5,6^{J81}* and *5,6^{J82}* deletions were recovered during an experiment aimed at isolating local hops of the *bluetail* transposon (Galloni et al., 1993; Mihaly et al., 1997). A chromosome (called J82.blt) containing the deletion present in *iab-5,6^{J82}* and the starting bluetail transposon was first isolated. In order to assign the phenotype associated with the deletion alone, we remobilized the transposon from J82.blt Among the progeny, we recovered *iab-5,6^{J82}* (a clean excision) and the *iab-5,6^{J81}*, which probably resulted from an aborted gene conversion event. The *iab-6,7^{IH}*, *Fab-6,7^{1}* and *Fab-6,7^{2}* deletions were recovered by imprecise excision of the *bluetail* transposon. Local hops and imprecise excision of the *iab-7^{blt}* transposon were screened on the basis of their failure of complementation with the *iab-4,5,6^{DB}* chromosome. The extents of the deletions were mapped by whole-genome Southern and by PCR analysis. *Fab-3,5^{DV}*was recovered on the basis of its dominant phenotype during an excision screen of the HCJ200 P-element present in *iab-4* (Bender and Hudson 2000). The *iab-7^{280}* deletion was recovered by mobilization of the *fs(3)5649* transposon, as described by Barges et al. (Barges et al., 2000).

PTSs deletions

iab-7^{R73}

As meiotic recombination attempts to recover the R73 deletion separated from the *Fab-7^{1}* deletion had failed, we generated a new R73 deletion by gene conversion using the *fs(3)5649* chromosome (Gloor et al. 1991). Convertant chromosomes carrying the R73 deletion were screened by PCR. The Δ330 deletion was also recovered during the screen, probably resulting from an abortive conversion event.

ΔHS^{*iab-6}

Gene conversion initiated by the excision of the P-element in the *iab-7^{blt}* line was used to direct specific mutations in the *Fab-7* boundary (for details, see Hogga et al., 2001). A deletion removing the sequence covering the minor nuclease hypersensitive site was created by first introducing two loxP sites in the *Nco*I and *Bsm*I sites at positions 84,827 and 85,028, respectively. The deletion was recovered after a cross to flies expressing Cre (Siegal and Hartl, 1996).

RESULTS

Isolation of internal BX-C deletions

In order to dissect genetically the 3′ cis-regulatory domain of the Abd-B gene, we isolated a number of deficiencies derived from the imprecise excisions of three P-elements [*iab-7^{blt}*, *fs(3)5649* and HCJ200; see, respectively, Galloni et al. (Galloni et al., 1993), Barges et al. (Barges et al., 2000) and Bender and Hudson (Bender and Hudson, 2000)]. Most of these deficiencies were recovered because of their homeotic transformations of the posterior abdominal segments.

The deficiency breakpoints were first determined by whole genome Southern analysis and then confirmed by PCR analysis. Their positions are reported along the DNA sequence (SEQ89E) (Martin et al., 1995). The homeotic transformations associated with these deficiencies are described below, specifically focusing on the features of the posterior abdominal segments in adult males. The *Abd-B* expression pattern observed in the ventral nerve chord of mutant embryos is also described. Fig. 1A shows the extents of these deficiencies. The *iab* cis-regulatory domains are positioned relative to the boundaries and data presented below.

Identification of the IAB6 and IAB7 initiators

Males homozygous for *iab-7^{280}* have their 7th abdominal segment completely transformed into a copy of A6 (Fig. 2B). In mutant embryos, the *Abd-B* expression pattern in PS12/A7 is replaced by the PS11/A6-specific pattern (Fig. 3). The homeotic transformations and the *Abd-B* expression patterns observed in *iab-7^{280}* are exactly the same as those seen in *iab-7^{Sz}*, a mutation that removes the entire *iab-7* cis-regulatory region between *Fab-7* and *Fab-8* (Galloni et al., 1993). In agreement with this, the *iab-7^{280}* mutation deletes 20 kilobases (kb) between sequence positions 64,370 and 84,012 (Fig. 1A), thus removing the entire *iab-7* cis-regulatory region. On the proximal side, a second smaller deletion occurred, removing DNA belonging to *iab-6*, between 89,392 and 86,650 (Fig. 1A) The wild-type appearance of PS11/A6 in adults and embryos indicate that no essential function of *iab-6* is affected by this deletion.

The *iab-6,7^{IH}* mutation deletes 26 kb (96,559-70,265; Fig. 1A), removing about three-quarters of the *iab-6* region and nearly two-thirds of the *iab-7* region. In flies homozygous for this deletion, both A6 and A7 are transformed to differing degrees towards A5, consistent with the removal of important *iab-6* and *iab-7* enhancers (Fig. 2D). The adult phenotype of this deletion shows that although *iab-6* activity is completely abolished (as seen by the complete transformation of A6 into A5), *iab-7* is still partially functional [revealed by the only partial transformation of A7 into A5 (Fig 2D)]. The *Abd-B* expression pattern in the embryonic nerve chord shows remarkable correspondence to these adult phenotypes (Fig. 3). In PS11/A6, where *iab-6* is inactivated, *Abd-B* expression follows the PS10/A5-specific pattern. If *iab*-7 were also completely inactive, we would predict that the PS10/A5-specific *Abd-B* expression pattern would also be reiterated in PS12/A7. Instead, we observe a weaker version of the normal PS12/A7-specific pattern that corresponds to a level of expression intermediate between that of normal PS11/A6 and PS12/A7.

Comparing *iab-6,7^{IH}* to *iab-7^{280}* allows us to map important regulatory elements within the *iab-6* and *iab-7* regions. The *iab-7^{280}* deletion does not alter *iab-6* function, while *iab-6,7^{IH}* deletion is

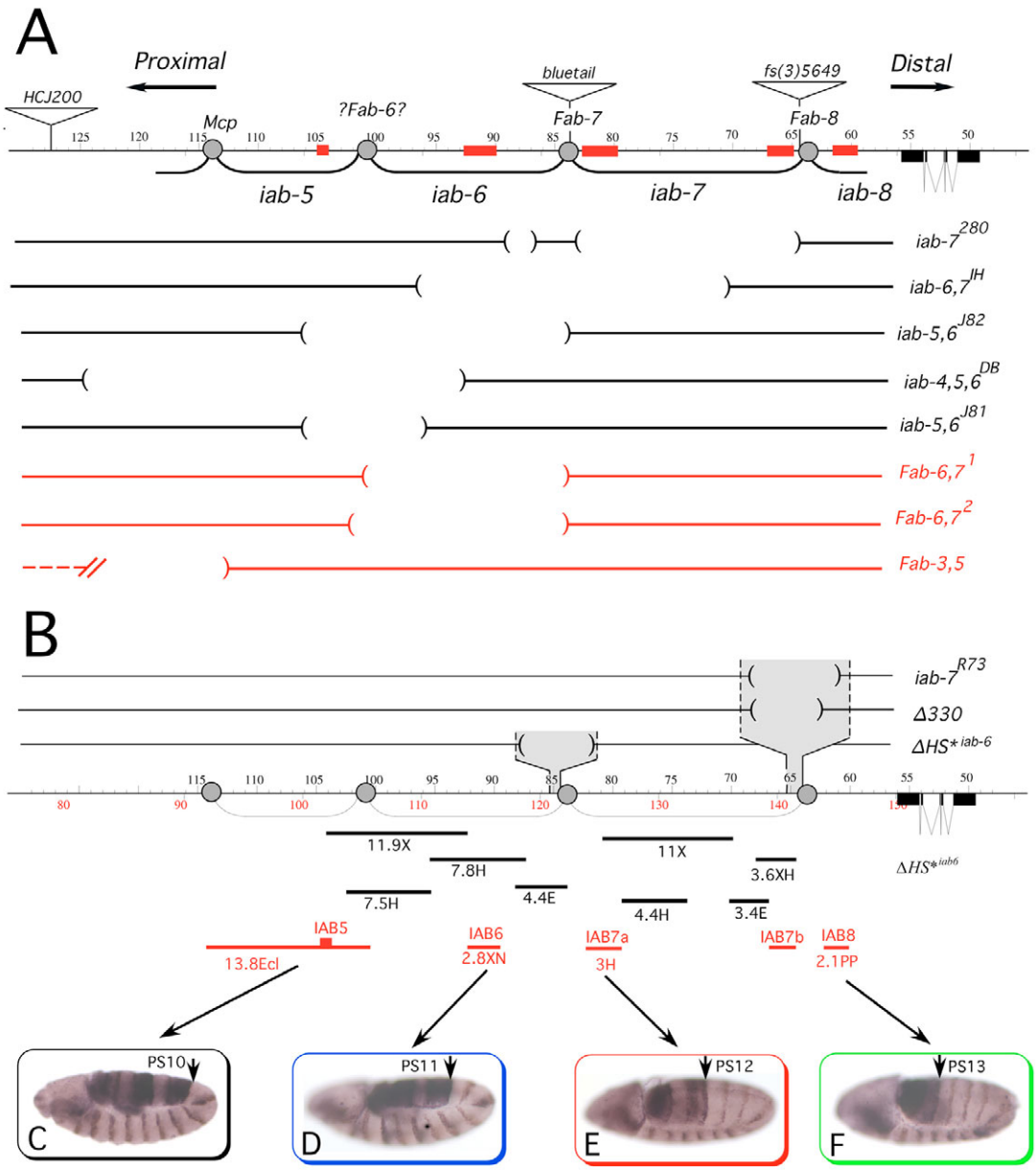

Fig. 1. Regulatory landscape of the *Abd-B* gene of the BX-C. (**A**) Genomic map of the 3′ cis-regulatory region of the *Abd-B* gene. The proximal and distal arrows point towards the centromere and telomere, respectively. The black numbers above the DNA line correspond to the nucleotide sequence marked in kb (SEQ89E) (Martin et al., 1995). The exons of the *Abd-B* transcription unit encoding the m variant are shown below the DNA line. The extent of the *iab-5, 6 and 7* cis-regulatory domains are indicated by brackets, with the domain boundaries in between shown by gray circles. The red boxes on the DNA line mark the IAB5, IAB6, IAB7a, IAB7b and IAB8 initiator elements (from proximal to distal, respectively). The extent of the deletions analyzed are shown below the DNA line, in black for deletions associated with loss-of-function of phenotypes and in red for gain-of-function phenotypes. (**B**) The DNA line with the scales as in A is drawn again. The extent of the different restriction fragments used in transgenic constructs are shown below, with the initiator fragments drawn in red. The expression patterns directed by these initiator fragments are presented in **C-F**, which show embryos at the extended germ band stage, doubled stained with antibodies directed against Engrailed (brown) and the β-Galactosidase (in blue). The parasegmental anterior border of *lacZ* expression (indicated by an arrow) shifts one parasegment posterior in each panel from left to right

null for *iab-6*. This indicates that sequences crucial for *iab-6* function lie between the proximal breakpoints of these two deletions (89,392 and 96,559; see Fig. 1A). Meanwhile, regarding *iab-7*, the comparison of these two deletions demonstrates that there are at least two separable elements important for *iab-7* activity. One element uncovered by the *iab-6,7^{IH}* deletion and one located in between the distal breakpoints of the two deletions. Consistent with this, we and others had previously isolated a weak PS12 specific initiator element mapping between the distal breakpoints of these two deletions (67,072 and 67,806) (Zhou et al., 1999; Barges et al., 2000). We postulate the existence of another PS12-specific initiator element.

In order to further narrow down the regions containing these enhancers, we cloned the regions implicated by our genetic analysis in front of a *Ubx-lacZ* reporter gene and studied the expression pattern of the resulting constructs in transgenic flies.

Genetic analysis had localized the new *iab-6* regulatory element to a ~7 kb region between the proximal break points of the *iab-6,7^{IH}* and the *iab-7^{280}* deletions. When a ~8 kb fragment approximating this region is placed in front of the *Ubx-lacZ* reporter construct (fragment 7.8H in Fig. 1B), it drives expression of the *lacZ* reporter in a parasegment-specific manner, with strong expression in PS11, PS13 and PS14 (data not shown, but see below). The parasegment-specific expression pattern driven by this element suggests that it is capable of reading the parasegmental address and is, therefore, an initiator element. By subcloning overlapping neighboring fragments into the same vector, we were able to narrow down the *iab-6* initiator to a ~3 kb region contained within fragment 7.8H. Consistent with this, a ~2.8 kb fragment (2.8XN in Fig. 1B) from this region is fully able to recapitulate the PS-specific pattern of fragment 7.8H (Fig. 1D). We have therefore, named this fragment IAB6.

The IAB6 fragment is capable of maintaining strong *lacZ* expression in PS11 and PS13 at later stages of embryogenesis (see Fig. S1B in the supplementary material), indicating that the fragment contains a maintenance element in addition to the initiator. The presence of a maintenance element is further supported by the observation that transgenic flies carrying the IAB6 construct show pairing sensitive repression of the mini-white reporter gene in 30% of the lines (three out of 10 lines), a hallmark characteristic of MEs (Kassis, 1994; Gindhart and Kaufman, 1995; Pirrotta and Rastelli, 1994).

To identify the second putative *iab-7* activating element, we once again cloned overlapping fragments from the implicated area into the *Ubx-lacZ* reporter construct. A ~3 kb fragment (3H; Fig. 1B) located at the proximal edge of the *iab-7* cis-regulatory region is able to direct the early expression of the *Ubx-lacZ* reporter gene in PS12

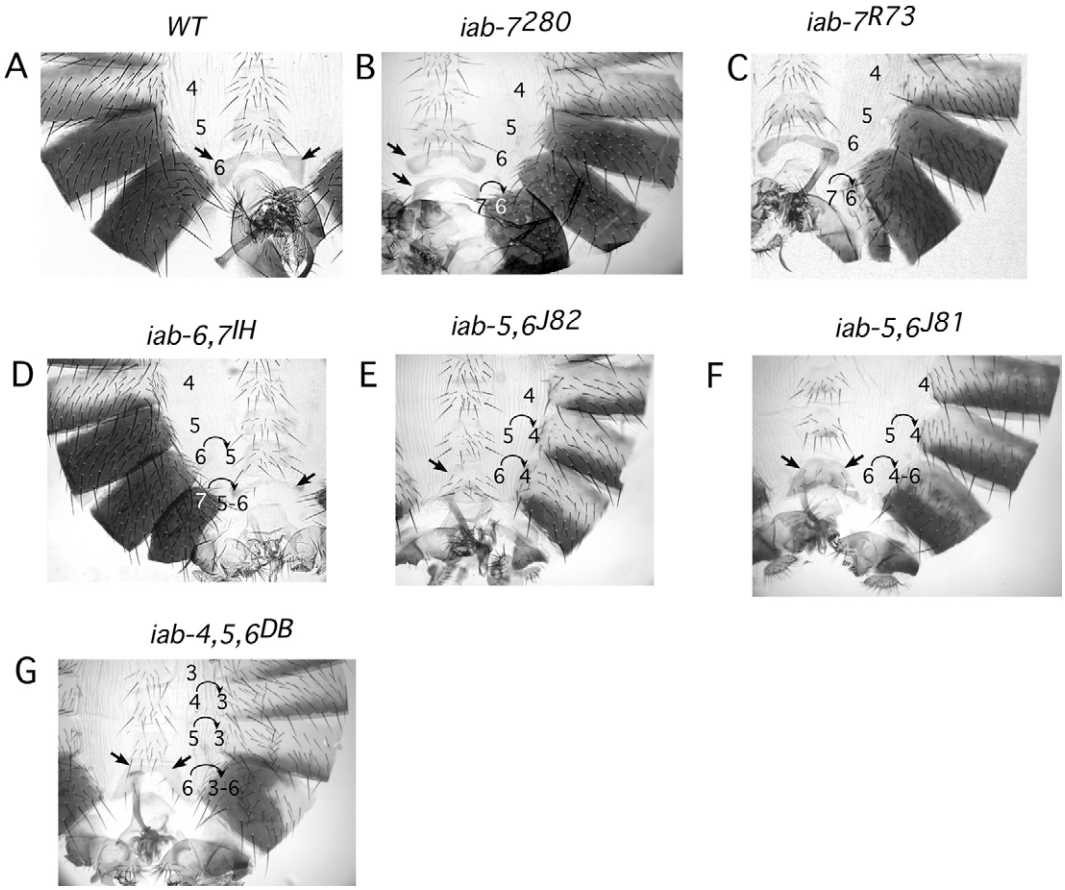

Fig. 2. Homeotic transformations in adult male. Male abdomens were cut along the dorsal midline and flattened on a slide. The dorsal surface of each abdominal segment has a rectangular plate of hard cuticle called the tergite. Only half of the tergites of the 4th, 5th and 6th abdominal segments (numbered) are visible, as well as the genitalia at the bottom. (**A**) In wild type, the 5th and 6th tergites are pigmented. The ventral surface of abdominal segments is composed of soft cuticle called the pleura. On the ventral midline of the pleura, there are small plates of harder cuticle called sternites. In wild type, the 6th sternite (shown by arrows) can easily be distinguished from the more anterior sternites by its different shape and by the absence of bristles. In wild-type males, the 7th abdominal segment present in embryos and larvae does not contribute to any adult structures after metamorphosis. (**B**) *iab-7^{280}*males harbor a complete transformation of A7 into A6, as revealed by the presence of a large 7th tergite and sternites (arrows). (**C**) In *iab-7^{R73}* males, however, there is only a weak transformation of A7 into A6, as revealed by the small 7th tergite and the absence of a 7th sternite on the ventral side. (**D**) In *iab-6,7^{IH}*, A6 is completely transformed into a copy of A5. However, A7 is only partially transformed into a segment of intermediate identity between A5 and A6, as seen by the shape of the 7th sternite, which resembles the 6th, but harbors a few bristles (A5 character). This indicates that in *iab-6,7^{IH}*, *iab-7* is not completely inactivated (**E**) In *iab-5,6^{J82}*, both A5 and A6 are transformed into a copy of A4. (**F**) In *iab-5,6^{J81}* the residual pigmentation on the 6th tergite, as well as the shape of the 6th sternite show that A6 is not fully transformed into A4, but instead, into a mixture of identity between A4 and A6, suggesting that the *iab-6* domain is not completely inactivated by the deletion. (**G**) In *iab-4,5,6^{DB}*, A5 is transformed into A3. However, as in *iab-5,6^{J81}*, the 6th segment harbors features of A6, suggesting that *iab-6* is also not completely inactivated by the deletion.

and PS14, indicating that this element is also an initiator element (Fig. 1E). This expression pattern is very similar to that already described for another PS12 initiator element located about 10 kb away, at the other edge of the *iab-7* cis-regulatory region (Zhou et al., 1999; Barges et al., 2000). We will refer to these two initiator elements as IAB7a and IAB7b, with IAB7a being the more proximal element. It should be noted that the level of PS12 and PS14-specific expression of IAB7a is much higher than that of the previously described IAB7b element. In transgenic lines carrying IAB7a, the *lacZ* expression pattern fades away at later stages of embryogenesis, indicating that no maintenance element is present within this fragment.

Initiators are not sufficient for *Abd-B* patterning

Homozygous *iab-5,6^{J82}* males have their A5 and A6 segments transformed into A4, indicating that both *iab-5* and *iab-6* are affected by this mutation (Fig. 2E). These adult homeotic transformations are paralleled by the absence of *Abd-B* staining in PS10/A5 and PS11/A6 in homozygous *iab-5,6^{J82}* embryos (Fig. 3). In agreement with the complete loss of *iab-6* function, the entire *iab-6* domain is deleted. However, despite the fact that *iab-5* function is inactive, the deletion affects only one-third of the *iab-5* region (Fig. 1A; 83,791-106,497). This observation indicates that sequences necessary for *iab-5* activity reside within the distal one-third of the *iab-5* region. Consistent with this finding, Busturia and Bienz (Busturia and Bienz, 1993) had previously identified an IAB5 initiator in this region that can drive *Ubx-lacZ* expression in PS10, PS12 and PS14 (see also Fig. 1C).

The *iab-5,6^{J81}* deletion shares its proximal endpoint with *iab-5,6^{J82}*, but it is much shorter and extends only 10.5 kb distally, removing the proximal one-third of *iab-6* (Fig. 1A; between 95,869 and 106,497). The transformation of PS10/A5 into PS9/A4 and the loss of *Abd-B* expression in PS10 corroborate that sequences covering the IAB5 initiator element are indispensable for *iab-5* function (Fig. 2F; Fig. 3). Interestingly, *iab-6* function is partly affected in *iab-5,6^{J81}*, even though the IAB6 initiator is untouched by this deletion. In embryos, the level of *Abd-B* expression appears normal in PS11/A6 (Fig. 3). However, in homozygous adult males, there is a partial transformation of A6 into A5 (Fig. 2F). Although

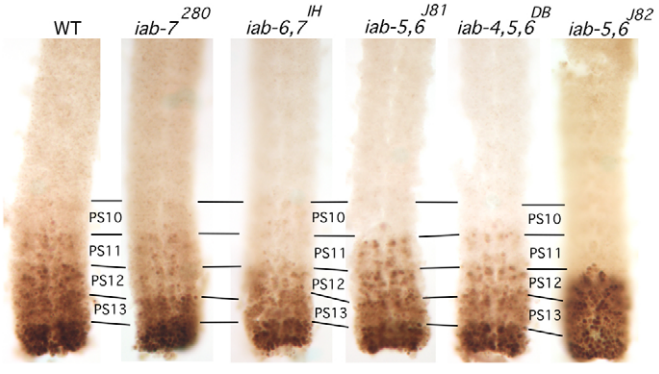

Fig. 3. *Abd-B* expression pattern in wild type and deficiency mutants. HRP staining with antibodies against *Abd-B*. After staining, the central nervous systems were dissected out from 12 hour-old embryos. In wild type, the typical *Abd-B* expression pattern is characterized by an anterior-to-posterior gradient from PS10 to 13 in the number of expressing nuclei per parasegment, as well as by the intensity in each nucleus. See text for the description of the expression patterns in the different mutants.

our previous data indicates that the IAB6 initiator is required for *iab-6* activity, we must conclude, based on *iab-5,6^{J81}*, that it is not sufficient for *iab-6* function and that there are other elements, probably adult tissue-specific enhancers that are required for full iab-6 activity.

The *iab-4,5,6^{DB}* deletion has already been described in previous reports (Karch et al., 1985; Celniker et al., 1990; Sanchez-Herrero, 1991; Crosby et al., 1993). It removes DNA between map positions 95,464 and 124,218 (Fig. 1A). Based on the PS-specific expression pattern of enhancer trap P-elements that are inserted in the BX-C (the HCJ200 line); Bender and Hudson (Bender and Hudson, 2000) have redefined the position of the *iab-4* cis-regulatory region at position 127,367. Thus, *iab-4,5,6^{DB}* appears to remove most of *iab-4*, the entire *iab-5* region and the proximal half of the *iab-6* cis-regulatory region (Fig. 1A). In agreement with this, homozygous *iab-4,5,6^{DB}* males have their 4th and 5th abdominal segments transformed into the 3rd (Fig. 2G) (Karch et al., 1985).

Interestingly, the 6th abdominal segment in homozygous *iab-4,5,6^{DB}* males is partly transformed towards A3 or A4 (Fig. 2G), indicating that the remaining half of *iab-6* is only partially functional even though the IAB6 initiator is still present. The latter observation supports the conclusions drawn from *iab-5,6^{J81}*, which suggests that the IAB6 initiator is necessary but not sufficient for iab-6 function. This conclusion is further corroborated by the similarity of the embryonic *Abd-B* expression pattern in PS10/A5 and PS11/A6 of embryos homozygous for either *iab-4,5,6^{DB}* or *iab-5,6^{J81}* (Fig. 3). In both cases, there is no expression in PS10/A5 (both deletions remove *iab-5*), and *Abd-B* expression in PS11/A6 is very similar to the wild-type pattern.

Deletion of the *iab-7* and *iab-6* PTSs

We have isolated two deletions in the *iab-7* region that are of special interest as they remove the region implicated in Promoter Targeting Sequence (PTS) activity. Zhou and Levine (Zhou and Levine, 1999) studied an element from the BX-C in reporter constructs. They suggested that this PTS element helps a distal enhancer bypass an intervening insulator to reach its target promoter. Zhou and Levine mapped the PTS to an 820 bp region deleted from the *Fab-7^{R73}* chromosome. Unfortunately, this chromosome was isolated as a revertant of *Fab-7^{1}* (Gyurkovics et al., 1990) and contains the *Fab-7^{1}* deletion, making phenotypic analysis difficult. We have recovered a chromosome carrying solely the 820 bp deletion from the *Fab-7^{R73}* chromosome implicated in PTS activity, that we named *iab-7^{R73}* (Fig. 1A; deletion of DNA between 64,229 and 65,049; see Materials and methods). Fig. 2C shows a homozygous male. A5 and A6 acquire normal identity, while A7 is weakly transformed into A6. Thus, a clean removal of the PTS sequence only weakly impairs *iab-7* function, with no effect on *iab-5* and *iab-6* function.

We also isolated a smaller deletion (*Δ330^{iab7}*) that shares the same proximal breakpoint with *iab-7^{R73}* but extends only ~670 bp (Fig. 1A). As homozygous *Δ330^{iab7}* males and females are indistinguishable from wild-type flies, sequences crucial for the *iab-7^{R73}* phenotype must lie within the 152 bp region on the distal side of the *iab-7^{R73}* deletion.

Recently, a second PTS element originating from *iab-6* has been described (PTS-6) (Chen et al., 2005). This second PTS element was mapped to the minor DNase I hypersensitive site of *Fab-7* (Karch et al., 1994) (from positions 84,827 to 85,028). During the course of site-directed mutagenesis studies on the *Fab-7* boundary and the *iab-6* cis-regulatory domain, we isolated a ~700 bp deletion (84,661-85,367) that removes the *Fab-7* minor hypersensitive site and ~500

bp of *iab-6* that we have named *ΔHS*^{iab6}*. Although *ΔHS*^{iab6}* entirely removes the new PTS-6 element, it is not associated with any abnormal phenotype.

Deletions associated with gain-of-function/boundary phenotypes

Fab-6,7^{1} and *Fab-6,7^{2}* are two deletions associated with gain-of-function phenotypes with very similar breakpoints and phenotypes. Both deletions share the same distal deficiency breakpoint at position 83,791 kb (the site of insertion of the *blt* transposon). On the proximal side, however, the deficiency breakpoint of *Fab-6,7^{1}* maps at position 101,096, while that of *Fab-6,7^{2}* maps to position 102,687 (Fig. 1A). Both of these deletions remove the *Fab-7* boundary element along with the entire *iab-6* domain. Interestingly, instead of showing a homeotic transformation of the 6th abdominal segment into the 5th, like *iab-6,7^{IH}*, *Fab-6,7^{1}* and *Fab-6,7^{2}* exhibit a striking dominant gain-of-function phenotype, in which, the 5th and 6th male abdominal segments are largely reduced in size (Fig. 4). In extreme cases (such as shown in Fig. 4C), homozygous males harbor only rudiments of the 5th and 6th abdominal segments. This phenotype corresponds to a strong transformation of A5 and A6 towards A7 (in wild-type males, the 7th abdominal segment present in embryos and larvae does not secrete any visible tergite or sternite cuticle). In the CNS of embryos, we find the same level and pattern of *Abd-B* expression in PS10/A5 and PS12/A7. These patterns are higher than the wild-type PS10/A5 expression pattern and lower than the pattern in PS12/A11 (Fig. 4F).

Based on the similarity of these phenotypes to the previously isolated *Fab-7* and *Fab-8* deletions, we believe that the *Fab-6,7* deletions must delete a putative *Fab-6* boundary element that normally separates *iab-5* from *iab-6*. The presence of this boundary element can be best observed by comparing the *Fab-6,7* mutations to the *iab-6,7^{IH}* deletion. Although in the *Fab-6,7* mutants, we observe a fusion between *iab-5* and *iab-7*, in *iab-6,7^{IH}* mutants, *iab-7* is still capable of behaving in an autonomous manner. These observations imply that there must be a boundary element mapping to the region between the proximal breakpoints of these deletions (96,559 and 101,096) that keeps these domains separate.

Comparing the extent of the *Fab-6,7^{1}* (or *Fab-6,7^{2}*) mutation with that of the *iab-5,6^{J82}* mutation sheds light on the dominant gain-of-function frontabdominal phenotype. All three deletions share the same distal endpoint, indicating that the origin of the *Fab-6,7^{1}* and *Fab-6,7^{2}* GOF phenotypes must lie within the DNA segment that remains present in *Fab-6,7^{1}* and *Fab-6,7^{2}*, and that is deleted in *iab-5,6^{J82}*. Interestingly, this DNA segment contains the IAB5 initiator element (see above) (Busturia and Bienz, 1993) that, in the *Fab-6,7* mutants, now becomes juxtaposed to the complete *iab-7* domain (the *Fab-7* boundary is removed in the *Fab-6,7* alleles, and in *iab-5,6^{J82}*). We believe that the dominant gain-of-function phenotype is caused by the IAB5 initiator element ectopically activating *iab-7* in PS10, an interpretation corroborated by the observation that in both mutations, the PS10/A5 and PS12/A7 Abd-B expression levels are similar and higher than the level normally found in PS10 of wild-type embryos.

Surprisingly, in both *Fab-6,7* deletion mutants, PS11 expression of *Abd-B* differs from that seen in PS10 and PS12. As the *iab-6* domain is entirely deleted in these flies, we expected to see an A7/A5 fusion pattern of expression similar to that seen in PS10 and PS12. Interestingly, this is not the case. The pattern of *Abd-B* expression in PS11 is greatly reduced from the level seen in its neighboring parasegments. As other large boundary mutants, such as the *Fab-3,5^{DV}* mutation described below, do not behave similarly, we cannot explain this result at this time.

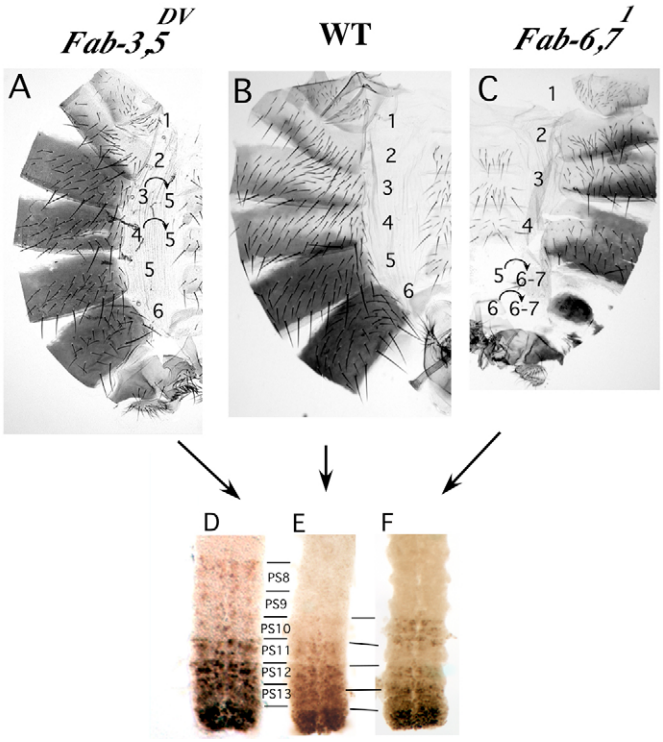

Fig. 4. Mutations associated with gain-of-function phenotype. (**A-C**) Cuticles from adult males of homozygous *Fab-3,5^{DV}*, wild type and *Fab-6,7^{1}*. See text and the legend of Fig. 2 for descriptions. (**D-F**) The corresponding *Abd-B* expression patterns in the CNS of 12-hour-old embryos (refer to legend of Fig. 3).

The third deletion we identified that has a gain-of-function phenotype is *Fab-3,5^{DV}*. *Fab-3,5^{DV}* is a deletion derivative of the HCJ200 enhancer trap line that lies at the distal edge of *iab-3* at position 127,367 (Bender and Hudson, 2000). An imprecise excision created a bidirectional deletion of 25 kb between positions 112,381 and 137,391, removing the *Mcp* boundary along with the entire *iab-4* region and about 10 kb of the distal side of *iab-3*. Thus, *Fab-3,5^{DV}* fuses the proximal side of the *iab-3* region to *iab-5*. The deletion does not affect the previously identified IAB3 initiator element (Simon et al., 1990). In *Fab-3,5^{DV}*, it appears that the juxtaposition of the IAB3 initiator element to *iab-5* results in the ectopic activation of *iab-5* in A3/PS8. Adult males have A3 and A4 transformed into a copy of A5 (Fig. 4A). Likewise, in embryos, the *Abd-B* expression pattern normally seen in PS10 appears ectopically in PS8 and PS9 (Fig. 4D).

Other regulatory elements in the Abd-B 3′ cis-regulatory region

We have extended the reporter gene analysis that allowed us to identify the IAB6 and IAB7a initiator elements, to scan the whole Abd-B 3′ cis-regulatory region for additional enhancers and maintenance elements. We did this by inserting overlapping DNA fragments (Fig. 1B) spanning most of the region from *iab-5* to *iab-8* in front of the *Ubx-lacZ* reporter gene in transgenic flies and studying either the expression pattern of the resulting constructs, or the pairing-sensitive repression of the mini-white cassette contained on the transgene (see Fig. S1 in the supplementary material).

DISCUSSION

The proper identity of PS10/A5 through PS13/A8 is determined by the anterior-to-posterior stepwise increase in the level of *Abd-B*. This pattern of expression is achieved through the action of four large cis-regulatory domains. Using reporter constructs, a number of groups have identified several types of regulatory elements from within the cis-regulatory region. These elements include initiator elements, maintenance elements (polycomb response elements/PREs), cell/tissue-type specific enhancers, chromatin insulators and promoter targeting sequences (PTSs) (for a review, see Maeda and Karch, 2006). Unfortunately, with the exception of the boundary elements, genetic proof for the role of each of these elements has been confused because of the lack of discrete mutations. Thus far, most of the mutations used for the genetic analysis were rearrangement breaks (Lewis, 1978; Karch et al., 1985; Sanchez-Herrero et al., 1985; Celniker et al., 1990). Because these breaks remove extremely large regions of the cis-regulatory region and place foreign DNA in its place, the phenotypes resulting from these mutants are often complex and difficult to interpret. Here, we have described a collection of internal deletions within the *Abd-B* region of the BX-C. Using these deletions, we have been able to infer genetically the role of many of the elements previously identified. Our results, when combined with previous data, point to four important conclusions regarding the *Abd-B* region of the BX-C. First, as already discussed in prior publications (Karch et al., 1985; Celniker et al., 1990; Sanchez-Herrero, 1991; Galloni et al., 1993; McCall et al., 1994; Bender and Hudson, 2000) (for reviews, see Peifer et al., 1987; Maeda and Karch, 2006), the *Abd-B* region is modular by nature and can be divided into discrete functional units or domains. Second, in a wild-type situation, each domain appears to be sufficient to specify the appropriate level of *Abd-B* expression in a particular parasegment. Third, each domain is itself modular and made up of a similar set of definable regulatory elements. Finally, the activity of each domain is absolutely dependent on the presence of an initiator element.

Abd-B is controlled by a single *iab* domain in each parasegment

In 1978, E. B. Lewis first proposed a model for BX-C patterning that stated that once segment-specific functions are activated along the anteroposterior axis, they remain active in more posterior segments and contribute to the identity of those segments (Lewis, 1978). His hypothesis was based primarily on the posterior to anterior transformations observed in flies lacking distal regions of the BX-C. For example, embryos that lacked all BX-C sequences distal to the *bxd* domain, had all abdominal segments posterior to the first abdominal segment (A1) transformed into copies of A1. The transformations toward A1 suggested that everything necessary for A1 development was present in all posterior segments and that it was an accumulation of other segment-specific factors that caused the deviation in segmental identity. In agreement with the Lewis model, we, and others, have previously shown that enhancer trap lines inserted in the BX-C and transgenic initiator constructs are expressed in a pattern that starts from a specific parasegment and continues in more posterior parasegments (Simon et al., 1990; Qian et al., 1991; Müller and Bienz, 1992; Galloni et al., 1993; McCall et al., 1994; Zhou et al., 1999; Barges et al., 2000; Bender and Hudson, 2000).

Although these findings strongly suggested that the domains that become active in the parasegment they specify remain active in the more posterior parasegments, our analysis of internal deletions in the BX-C indicate that anterior domains are not required for the specification of more posterior parasegments. This is best illustrated by deletions that remove most or all of a single cis-regulatory domain. For example, the $iab\text{-}7^{Sz}$ and $iab\text{-}7^{280}$ deficiencies both delete the entire *iab-7* domain. If *iab-7* was required for the identity of the more posterior parasegment, PS13/A8, as predicted by the Lewis model, one might expect that deleting *iab-7* would affect PS13 identity. This is not what is seen. Flies homozygous for either of these mutations have PS12/A7 transformed into PS11/A6, while PS11/A6 and, more importantly, PS13/A8 are not affected in any detectable way. This phenotype indicates that although *iab-7* is absolutely required in PS12/A7, it is dispensable for the identity of all other parasegments. However, PS12 is still transformed into a perfect copy of PS11, indicating that *iab-6* remains capable of functioning in PS12/A7. Therefore, we believe that more anterior domains remain capable of functioning in more posterior parasements, but only in the absence of an active posterior domain. This is consistent, for example, with the observation that bxd^{+} is required in segments more posterior to PS6/A1, as *bxd* is the most posteriorly activated regulatory domain of the *Ubx* gene.

Internal deletions that affect more than one cis-regulatory region confirm these conclusions. For example, in $iab\text{-}6,7^{IH}$, where both the *iab-6* and the *iab-7* domains are deleted, both A6 and A7 acquire an A5 identity. However, PS13/A8 identity is again not affected by the deletion even though, in this case, two more-anterior domains are missing. Once again, the more-anterior *iab-5* domain remains capable of acting posterior to PS10/A5, but only in the absence of *iab-6* and *iab-7*. Similar results are seen with both the $iab\text{-}5,6^{J81}$ and the $iab\text{-}5,6^{J82}$ deletions, where PS12/A7 identity is unaffected in the absence of *iab-5* (and also *iab-6* for $iab\text{-}5,6^{J82}$).

Although it is possible that deletions of anterior domains lack a visible phenotype because of the masking effect of the stronger enhancers in more posterior domains, it is clear from our results that the graded *Abd-B* expression pattern cannot be accounted for solely based on the summation of individual regulatory domains. Instead, each regulatory domain is, by itself, sufficient to generate an appropriate pattern of *Abd-B* expression in a specific parasegment (see also Crosby et al., 1993).

Initiators work as domain control regions

The autonomous nature of the cis-regulatory domains implies the existence of elements within each domain that can independently sense and respond to positional cues. At present, the best candidates for serving these functions are among the enhancers identified by transgenic assays. Two basic types of enhancers have been found within the cis-regulatory regions of the BX-C. Some enhancers are capable of directing the expression of reporter genes in specific types of cells or tissues, irrespective of parasegmental boundaries. These enhancers are usually turned on relatively late during embryogenesis (Simon et al., 1990; Busturia and Bienz, 1993; Pirrotta et al., 1995). An example of this kind of enhancer is seen in fragment 4.4E from the *iab-6* cis-regulatory region that is capable of driving *Abd-B* expression in the visceral mesoderm (see Fig. S1 in the supplementary material). Other enhancers, however, direct expression of reporter genes from the end of cellular blastoderm stage in a segmentally restricted manner, indicating that they are capable of responding to a specific segmental address (Simon et al., 1990; Qian et al., 1991; Müller and Bienz, 1992; Shimell et al., 2000). These include the previously identified IAB5, IAB7b and IAB8 enhancers that express from PS10, PS12 and PS13, respectively (Fig. 1C-F) (Busturia and Bienz, 1993; Zhou et al., 1999; Barges et al., 2000). Here, we described two new elements of this type: IAB6 with a PS11 specificity and IAB7a with a PS12

specificity. Taken together, these findings demonstrate that each cis-regulatory region contains at least one PS specific enhancer. These specific enhancers have been previously referred to as 'initiators'.

Analysis of the phenotype of internal deletions suggests that the removal of the IAB5 or IAB6 initiator completely abolishes the function of *iab-5* or *iab-6*, respectively. For example, although the *iab-5,6^{J81}* deletion leaves intact about two-thirds of the *iab-5* domain, it deletes the IAB5 initiator element and is completely null for *iab-5* function. The *iab-6,7^{IH}* is another example. This deletion removes the IAB6 initiator and, despite the fact that one quarter of *iab-6* is not affected by the deletion, *iab-6* is completely inactivated. These results suggest that the IAB5 and IAB6 initiator elements are indispensable for *iab-5* and *iab-6* function, respectively.

Although the removal of initiator elements completely abolishes the function of a regulatory domain, the presence of an initiator is not sufficient to recapitulate the activity of the whole domain. This is clearly seen in the *iab-5,6^{J81}* and *iab-4,5,6^{DB}* deletions. In both of these mutants, the IAB6 initiator is still present, yet there is a partial transformation of A6 towards A5. These results highlight the importance of other regulatory elements, such as cell-type-specific enhancers, within the *iab* regions that help control *Abd-B* expression.

Thus far, all available data suggest that *iab-5* and *iab-6* each harbor only one initiator element. The situation in *iab-7* and *iab-8*, however, appears more complex. In both cases, two initiator elements have been identified in each domain. Comparison of the *iab-7^{280}* and the *iab-6,7^{IH}* phenotypes suggests that within *iab-7*, the two initiators contribute to the final *Abd-B* expression pattern. Although *iab-7^{280}* is completely null for *iab-7*, the *iab-6,7^{IH}* mutation leaves the IAB7b initiator intact and does not completely inactivate *iab-7*. This is consistent with the idea that complete loss of *iab-7* function can be achieved by only the removal of both PS12 initiators in cis.

iab-8 may represent another example of a regulatory region containing more than one initiator element. An IAB8 initiator has been identified between the *Fab-8* boundary and the 3′end of the *Abd-B* transcription unit (Fig. 1) (Zhou et al., 1999; Barges et al., 2000). However, mutations that separate the IAB8 element from the *Abd-B* transcription unit have no detectable effect on *iab-8* function, suggesting the presence of a second element within *iab-8* that can compensate for the loss of the IAB8 initiator. Indeed, Estrada et al. (Estrada et al., 2002) described the existence of a second *iab-8*-specific initiator element upstream of the *Abd-Bm* promoter.

Within the BX-C, *Abd-B* expression is strictly restricted to specific parasegments in all tissue. However, in transgenic constructs, only initiator elements seem to sense a parasegmental address. Outside the complex, the cell- and tissue-specific enhancers found within the BX-C do not. For example, although an isolated fragment, 11X, from the *iab-7* region directs the expression of a *Ubx-lacZ* construct in the epidermis of each parasegment (see Fig. S1 in the supplementary material), normal *Abd-B* expression in the epidermis is restricted to the parasegments posterior to PS12. The discrepancy between the transgenic and the native expression of *Abd-B* implies that something within each domain must act to control the activity state of the entire domain. Because the initiator elements seem to be required for the function of each domain and can sense parasegmental position, we believe that initiator elements act as domain control regions that sense a parasegmental address and relay this information to all other elements within the domain. Although the exact mechanism of this transmission is unknown, it is clear that a part of the mechanism involves the regulation of maintenance elements.

Maintenance elements

The activity pattern of cis-regulators is set up early in embryonic development by the gap and pair rule genes that specifically instruct initiator elements (Irish et al., 1989; Müller and Bienz, 1992; Qian et al., 1991; Shimell et al., 1994; Casares et al., 1995). By the time of mid-embryogenesis, the products of the gap and pair rule genes disappear. However, the activity of cis-regulatory elements is continuously required until the end of development. The activity state of each regulatory domain is maintained throughout the rest of development by maintenance elements (Müller and Bienz, 1991; Simon et al., 1993; Chan et al., 1994; Chiang et al., 1995; Fritsch et al., 1999) [for definition of ME see Brock and van Lohuizen (Brock and van Lohuizen, 2001)]. We believe that maintenance elements respond to the activity state of initiator elements and modify the chromatin structure of each domain accordingly. Consistent with this, maintenance elements (MEs) have generally been found to contain binding sites for *Polycomb*-Group (*Pc*-G) and *trithorax*-Group proteins (*Trx*-G) (Strutt et al., 1997; Brown et al., 1998; Fritsch et al., 1999; Tillib et al., 1999; Horard et al., 2000; Busturia et al., 2001; Mishra et al., 2001; Hur et al., 2002; Dejardin et al., 2005). Both *Pc-G* and *trx-G* proteins are known to be involved in modifying chromatin structure, with *Pc*-G proteins being involved in compacting chromatin (silencing) and *Trx*-G proteins being involved in opening chromatin (for reviews, see Ringrose and Paro, 2004; Brock and Fisher, 2005).

Here, we have described two new maintenance elements in *iab-6*. The occurrence of multiple MEs/PREs in a single cis-regulatory region has been documented before and may represent the normal situation, rather than the exception (Chiang et al., 1995). The *iab-5* cis-regulatory region, for example, also contains at least two MEs (Busturia and Bienz, 1993; Busturia et al., 1997; Müller et al., 1999). Although there has been only one ME identified within each of the *iab-7* and *iab-8* cis-regulatory regions (Hagstrom et al., 1997; Mihaly et al., 1997; Barges et al., 2000), it is likely that they too, contain more than one ME. Supporting this, a deletion of the single known *iab-7* ME (PRE) has very little consequence on the phenotype of PS12/A7, suggesting a functional redundancy with another ME within *iab-7* (Mihaly et al., 1997).

Like cell-type-specific enhancers, maintenance elements in transgenic constructs do not seem to be parasegmentally regulated. Indeed, one of the most common assays used to identify new maintenance elements requires them to maintain a pattern of expression initiated by the *bx* initiator (Müller and Bienz, 1991; Simon et al., 1993; Chan et al., 1994; Fritsch et al., 1999; Busturia et al., 2001). Therefore, like that of cell-type specific enhancers, ME function seems to be dictated by initiator elements.

Domain boundaries and PTS

Confirmation of the role of initiator elements in the control of both cell-type specific enhancers and MEs, comes from the analysis of a specific class of gain-of-function mutants. These mutations result from the simple deletions of regions located between the cis-regulatory domains. As a result of these deletions, neighboring cis-regulatory domains fuse into single units of gene regulation. Thus, these regions have been called domain boundaries to highlight their role in separating domains. An example of a boundary deletion is the *Fab-7* deletion (Gyurkovics et al., 1990; Mihaly et al., 1997). In the *Fab-7* deletion, less than 800 bp are deleted yet there is an almost complete transformation of PS11/A6 into PS12/A7. Surprisingly, the cells not transformed towards a PS12-type fate do not become

PS11 like, but PS10 like. Normally in PS11/A6, *iab-6* is active while *iab-7* is silent. In *Fab-7* mutants, one gets a fusion of the two domains. The result of this fusion is that there are two different parasegment specific initiator elements in the same domain. We believe that the resulting phenotypes stem from the competition between these two initiator elements on the various enhancers and maintenance elements. In most cells in PS11, the *iab-6* initiator correctly reads the parasegmental address as PS11 and instructs all enhancers and maintenance elements (including the stronger iab-7/PS12 enhancers) to turn on *Abd-B* expression. Because of the *iab-7* enhancers, there is an overexpression of *Abd-B* in PS11 to the level of PS12. Meanwhile, in other cells, the *iab-7* initiator(s) correctly reads the parasegmental address as PS11, remains silent, along with other elements of the domain (including the elements originating from *iab-6*).

Although the genetic evidence highlighting a role for the boundary regions in *Abd-B* gene regulation is quite clear, the mechanism by which boundaries function is still mysterious. In transgenic constructs, boundaries act as insulator elements: elements capable of blocking enhancer-promoter interactions when intercalated between them (Hagstrom et al., 1996; Zhou et al., 1996; Zhou et al., 1999; Barges et al., 2000). The role of boundaries as elements capable of separating domain specific enhancers and silencers may allude to the validity of this transgenic assay. However, it is clear that within the BX-C, boundaries cannot solely act as insulator elements, and that there must be some mechanism by which these insulators can be bypassed when in the proper context.

An element deriving from the region next to the *Fab-8* boundary has been proposed to aid distal enhancers in bypassing intervening boundaries. This element has been termed the promoter targeting sequence (PTS) (Zhou and Levine, 1999). In transgenic constructs, the PTS permits distal enhancers to overcome the insulating effect of *Fab-8*. However, the in situ function of the PTS is much less clear. Part of the reason for this is that some of the phenotypes originally attributed to a PTS deletion, such as the partial transformation of A5 towards A4, are, in fact, the consequence of the haplo-insufficiency of the *Abd-B* deletion used by Zhou and Levine. Another factor complicating the evaluation of the function of the PTS in the BX-C is that, until now, the only available mutation that deletes the PTS region was a revertant of *Fab-7*^{1} (*Fab-7*^{R73}), and therefore carried a second deletion in the *Fab-7* region.

Here, we described two mutations that remove putative PTS elements. The first is a mutation that deletes the R73/PTS-7 region without affecting the rest of the BX-C. And the second is a deletion that removes the newly described PTS-6 element from iab-6 (Chen et al., 2005). In both cases, the phenotypes associated with the PTS deletions are relatively small. In contrast to the phenotype of the double deletion, *Fab-7*^{R73}, flies homozygous for the single *iab-7*^{R73} deletion exhibit only a rather mild transformation of A7. The morphology of A6, however, is wild type in these flies, indicating that the R73 region has only a weak regulatory activity that is restricted to *iab-7*. Meanwhile, the *ΔHS*^{*iab6} deletion that removes the entire PTS-6 element is completely wild type in appearance. Taken together, our results indicate that if the PTS hypothesis is correct, each domain must contain multiple, redundant PTS elements.

The domain model

Based on our data and the data of others (Simon et al., 1993; Crosby et al., 1993; Pirrotta et al., 1995), we propose a model to summarize our current understanding of how the BX-C functions (Fig. 5). We believe that each domain autonomously controls the expression of *Abd-B* within one parasegment. Accordingly, each regulatory domain in the BX-C is a modular array of all of the elements necessary for *Abd-B* expression in a particular parasegment. Key to the functioning of each domain is the initiator element. Acting as a centralized domain control region, the initiator element determines whether the appropriate gap and pair-rule gene products are present to justify the activation of the domain. If the proper positional address is read, the initiator then sends a signal to the rest of the domain to activate various enhancers. Because a domain lacking an initiator seems to be silenced, we imagine that this signal is made up primarily of an inhibitory signal to the ME/PREs. With the PREs placed into a non-silencing state, the enhancers become free to activate *Abd-B*. If the proper parasegmental address is not found,

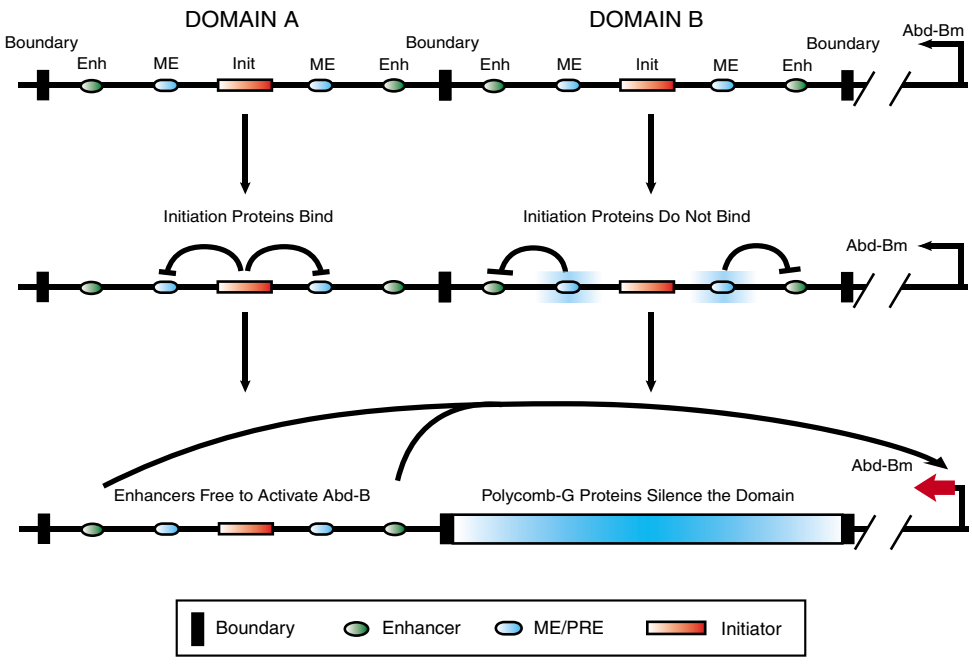

Fig. 5. Initiators control the activity of whole domains. Two adjacent domains are drawn, as well as the target *Abd-B* gene further to the right. The thick blue rectangles symbolize silencing by the *Pc-G* complex(es). The example on the left shows an initiation domain, while the example on the right shows a non-initiated domain.

then the Pc-G proteins silence the domain. Throughout all of this, the domain boundaries keep each domain independent and free from competing influences.

Despite the fact that a rough map of the 'regulatory landscape' of the *Abd-B* domain is now available, a number of questions regarding the function of the regulatory elements remain to be answered. These include the problem of how different regulatory elements within individual domains interact and communicate. For example, what is the nature of the signal from initiator to ME? One possibility currently being discussed is the idea that transcription across PREs might be the inhibitory signal (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005). Although it is still unclear whether transcription plays an instructive role in BX-C regulation, it is interesting to speculate that initiators may in fact generate transcription across PREs to inactivate them. Until recently, answering these types of questions within the BX-C have been unreasonable. Perhaps now, through the use of targeted mutagenesis and gene conversion, we will finally be able to tackle precise questions in the BX-C. Indeed, our results raised many questions, now ripe for investigation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/15/2983/DC1

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